A kinetic study of metabolite transfer in coupled two-enzyme reactions

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Abstract

There are two fundamentally different ways by which a metabolite can be transferred between enzymes in metabolic pathways. If the two sequential enzymes cannot form a complex with each other, the metabolite must first be released from the producing enzyme to the reaction medium and then be transported to the consuming enzyme, via the reaction medium, by free diffusion. If the two sequential enzymes are capable of forming a complex with each other, an alternative metabolite transfer mechanism could be operative. The metabolite might then be directly transferred from the producing enzyme to the consuming one without prior release to solution. The term 'channelling' refer to such a mechanism of direct metabolite transfer. The mechanism by which this metabolite transfer takes place is a main determinant of the kinetic characteristics and control properties of the sequential metabolic enzyme reactions. Establishing what mechanism of metabolite transfer is applicable in specific cases, or in general, is therefore a matter of outstanding importance for our understanding of the behaviour and regulation of metabolic pathways. This thesis deals with the hypothesis concerning metabolite channelling in systems that may form dynamic bi-enzyme complexes in vitro.

Some crucial steady-state and transient-state kinetic experiments were carried out, aiming at testing if there is any general difference in the mechanism of NADH transfer between dehydrogenases depending on the chiral coenzyme specificity of the enzymes. The next investigation was initiated to examine the unchallenged proposal of a channelled transfer of dihydroxyacetone phosphate in the reaction system of aldolase and glycerol 3-phosphate dehydrogenase, just as the unchallenged isotope dilution data taken to be indicative of a channelled transfer of glyceraldehyde-3-phosphate in the reaction system of aldolase and glyceraldehyde-3-phosphate dehydrogenase. In the final two studies the coupled reactions catalysed by the fusion protein of malate dehydrogenase and citrate synthase, and of beta-galactosidase and galactose dehydrogenase were examined, by using a new and more direct approach where all predictions on the expected free diffusion behaviour of the fusion protein were based upon the kinetic properties of the fusion protein itself. From the results achieved it was concluded that no tenable evidence is available for metabolite channelling in systems involving glycolytic enzymes; and the results obtained also provide clear evidence that channelling due to proximity effects either is absent or of negligible quantitative significance.

Key words: Enzyme kinetics, Free diffusion, Channelling, Dehydrogenase, Fusion protein, Proximity effect

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TABLE OF CONTENTS

LIST OF PAPERS V

ABBREVIATIONS VI

INTRODUCTION 1

BACKGROUND 5

Metabolite transfer mechanisms 5

Enzyme-enzyme complexes 6
Non-dissociable complexes 6
Dissociable complexes 7

Evidence for channelling in systems involving glycolytic enzymes 7

The channelling controversy 9

Proximity effects 10
Fusion proteins 11

PRESENT INVESTIGATION 12

Paper I 12

Paper II 15

Paper III 17

Paper IV 20

CONCLUSIONS 23

POPULÄRVETENSKAPLIG SAMMANFATTNING PÅ SVENSKA 24

ACKNOWLEDGEMENTS 26

REFERENCES 27

LIST OF PAPERS

This thesis is based on the following papers, referred to in the text by their roman numerals:

I. **Mechanism of NADH transfer among dehydrogenases**
Wilma Martínez Arias, Henrik Pettersson and Gösta Pettersson
Biochimica et Biophysica Acta (1998) 1385:1, 149-156

II. **Mechanism of metabolite transfer in coupled two-enzyme reactions involving aldolase**
Henrik Pettersson and Gösta Pettersson
European Journal of Biochemistry (1999) 262:2, 371-376

III. **Kinetics of the coupled reaction catalysed by a fusion protein of yeast mitochondrial malate dehydrogenase and citrate synthase**
Henrik Pettersson, Peter Olsson, Leif Bülow and Gösta Pettersson
European Journal of Biochemistry (2000) 267:16, 5041-5046

IV. **Kinetics of the coupled reaction catalysed by a fusion protein of β-galactosidase and galactose dehydrogenase**
Henrik Pettersson and Gösta Pettersson
Biochimica et Biophysica Acta (2001) 1549:2, 155-160

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ABBREVIATIONS

Ac-CoA	Acetyl Coenzyme A
Aldolase	Fructose 1,6-bisphosphate aldolase
ATP	Adenosine triphosphate
Galactonolactone	D-galactono-1,4-lactone
CoA	Coenzyme A
D-Galactose	D-Galactopyranose
FAD	Flavin adenine dinucleotide
NAD^+	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide, reduced form

Enzyme nomenclature according to *Enzyme Nomenclature 1992* [1].

INTRODUCTION

Metabolism is the process of integrated enzyme-catalysed chemical reactions that contribute to the maintenance of the cells in which they occur [2, 3]. In animals, like us, metabolic reactions account for the degradation of food to provide not only energy, but also chemical intermediates for synthesis of molecules necessary for growth and reproduction [3, 4]. Similarly, every cell of every organism either produces or gathers from its surroundings small molecules and combines these chemical building blocks to form larger molecules. At the same time, cells contain enzymes for decomposing all of the molecules that have been made. Thus, we have a complex network of metabolic reactions for synthesis and breakdown of substances required for self-renewal of cells and tissues, reactions that serve the ultimate purpose of enabling a cell or multicellular organism to reproduce itself [4].

The set of metabolic reactions that participate in the synthesis or degradation of a certain substance is usually referred to as a metabolic pathway [3]. A pathway of particular metabolic importance is the glycolytic one, which accounts for the conversion of glucose into pyruvate through the sequence of enzymically catalysed reactions shown in Figure 1 [4, 5].

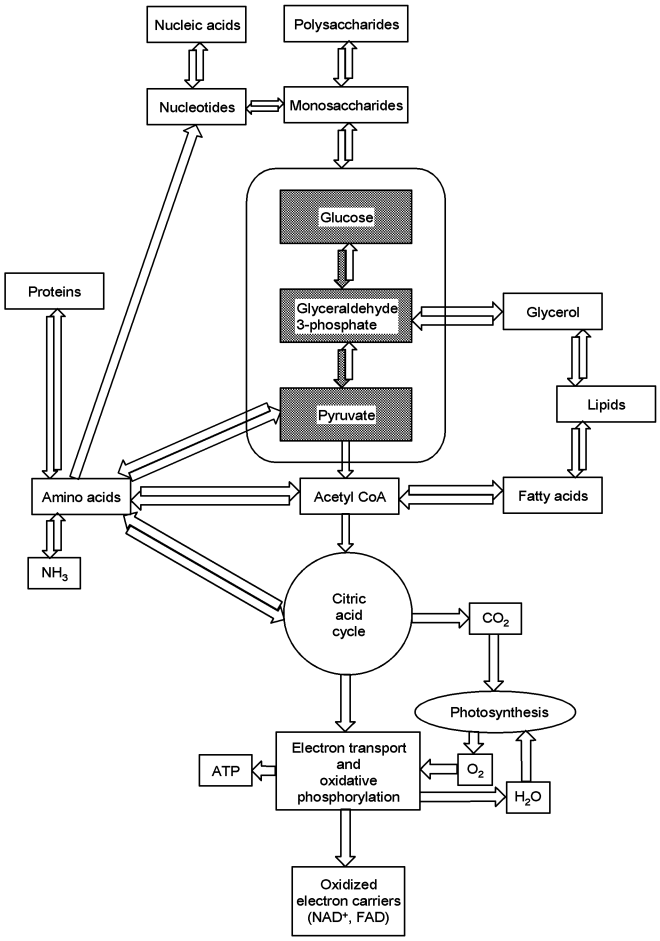

Figure 1. This overview of metabolism highlights the glycolytic pathway, which accounts for the conversion of glucose into pyruvate.

Glycolysis (from the Greek *glykys*, meaning "sweet", and *lysis*, meaning "splitting") occurs in all living cells [5]. It may provide cells and organisms with energy under both aerobic and anaerobic conditions. Furthermore, glycolytic intermediates participate in additional pathways of central metabolic importance, such as those leading to the synthesis of lipids and, in photosynthesising organisms, to carbon dioxide fixation and carbohydrate production [6]. In aerobic organisms, the glycolytic product pyruvate is converted into acetyl-CoA, a central metabolite that may enter the citric acid cycle and

hence becomes linked to a variety of biosynthetic processes (Figure 2) [3, 6].

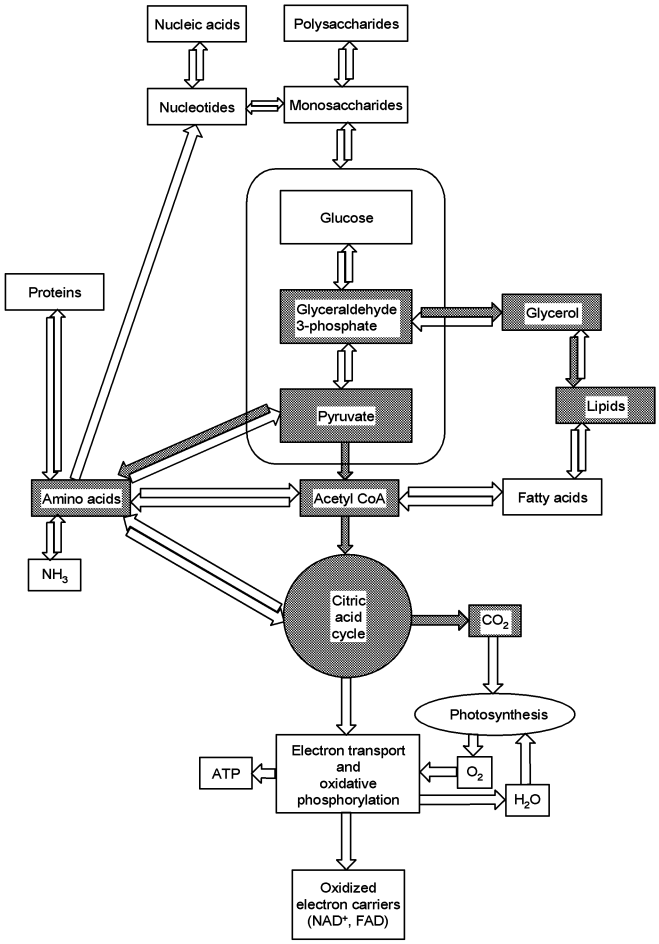

Figure 2. This overview highlights some of the metabolic pathways that glycolytic intermediates participate in.

A common feature of virtually all metabolic pathways is that they involve sequences of enzymically catalysed reactions [3]. The product of one enzyme reaction is the substrate for one or several subsequent reactions and must therefore be transferred from the producing enzyme to the consuming one. The mechanism by which this metabolite transfer takes place is a main determinant of the kinetic characteristics and control properties of the sequential metabolic

enzyme reactions [6]. Establishing what mechanism of metabolite transfer is applicable in specific cases, or in general, is therefore a matter of outstanding importance for our understanding of the behaviour and regulation of metabolic pathways.

The present thesis is concerned with this problem.

BACKGROUND

Metabolite transfer mechanisms

There are two fundamentally different ways by which a metabolite can be transferred between enzymes in metabolic pathways [7]. If the two sequential enzymes cannot form a complex with each other, the metabolite (M) must first be released from the producing enzyme (E_1) to the reaction medium and then be transported to the consuming enzyme (E_2) via the reaction medium by free diffusion (Figure 3).

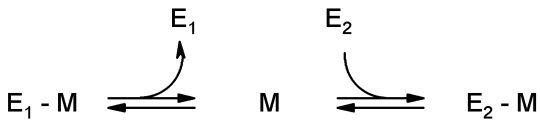

Figure 3. Reaction scheme for a free-diffusion mechanism.

Metabolite transfer between consecutive enzymes in metabolic pathways has traditionally been assumed to proceed by such a free-diffusion mechanism [8-10].

If the two sequential enzymes are capable of forming a complex with each other, however, an alternative metabolite transfer mechanism could be operative. The metabolite might then be directly transferred from the producing enzyme to the consuming one without prior release to solution (Figure 4).

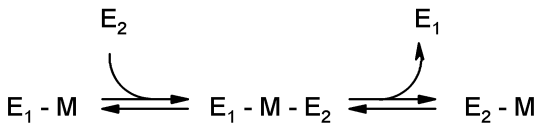

Figure 4. Reaction scheme for a direct transfer mechanism.

The term 'channelling' has been coined to refer to such a mechanism of direct metabolite transfer [11, 12].

Proponents of the channelling idea have drawn attention to several potential advantages of a direct metabolite transfer. Ovádi [11] summarised the main benefits of channelling as follows:

- It prevents or impedes loss of intermediates by diffusion
- It decreases the transit time required for an intermediate to reach the active site of the next enzyme
- It reduces the transient time for the system to reach the new steady state
- It protects chemically labile intermediates
- It circumvents unfavourable equilibrium
- It segregates the intermediates of competing chemical and enzymic reactions

In addition to the above six points, channelling has been considered advantageous since it may lower the bulk concentration of intermediates [11-14]. This would solve the solubility problem arising from the coexistence of an enormous number of small molecules [11, 12, 14].

Enzyme-enzyme complexes

Non-dissociable complexes

A direct transfer of metabolites requires that the producing and consuming enzymes are able to form a complex with each other in solution. For instance, sequential enzymes in metabolic pathways have been found to form static non-dissociable bi- or multienzyme complexes that can be readily isolated and show no tendency to dissociate in solution [12, 15-18].

A classical example of a stable multienzyme complex is the mammalian fatty acid synthase that catalysis the synthesis of saturated fatty acids from malonyl-CoA [19-23]. In this multienzyme complex, the intermediately formed metabolites remain covalently bound to the complex throughout the pathway reactions [9, 24].

An example of a stable bi-enzyme complex is the tryptophan synthase $\alpha_2\beta_2$ complex from *Salmonella typhimurium* [10, 25-28]. The four subunits have a spatial organisation forming a 25 Å long hydrophobic tunnel [29]. This tunnel is suggested to facilitate the diffusion of indole from its site of production to the site of tryptophan synthesis, and to prevent its escape to the solvent during catalysis.

Dissociable complexes

The enzymes catalysing reactions of most central metabolic pathways (e.g. glycolysis) do not form any stable multienzyme complexes, but have since long been considered to be free and 'soluble' [9, 30]. During the last few decades, evidence has been presented to show that consecutive glycolytic enzymes may form weak dissociable complexes if the enzyme concentrations are high enough [2, 12, 31]. These weak complexes might be of biological significance, because glycolytic enzymes in some tissues are present at very high concentrations [32-36], even higher than those of the substrates that the enzymes act upon [11, 37-40]. This has led to the idea that weak dissociable bi-enzyme complexes, formed transiently during metabolic reactions, might play an essential role as mediators of a direct metabolite transfer between soluble enzymes in central metabolic pathways [2, 9, 11, 12, 41, 42]. The term 'dynamic channelling' has been introduced to distinguish such a mechanism of direct metabolite transfer from that (static channelling) occurring in stable bi- or multienzyme complexes [9, 11].

Evidence for channelling in systems involving glycolytic enzymes

Observations that consecutive enzymes may form a bi-enzyme complex [43-53] demonstrate that metabolite transfer by dynamic channelling is a mechanistic possibility, but do not imply that the actual transfer of metabolites is channelled; it might well occur by free diffusion also in a bi-enzyme complex [54-57]. Discrimination between the two alternative mechanisms for metabolite transfer can be made only by methods that provide information on the metabolite

transfer process. This has led several research groups to carry out kinetic studies of the coupled reactions catalysed by pairs of consecutive 'soluble' enzymes in metabolic pathways [51, 58, 59]. For a start, such studies were directed mainly towards systems involving enzymes of the glycolytic pathway [30, 54, 60-62].

Thus, Ovádi and Keleti in 1978 presented kinetic results leading them to conclude that D-glyceraldehyde 3-phosphate is directly transferred from aldolase to glyceraldehyde-3-phosphate dehydrogenase without prior release to solution [45]. Orosz and Ovádi [63] later obtained additional kinetic evidence that they claimed supported such a transfer mechanism.

Weber and Bernhard drew attention to the kinetic differences between the two mechanistic alternatives shown in Figure 3 and Figure 4 [64]. In a kinetic study of the coupled reaction catalysed by glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase, they claimed to have found conclusive evidence that the metabolite 1,3-bisphosphoglycerate is directly transferred between the two enzymes in an enzyme-substrate-enzyme complex.

Bernhard and co-workers [32, 38, 65] later presented kinetic evidence leading to the conclusion that there is a channelled transfer of NADH from glyceraldehyde-3-phosphate dehydrogenase to glycerol-3-phosphate dehydrogenase and L-lactate dehydrogenase. In further studies, they found that NADH channelling occurs between dehydrogenases in general, provided that the enzymes show distinct stereospecificities (A- vs. B-side specificity) towards NADH [38, 66-68].

Kinetic evidence claimed to be indicative of a direct transfer of metabolites has been presented also for the coupled reaction involving aldolase and glycerol-3-phosphate dehydrogenase [69].

The channelling controversy

There seems to be a consensus that metabolite channelling may occur in systems involving non-dissociable bi- or multienzyme complexes [17]. The idea has been strongly questioned, however, that metabolite channelling is of significance also in systems involving dissociable complexes of glycolytic and other 'soluble' enzymes [51]. In particular, the kinetic evidence put forward to support such proposals has received strong criticism.

For example, Kvassman and Pettersson [70] found that the kinetic behaviour of the aldolase/glyceraldehyde-3-phosphate dehydrogenase system can be fully explained in terms of a free-diffusion mechanism when adequate consideration is taken to the hydration that D-glyceraldehyde 3-phosphate undergoes in aqueous solution.

Kvassman and Pettersson [71, 72] also disputed the conclusion of Weber and Bernhard [64] that there is a direct transfer of 1,3-bisphosphoglycerate from glyceraldehyde-3-phosphate dehydrogenase to phosphoglycerate kinase. Transient-state kinetic data reported by Kvassman and Pettersson [71, 72] provided evidence that the exchange of 1,3-bisphosphoglycerate between the two enzymes is consistent with a free-diffusion mechanism, and the experimentally based arguments leading Bernhard and co-workers [64] to conclude otherwise were shown to be untenable.

The proposal of Bernhard and co-workers [64] that there is a channelled transfer of NADH between glycerol-3-phosphate dehydrogenase and L-lactate dehydrogenase was challenged by Chock and Gutfreund [73], who presented transient-state kinetic data consistent with a free-diffusion mechanism of NADH transfer. Bernhard and co-workers in a rebuttal to the latter work [74] reiterated their conclusion that the transfer of NADH is channelled.

Controversial reports on the mechanism of metabolite transfer accumulated also with regard to other enzymic systems and metabolic pathways [59, 75-85]. The channelling controversy became one of the major issues in the enzymological field during the 1980's, affecting also the scientific debate in adjacent fields such as those dealing with

control analysis and metabolic regulation [17, 51, 86-93]. The outstanding scientific interest of this issue was recognised by Cornish-Bowden, who as an editor of *Journal of Theoretical Biology* took the valuable initiative of devoting an entire volume (volume **152**, 1991) of this journal to the channelling controversy in the form of a target review (written by Ovádi [11]) with commentaries from researchers in the field.

Ovádi in her review summarised the available evidence regarding channelling in various systems. Table 1 represents an extract from her review.

Table 1. A summary of the available evidence regarding channelling according to Ovádi [11].

Enzymes involved	Mechanism	References
Aldolase/Glyceraldehyde-3-phosphate dehydrogenase	Leaky channel	*pro*: [45, 63] *contra*: [70]
Phosphoglycerate kinase/Glyceraldehyde-3-phosphate dehydrogenase	Direct transfer	*pro*: [37, 65, 94] *contra*: [71, 95]
Aldolase/Glycerol-3-phosphate dehydrogenase	Direct transfer	*pro*: [37, 96] *contra*: [73]
Glyceraldehyde-3-phosphate dehydrogenase/Alcohol dehydrogenase	Direct transfer	*pro*: [38, 97]
Glyceraldehyde-3-phosphate dehydrogenase/L-lactate dehydrogenase	Direct transfer	*pro:* [38]
Glycerol-3-phosphate dehydrogenase/L-lactate dehydrogenase	Direct transfer	*pro*: [37, 74, 98] *contra*: [73]

Proximity effects

The term 'channelling' was originally introduced to denote metabolite exchange by the direct mechanism in Figure 4, where the metabolite is transferred without prior release to the reaction medium (a perfect channel). Some authors have preferred to use the term in a wider sense to denote any situation where a metabolite is transferred from one enzyme to another without prior equilibration with the reaction medium [11, 18, 23, 42, 99]. It has been argued that if the producing and consuming enzymes are located in close proximity to each other (e.g. through formation of a bi-enzyme complex or by binding to a

cellular structure), surface diffusion may occur or local concentrations gradients may be set up which enhance the rate of transfer of the metabolite [10, 12, 51, 99-106].

Fusion proteins

Several authors have argued that metabolite channelling is likely to occur due to 'proximity effects' once two sequential enzymes are brought close together, e.g. through the dynamic or static formation of a bi-enzyme complex [9, 11, 12, 51, 101, 107, 108]. Such complex formation can be anticipated to decrease the distance between the metabolite-producing enzyme and metabolite-consuming enzymic sites, and this has been stated to offer catalytic advantages in the form of enhanced steady state and/or transient reaction rates [12, 51, 101, 109, 110].

The significance of proximity effects has been experimentally tested by kinetic studies of sequential enzymes that have been covalently fused to each other by chemical modification or genetic engineering [105, 111-116]. The transient lag time for product formation was found to be shorter for the fusion proteins than for free enzymes. This was taken to indicate that there is a channelled transfer of metabolites in the fusion proteins.

Elcock and McCammon [117, 118] found that a positive electrostatic surface exists between the two enzymic sites of malate dehydrogenase and citrate synthase, and that the inclusion of electrostatic interactions in the Brownian dynamics simulations increased the transfer efficiency. This lead them to propose that electrostatic surface diffusion accounts for a highly efficient channelling of oxaloacetate in the fusion protein.

PRESENT INVESTIGATION

This thesis deals with the hypothesis concerning metabolite channelling in systems that may form dynamic bi-enzyme complexes *in vitro*. It summarises the following papers, I-IV.

Paper I

Srivastava and Bernhard examined the transfer of NADH among metabolically central dehydrogenases with the so-called enzyme buffering method [65-67]. This method is based on the use of high concentrations of a buffering dehydrogenase (E_1) to decrease the concentration of free NADH available for the enzymic steady-state action of a second dehydrogenase (E_2) present in catalytic amounts (Figure 5). The expected decrease in concentration of NADH (and consequent inhibition of the catalytic dehydrogenase) caused by the buffering dehydrogenase was calculated using experimental estimates of the equilibrium constant for its complex formation with NADH.

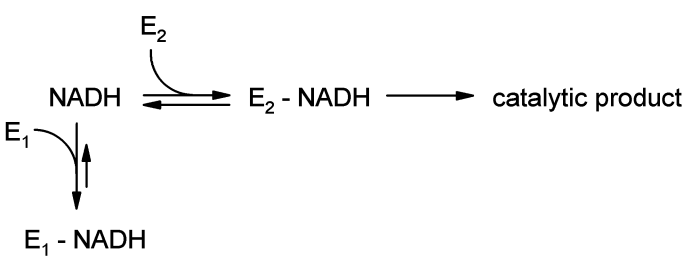

Figure 5. Reaction scheme showing the enzyme buffering method.

By this approach, Srivastava and Bernhard found that the inhibition caused by the buffering enzyme was consistent with its coenzyme binding capacity when the two dehydrogenases exhibited the same (A-side or B-side) chiral coenzyme specificity. With pairs of dehydrogenases of distinct chiral specificity, however, the inhibition caused by the buffering enzyme was found to be less than expected from its estimated coenzyme binding capacity. This was taken to

indicate that the complex of NADH with the buffering enzyme might substitute for free NADH in the reaction catalysed by the second dehydrogenase, such that there is a direct (channelled) transfer of NADH from one enzyme to the other.

The proposal that NADH is directly transferred between dehydrogenases of distinct chiral coenzyme specificity initiated a vivid debate. Chock and Gutfreund [73] challenged the proposal in a report leading them to conclude that the transfer of NADH between glycerol-3-phosphate dehydrogenase (B-side specific) and L-lactate dehydrogenase (A-side specific) occurs by free-diffusion. In a rebuttal to that report, Bernhard and co-workers [74] maintained that the transfer of NADH is channelled, but Gutfreund and co-workers responded to the rebuttal by performing a transient-state kinetic study providing additional evidence for a free-diffusion mechanism of NADH transfer between glycerol-3-phosphate dehydrogenase and L-lactate dehydrogenase [7, 119].

The criticism raised by Chock and Gutfreund [73] was supported by Martínez Arias and Pettersson [120], who found that the transfer of NADH between glyceraldehyde-3-phosphate dehydrogenase (B-side specific) and alcohol dehydrogenase (A-side specific) is consistent with a free-diffusion mechanism with no detectable contributions from a channelled NADH transfer. However, reports from other research groups also appeared [10, 39, 41, 121-123], that gave support to the steady-state kinetic observations made by Bernhard and co-workers.

We therefore decided to carry out some crucial steady-state and transient-state kinetic experiments aiming at testing if there is any general difference in the mechanism of NADH transfer between dehydrogenases depending on the chiral coenzyme specificity of the enzymes.

In a first series of transient-state kinetic experiments, we examined the transfer of NADH from alcohol dehydrogenase (A-side specific) to other dehydrogenases (A-side as well as B-side specific), chosen among those investigated in the enzyme buffering experiments reported by Srivastava and Bernhard [66]. Alcohol dehydrogenase was chosen as the donor enzyme because of the slow rate of dissociation of its binary complex with NADH (k_{off} = 5 s^{-1} [120]). A

rate-limiting contribution from this slow step should be easy to detect and to distinguish from the high rates (150 – 250 s^{-1}) proposed by Srivastava and Bernhard to govern direct NADH transfer between dehydrogenases with opposite stereospecificity [65]. The results in Paper I establish that NADH transfer from alcohol dehydrogenase to all the acceptor enzymes listed in Table 2 indeed is rate-limited by a process with a first-order rate constant of about 5 s^{-1}.

Table 2. Limiting values of first-order rate constants for the transfer of NADH from alcohol dehydrogenase to other dehydrogenases.

Acceptor enzyme (E_2)	Specificity	Concentration [μM]	Rate constant [s^{-1}]
Glycerol-3-phosphate dehydrogenase	B-side	50	5 (±1)
Glyceraldehyde-3-phosphate dehydrogenase	B-side		
Rabbit muscle		140	7 (±2)
Halibut muscle [120]		75	5 (±1)
L-lactate dehydrogenase	A-side	47	6 (±2)
Malate dehydrogenase	A-side	180	10 (±2)

This provides clear evidence that, irrespective of the stereospecificity of the acceptor enzyme, NADH dissociation from the donor enzyme represents an obligatory step in the process of coenzyme transfer, i.e. that NADH is transferred by the free-diffusion mechanism in Figure 6.

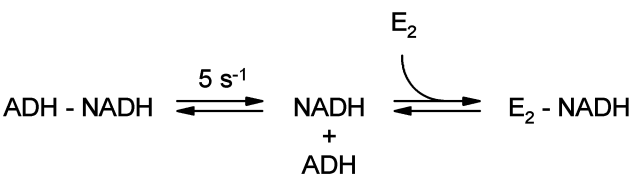

Figure 6. The transfer of NADH from alcohol dehydrogenase (ADH) to other dehydrogenases (E_2) is rate limited by NADH dissociation from alcohol dehydrogenase.

In a second series of steady-state kinetic experiments, the enzyme buffering method was used to examine the effect of glyceraldehyde-3-phosphate dehydrogenase (B-side specific) on the activity of glycerol-3-phosphate dehydrogenase (B-side specific). As previously found for an A-side specific acceptor enzyme [67, 120], the inhibitory effect of the buffering enzyme was lower than expected according to

calculations based on the NADH dissociation constant of K_d = 1.8 μM reported by Srivastava and Bernhard [67]. The discrepancy that the latter authors attributed to NADH channelling is present irrespective of the stereospecificity of the catalytic enzyme, but derives from the use of an inadequate K_d value that refers to NADH binding to the monomeric form of the buffering enzyme. At the enzyme concentrations used in the buffering experiments, glyceraldehyde-3-phosphate dehydrogenase is predominantly tetrameric, however, with a NADH dissociation constant of the order of 10 μM [120], which is in satisfactory agreement with the K_d value reported by Ovádi [61]. Basing the calculations on the latter K_d value, the inhibition caused by D-glyceraldehyde 3-phosphate was found to correspond well to that expected from the NADH binding capacity of the enzyme.

The results in Paper I therefore led us to conclude that NADH transfer among the examined dehydrogenases occurs by the free-diffusion mechanism in Figure 6 irrespective of the stereospecificity of the enzymes.

Paper II

Gutfreund and Chock already in 1991 concluded that proposals of substrate channelling had failed tests in systems involving glycolytic enzymes [124]. Ovádi, however, maintained as late as 1997 that channelling occurs among glycolytic enzymes [2], giving reference to two specific cases involving aldolase as one of the enzymic components. One of these cases concerns the transfer of dihydroxyacetone-phosphate from aldolase to glycerol-3-phosphate dehydrogenase (Figure 7), proposed to be channelled on the basis of kinetic data indicating that the transient rate of dihydroxyacetone-phosphate reduction is lower with the free substrate than with substrate produced through the action of aldolase on β-D-fructose 1,6-bisphosphate [96].

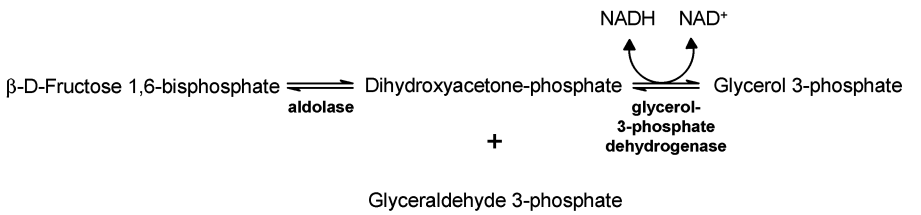

Figure 7. Reaction scheme for conversion of β-D-fructose 1,6-bisphosphate to glycerol 3-phosphate.

The second case concerns the transfer of D-glyceraldehyde 3-phosphate from aldolase to glyceraldehyde-3-phosphate dehydrogenase (Figure 8), which was claimed to be channelled on the basis of similar kinetic observations and the results of isotope dilution experiments [63].

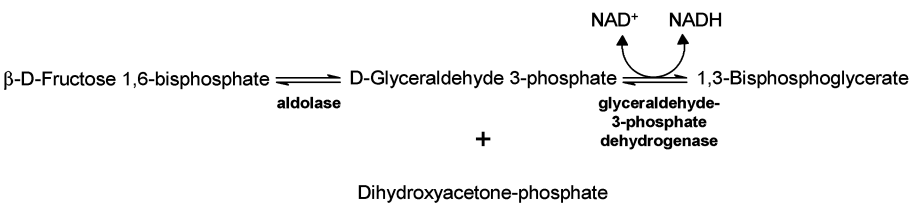

Figure 8. Reaction scheme for conversion of fructose 1,6-bisphosphate to 1,3-bisphosphoglycerate.

The reaction systems in Figure 7 and Figure 8 have one important mechanistic characteristic in common: the intermediate produced by aldolase (dihydroxyacetone-phosphate and D-glyceraldehyde 3-phosphate, respectively) undergoes reversible non-enzymatic hydration in aqueous solution to yield a geminal diol that does not act as a substrate for the subsequent dehydrogenase reaction [125, 126]. Pettersson and co-workers [70, 127] drew attention to the kinetic consequences of this non-enzymic hydration step with regard to the coupled aldolase/glyceraldehyde-3-phosphate dehydrogenase reaction, showing that the transient rate behaviour of the reaction actually is fully consistent with a free-diffusion mechanism of D-glyceraldehyde 3-phosphate transfer.

The investigation in Paper II was initiated to examine the consequences of the non-enzymic substrate hydration step with regard

to the unchallenged proposal of a channelled transfer of dihydroxyacetone-phosphate in the reaction system in Figure 7, just as to the unchallenged isotope dilution data taken to be indicative of a channelled transfer of glyceraldehyde-3-phosphate in the reaction system in Figure 8. In the former case, kinetic measurements were performed to obtain estimates of the rate constants for hydration and dehydration of dihydroxyacetone-phosphate. Using these rate constant estimates, the rate behaviour expected for dihydroxyacetone-phosphate transfer by free-diffusion was calculated and found to agree with that observed experimentally. As concerns the reaction shown in Figure 8, relationships were derived to include consideration of the hydration step for calculation of the isotope effect expected for D-glyceraldehyde 3-phosphate transfer by free-diffusion. The isotope effect thus calculated for the experiment reported by Ovádi [63] agreed excellently with that observed experimentally (Table 3).

Table 3. Isotope dilution factors determined by different methods.

Calculated by using our estimates of rate constants.	Determined experimentally by Ovádi [63].	Calculated for a free-diffusion mechanism using Ovádi's [63] values.
0.75	0.75	0.76

The results in Paper II, therefore, led us to conclude that experimental data so far reported for reactions in Figure 7 and Figure 8 are fully consistent with a free-diffusion mechanism of metabolite transfer. Jointly with the results in Paper I, they make it justified to fully support the statement of Gutfreund and Chock [124] that no tenable evidence is available for metabolite channelling in systems involving glycolytic enzymes.

Paper III

Malate dehydrogenase and citrate (*si*)-synthase catalyse consecutive reactions of the citric acid cycle (Figure 9). A fusion protein of the yeast form of these two enzymes was biosynthesised by Bülow and co-workers [115] and examined kinetically to test if there are any proximity effects in the transfer of oxaloacetate from malate

dehydrogenase to citrate synthase. They found the transient lag time for product formation in the coupled reaction catalysed by the two enzymes to be shorter for the fusion protein than for the free enzymes. This was taken to be indicative of a channelled transfer of oxaloacetate in the fusion protein. The interpretation was supported by the observation that inhibition of the coupled reaction by aspartate transaminase (which competes with citrate (*si*)-synthase for oxaloacetate) was less with the fusion protein than with the free enzymes.

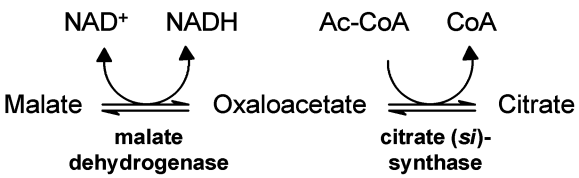

Figure 9. Reaction scheme for conversion of malate to citrate.

Similar results were later obtained by Srere *et. al.* with a fusion protein of the porcine form of the enzymes [128]. Brownian dynamics simulations performed with a hypothetical model of the fusion protein led Elcock and McCammon [118] to propose that the channelling of oxaloacetate is due to electrostatic surface diffusion guiding oxaloacetate from one enzymic site to the other. Theoretical calculations based on this idea [117] were found to fit well to the channelling efficiencies indicated by the kinetic results of Bülow and co-workers.

Unfortunately, the kinetic approach chosen by Bülow and co-workers is open to serious criticism in one fundamental respect: kinetic parameter values for malate dehydrogenase and citrate (*si*)-synthase activities of the fusion protein differ somewhat from those of the free enzymes. The free enzymes, therefore, cannot be unambiguously used as models for prediction of the expected free-diffusion behaviour of the fusion protein. Hence, it is uncertain to what extent the observed differences in rate behaviour of the fusion protein and the free enzymes actually are attributable to deviations from a free-diffusion behaviour of the fusion protein.

The investigation in Paper III was undertaken to eliminate this uncertainty and was performed in collaboration with Bülow and a member of his research group. We decided to use a new and more direct approach where all predictions on the expected free-diffusion behaviour of the fusion protein were based upon the kinetic properties of the fusion protein itself. Kinetic parameter values were determined for the citrate (*si*)-synthase and malate dehydrogenase activities of the fusion protein, as well as for the inhibitory effect of oxaloacetate on the latter activity. Using these parameter values, progress curves calculated for the coupled free-diffusion reaction in Figure 9 were found to account (within experimental precision) for those observed experimentally in studies of the coupled reaction catalysed by the fusion protein (Figure 10). The inhibitory effect of aspartate transaminase on the coupled reaction was similarly shown to agree with that expected for the free-diffusion mechanism in Figure 9.

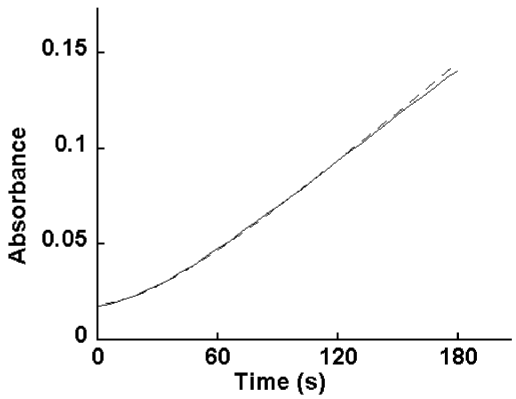

Figure 10. Time-course for the fusion-protein-catalysed coupled reaction. The experimental trace is represented by a plain curve, and the dashed curve indicates the absorbance changes expected for a free diffusion mechanism.

The results in Paper III thus led us to conclude that the rate behaviour of the fusion protein is fully consistent with a free-diffusion mechanism and fails to provide evidence for any significant channelling of oxaloacetate.

Paper IV

The significance of proximity effects in the transfer of metabolites between enzymes has been kinetically probed with several fusion proteins, invariably by the approach of using the behaviour of the free enzymes as a model for the expected free-diffusion behaviour of the fusion protein. Since the usefulness of that approach is rendered questionable by the results in Paper III, we decided to re-examine yet another fusion protein reported to exhibit kinetic properties indicative of metabolite channelling.

The fusion protein chosen was that between β-galactosidase and galactose 1-dehydrogenase, which catalyses a coupled reaction converting lactose into galactonolactone with intermediate formation of D-galactose (Figure 11). Examining the kinetics of enzymic catalysis of this coupled reaction, Ljungcrantz *et. al.* [114] found that the transient lag times for product formation were shorter with the fusion proteins than with an adequately composed mixture of the free enzymes. This was taken to be indicative of a channelled transfer of galactose, and the system appears to be of particular interest because the proposed channelling was reported to manifest itself also in increased steady-state rates of product formation.

Our examination of the coupled reaction catalysed by the fusion protein of β-galactosidase and galactose 1-dehydrogenase was based on the same approach as in Paper III.

In a first series of experiments, kinetic parameter values were determined for the β-galactosidase and galactose 1-dehydrogenase activities of the fusion protein itself. Consideration was taken to the complication that D-galactose is present in two isomeric forms (α and β), only one of which (the β-form) is produced and consumed by the fusion protein. Rate constants for the interconversion of the α- and β-forms of D-galactose were determined by standard polarimetric methods, and the corresponding reaction step was included in the reaction scheme considered (Figure 11).

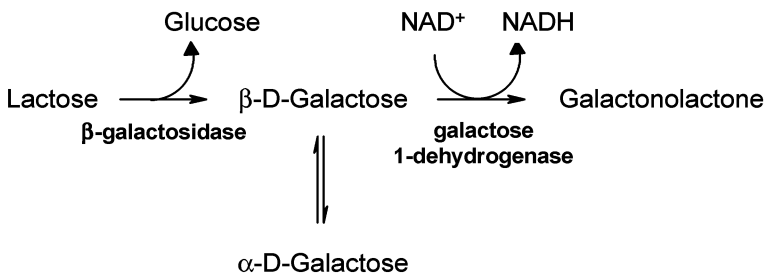

Figure 11. Reaction scheme for conversion of lactose to galactonolactone.

In a second series of experiments, progress curves for product formation in the coupled reaction catalysed by the fusion protein were examined and compared to those calculated theoretically for the reaction scheme in Figure 11, with the assumption that transfer of D-galactose occurs by free-diffusion. As illustrated by the results in Figure 12, we found that the experimentally observed time-course of product formation can be most satisfactorily accounted for in terms of a free-diffusion mechanism of D-galactose transfer. Our results lend no support to the proposal that there is a channelled transfer of D-galactose in the coupled reaction catalysed by the fusion protein.

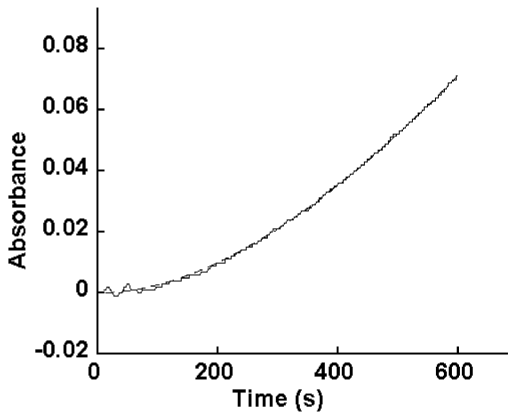

Figure 12. Time-course of the coupled reaction catalysed by the fusion protein. The experimental trace is represented by a plain curve, and the dashed curve indicates the absorbance changes expected for a free diffusion mechanism.

This was the second time the application of strict kinetic tests failed to confirm claims for detection of a channelled transfer of metabolites in coupled reactions catalysed by fusion proteins. Since such claims invariably have been based on the approach of using the free enzymes as a model for expected free-diffusion rate behaviour of the fusion proteins, we issued a warning that the inferences drawn on basis of the latter approach cannot be trusted.

More important, the results in Paper III and IV provide clear evidence that bringing two consecutive enzymes together in a fusion protein (or consequently, in a bi-enzyme complex through adjacent attachment to cell structures) does not lead to any significant kinetic advantages attributable to proximity effects. Such effects, if existing, are obviously too small to be detected at the level of precision of standard kinetic measurements. This conclusion is consistent with the results of Brownian dynamics simulation studies [118, 129].

CONCLUSIONS

As was mentioned in the Background, the biological significance of so-called dynamic metabolite channelling has been a matter of great dispute, particularly concerning systems involving glycolytic enzymes [2, 9, 11, 41]. The results in Paper I and II lead us to fully support the conclusion of Gutfreund and Chock [124] that no tenable evidence is available for metabolite channelling in systems involving glycolytic enzymes. The results in Paper III and IV provide clear evidence that channelling due to proximity effects either is absent or of negligible quantitative significance.

Proponents of the channelling idea have emphasized the kinetic advantages claimed to characterise channelling by direct metabolite transfer [11]. As pointed out by Kvassman and Pettersson [72] and supported by subsequent theoretical analyses [41, 130], such advantages might be associated with static channelling, but not likely with dynamic channelling. Metabolite transfer by the free-diffusion mechanism shown in Figure 3 occurs through a diffusional transport of the metabolite alone from the producing enzyme to the consuming enzyme. Metabolite transfer by the dynamic channelling mechanism shown in Figure 4 occurs through a diffusional transport of the complex of the metabolite with the producing enzyme to the consuming enzyme. There is no reason to believe that the diffusional transport of a macromolecular complex should proceed at a higher rate than the diffusional transport of a low-molecular-weight metabolite. On the contrary, the available evidence would seem to establish that diffusional processes involving macromolecules are strongly disfavoured in the crowded milieu of a cell cytosol [9, 55, 131, 132].

Biocatalysts such as enzymes have primarily evolved in response to a selective pressure in the direction of increased metabolic reaction fluxes. The absence of kinetic advantages of metabolite transfer by a mechanism of dynamic channelling therefore may be a main reason why no such mechanistic cases have been detected during several decades of extensive search for them in biological systems.

POPULÄRVETENSKAPLIG SAMMANFATTNING PÅ SVENSKA

I vår kropp står ämnesomsättningen för nedbrytning av mat till kemiska ämnen som är nödvändiga för vår energiförsörjning, kroppsuppbyggnad och fortplantning. Varje cell i varje organism tillverkar eller tar upp små molekyler, som de sedan sätter ihop till stora molekyler. Samtidigt som detta sker så bryter cellerna ner molekyler som har blivit tillverkade. Ur detta får vi ett komplicerat nätverk av metaboliska reaktioner för framställning eller nedbrytning av ämnen som behövs för förnyelse av celler och organ. Reaktionerna som har hand om den viktiga uppgiften att få celler eller multicellulära organismer att reproducera sig själva. De metaboliska reaktioner som medverkar i syntes eller nedbrytning av specifika ämnen kallas vanligtvis en metabolisk väg.

En central metabolisk väg är den glykolytiska, som omvandlar glukos till pyruvat genom en serie av enzymkatalyserade reaktioner. Glykolysen sker i alla levande celler. Den förser cellen och organismen med energi både under anaeroba (= utan syre) och aeroba (= med syre) förhållanden. Glykolytiska mellanprodukter deltar i en mängd andra metaboliska vägar som har stor betydelse för ämnesomsättningen, bland annat bildande av fett samt i gröna växter koldioxid och kolhydrater. I aeroba organismer omvandlas den glykolytiska produkten pyruvat till acetyl-CoA, en viktig metabolit som går in i citronsyracykeln och därifrån länkas vidare till en mängd olika biosyntetiska processer.

Gemensamt för alla metaboliska vägar är att de innehåller sekvenser av enzymkatalyserade reaktioner. Produkten från ett enzym är substrat till en eller flera på varandra följande reaktioner. Därför måste denna produkt överföras från det producerande enzymet till det konsumerande. Mekanismen bakom en sådan metabolitförflyttning är av avgörande betydelse för hur metaboliska vägar beter sig och regleras.

Det finns två väsentligt olika sätt som en metabolit kan överföras mellan enzymer i metaboliska vägar. Om enzymen inte kan bilda komplex med varandra, måste metaboliten först frigöras från det producerande enzymet och sedan förflyttas till det konsumerande enzymet via reaktionslösningen genom fri diffusion. Om de två sekventiella enzymen är kapabla att bilda komplex med varandra så finns det en alternativ metabolitöverföringsmekanism som kan vara gångbar. Metaboliten kan då överföras direkt mellan det producerande enzymet och det konsumerande, utan att metaboliten frigörs ut i reaktionslösningen. Termen kanalisering har myntats för att referera till en sådan direkt metabolitöverföring. Att fastställa vilken av dessa två överföringsmekanismer som (generellt eller i enskilda fall) förekommer är av stor betydelse för vår förståelse av enzymers samverkan och metaboliska processers beteende och reglering.

Denna avhandling sammanfattar fyra stycken studier som handlar om hypotesen att metaboliter kan kanaliseras i system som kan bilda bienzymkomplex.

Vi har utfört några avgörande kinetiska experiment för att utröna om det finns någon skillnad i överföringsmekanismen av NADH mellan dehydrogenaser beroende av den kirala ko-enzym specificiteten hos enzymen. Vidare undersökte vi den påstådda kanaliseringen av dihydroxyacetonfosfat mellan aldolas och glycerol-3-fosfatdehydrogenas och även de isotoputspädningsdata som påstås svara mot en kanaliserad överföring av glyceraldehyd-3-fosfat mellan aldolas och glyceraldehyd-3-fosfatdehydrogenas. I våra undersökningar av de kopplade reaktionerna katalyserade av fusionsproteinen malatdehydrogenase och citratsyntas samt β-galaktosidas och galaktosdehydrogenas använde vi en ny teoretisk modell som bygger på den förväntade fria diffusionsmekanismens uppförande på fusionsproteinet då alla kinetiska egenskaper baseras på fusionsproteinet självt.

Våra resultat ledde till slutsatsen att det inte finns några hållbara bevis för metabolit kanalisering i system som innehåller glykolytiska enzym. Vi fann också att kanalisering genom så kallade närhetseffekter är av försumbar betydelse.

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Mechanism of NADH transfer among dehydrogenases

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Abstract

Steady-state and transient-state kinetic experiments have been performed to test the proposal that there is a direct (channelled) transfer of NADH from one dehydrogenase to another if the two enzymes exhibit distinct chiral coenzyme specificity (A-side vs. B-side). The results lend no support to this proposal, but are fully consistent with a free-diffusion mechanism of NADH transfer irrespective of the chiral specificity of the enzymes.

Keywords: Enzyme kinetics; Channelling; Dehydrogenase

1. Introduction

Srivastava and Bernhard [1–3] in a series of investigations have examined the mechanism of NADH transfer among metabolically central dehydrogenases by the so-called enzyme buffering method. This method is based on the use of high concentrations of a buffering dehydrogenase to decrease the concentration of free NADH available for the enzymic action of a second dehydrogenase present in catalytic amounts. They found with certain pairs of dehydrogenases that the inhibitory effect of the buffering enzyme was lower than expected from its NADH binding capacity. This was taken to indicate that the complex of NADH with the buffering enzyme may substitute for free NADH in the reaction catalysed by the second dehydrogenase, such that there is a direct (channelled) transfer of NADH from one enzyme to the other.

With other pairs of dehydrogenases, the inhibitory effect of the buffering enzyme was found to be consistent with its coenzyme binding capacity. The latter observation was rationalised in terms of the proposal that there is a direct transfer of NADH from one dehydrogenase to another if, and only if, the two dehydrogenases exhibit distinct (A-side vs. B-side) chiral coenzyme specificity [2]. This proposal has been challenged by Chock and Gutfreund [4], who presented evidence leading them to conclude that the kinetics of NADH transfer between α-glycerolphosphate dehydrogenase (B-side specific) and lactate dehydrogenase (A-side specific) are fully consistent with a mechanism of non-channelled NADH transfer by free diffusion. The conclusion of Chock and Gutfreund initiated a vivid debate [5–10], and the controversy would not yet seem to be considered as having been definitely settled [11,12].

The criticism raised by Chock and Gutfreund [4] received strong support by a recent study in our laboratories [13], which failed to confirm the report of Srivastava and Bernhard [1] that there is a direct

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transfer of NADH between glyceraldehyde-3-phosphate dehydrogenase (B-side specific) and alcohol dehydrogenase (A-side specific). We have now performed some crucial steady-state and transient-state kinetic experiments aiming specifically at testing if there is any general difference in the mechanism of NADH transfer between dehydrogenases depending on the chiral coenzyme specificity of the enzymes. The results provide clear evidence that no such difference exists.

2. Materials and methods

2.1. Materials

NADH (grade I) and NAD^+ (grade I) were obtained from Boehringer (Mannheim), dihydroxyacetone phosphate (the lithium salt), pyruvic acid and glyceraldehyde-3-phosphate from Sigma, and tetra sodium pyrophosphate decahydrate from Fluka Biochemica. Other reagents were of analytical grade or the highest quality commercially available.

2.2. Enzymes

Liver alcohol dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase were prepared and assayed as described previously [13]. Rabbit muscle glyceraldehyde-3-phosphate dehydrogenase, rabbit muscle α-glycerolphosphate dehydrogenase (type I), bovine heart lactate dehydrogenase (type III) and porcine heart cytoplasmic malate dehydrogenase were purchased from Sigma Chemical Co. Crystals of the suspensions of the latter three enzymes were collected by centrifugation and dissolved in 50 mM sodium pyrophosphate buffer, pH 7.5, containing 0.1 mM EDTA and 0.35 mM 2-mercaptoethanesulfonic acid.

Concentrations of the enzymes were determined as described by Srivastava and Bernhard [1,2] and are reported throughout as active-site concentrations.

2.3. Methods

All experiments were carried out at 25°C in 50 mM sodium pyrophosphate buffer, pH 7.5, containing 0.1 mM EDTA and 0.35 mM 2-mercaptoethanesulfonic acid. Enzyme solutions in this buffer were prepared immediately before use by passing the enzyme stock solutions through a HiTrap or PD-10 desalting column pre-equilibrated with the pyrophosphate buffer.

Steady-state kinetic measurements of α-glycerolphosphate dehydrogenase catalysis were made in a Shimadzu UV-160 spectrophotometer as described by Srivastava and Bernhard [2]. Expected reaction rates in such measurements were calculated as detailed previously [13]. Transient-state kinetic experiments were performed fluorimetrically in a SX-18 MV stopped-flow apparatus from Applied Photophysics, UK. Excitation was at 340 nm, the emission intensity being monitored at wavelengths above 375 nm by the use of a cut-off filter. Steady-state and transient-state kinetic parameter values were determined statistically by iterative nonlinear regression analysis of the experimental data.

3. Results

3.1. NADH transfer from alcohol dehydrogenase to α-glycerolphosphate dehydrogenase

The transfer of NADH from alcohol dehydrogenase (A-side specific donor enzyme) to α-glycerolphosphate dehydrogenase (B-side specific acceptor enzyme) was examined by stopped-flow techniques. NADH (10 μM) was preincubated in one syringe with alcohol dehydrogenase (20 μM) and mixed with an excess of α-glycerolphosphate dehydrogenase (32 μM before mixing) from the second syringe. Under such conditions, it follows from reported estimates of the equilibrium constants for NADH binding to the two enzymes [1] that the coenzyme will be predominantly (93%) in the form of its binary complex with the donor enzyme at the start of the reaction and predominantly in the form of a complex containing the acceptor enzyme at the end of the reaction.

As illustrated by the typical result in Fig. 1 (trace B), fluorescence changes associated with the transfer of NADH from alcohol dehydrogenase to α-glycerolphosphate dehydrogenase in the above reaction conformed to a single-exponential transient with a first-order rate constant of 7 ($\pm$1) s^{-1}. The ampli-

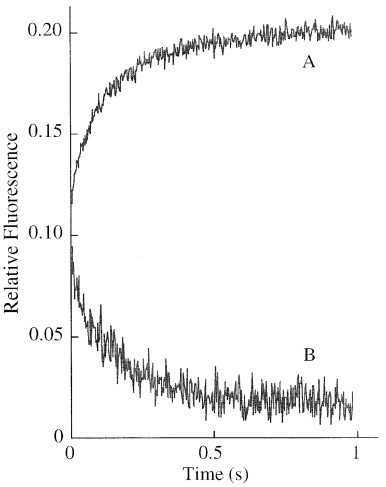

Fig. 1. Time course of NADH transfer from alcohol dehydrogenase to other dehydrogenases. Fluorescence changes recorded on mixing alcohol dehydrogenase (20 μM), preincubated with 10 μM NADH, with (A) 47 μM lactate dehydrogenase (A-side specific) and (B) 32 μM α-glycerolphosphate dehydrogenase (B-side specific) in 50 mM pyrophosphate buffer, pH 7.5.

tude of the transient agreed within experimental precision with the total fluorescence change occurring during reaction, excluding that any significant NADH transfer took place during the dead-time of the stopped-flow apparatus.

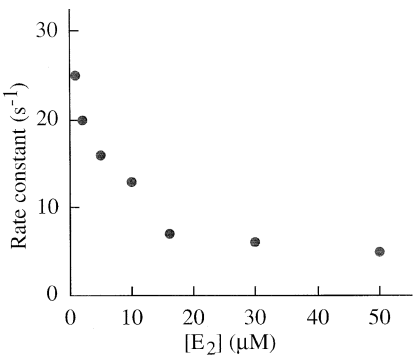

Fig. 2. Dependence of the first-order rate constant for NADH transfer from alcohol dehydrogenase to α-glycerolphosphate dehydrogenase on the concentration of the latter enzyme (E_2). Conditions as in Fig. 1, except that the E_2 concentration was varied.

Fig. 2 shows that the transient rate constant for NADH transfer in the above reaction steadily decreases with increasing concentrations of α-glycerolphoshate dehydrogenase, tending towards a saturation value of about 5 s^{-1}.

3.2. Transfer of NADH from alcohol dehydrogenase to other dehydrogenases

Similar examination of the transfer of NADH from alcohol dehydrogenase to other dehydrogenases (A-side specific as well as B-side specific) gave entirely analogous results. This is illustrated by trace A in Fig. 1, which was obtained using lactate dehydrogenase (A-side specific) as the acceptor enzyme. Table 1 lists the observed saturation values of the transient rate constants for NADH transfer, as indicated by the values obtained with the highest acceptor enzyme concentration tested. Inspection of these data shows that transient rate constants for the transfer of NADH from alcohol dehydrogenase (A-side specific) to three of the acceptor dehydrogenases tested (including the two B-side specific enzymes) tend towards saturation values agreeing within experimental precision with that of the rate constant for NADH dissociation from alcohol dehydrogenase to solution (5 s^{-1} [13]). There is strong reason to believe [10] that this value is approached also in the case of malate dehydrogenase.

3.3. Enzyme buffering experiments

The effect of high concentrations of halibut glyceraldehyde-3-phosphate dehydrogenase on the steady-state rate of α-glycerolphosphate dehydrogenase catalysis of the reduction of 0.5 mM dihydroxyacetone phosphate by 20 μM NADH was examined by monitoring the consumption of NADH spectrophotometrically. The results are given in Fig. 3. They are qualitatively consistent with the expectation that glyceraldehyde-3-phosphate dehydrogenase should act as an inhibitor of the examined catalytic reaction due to its coenzyme binding capacity and consequent lowering of the concentration of free NADH available for catalytic dihydroxyacetone phosphate reduction.

Table 1
First-order rate constants for the transfer of NADH from alcohol dehydrogenase to other dehydrogenases, determined fluorimetrically at 25°C in 50 mM pyrophosphate buffer, pH 7.5

Acceptor enzyme	Specificity	Concentration [μM]	Rate constant [s^{-1}]
α-Glycerolphosphate dehydrogenase	B-side	50	5 (±1)
Glyceraldehyde-3-phosphate dehydrogenase	B-side		
Rabbit muscle		140	7 (±2)
Halibut muscle [13]		75	5 (±1)
Lactate dehydrogenase	A-side	47	6 (±2)
Malate dehydrogenase	A-side	180	10 (±2)

Curves drawn in Fig. 3 represent the expected outcome of the experiment, as predicted by the kinetic parameter values (k_{cat} = 155 s^{-1} and K_m = 2.74 μM) reported by Srivastava and Bernhard [2] for the catalytic reduction of 0.5 mM dihydroxyacetone phosphate by NADH at pH 7.5. The dashed curve was calculated with the assumption that NADH binding to glyceraldehyde-3-phosphate dehydrogenase occurs at equivalent sites with the dissociation equilibrium constant K_d = 1.8 μM determined by Srivastava and Bernhard [1] and fails to confirm their report that the observed inhibition can be quantitatively accounted for in such terms. The dotted curve was similarly calculated using K_d = 10 μM [13] and provides a satisfactory fit to the experimental data. The best-fit value of the dissociation constant obtained by regression analysis of data in Fig. 1 was K_d = 14 μM (full curve).

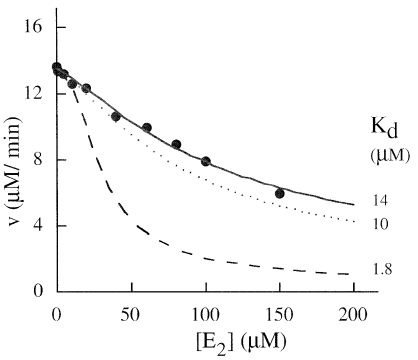

Fig. 3. Effect of glyceraldehyde-3-phosphate dehydrogenase (B-side specific) on the catalytic activity of α-glycerolphosphate dehydrogenase (B-side specific). Steady-state reaction velocities (●) determined for the reduction of dihydroxyacetone phosphate (0.5 mM) by NADH (20 μM) in the presence of 1.65 nM α-glycerolphosphate dehydrogenase and varied concentrations of halibut glyceraldehyde-3-phosphate dehydrogenase (E_2). Curves drawn show the expected outcome of the experiment as calculated using previously reported kinetic parameter values [2] and the indicated values of the equilibrium constant (K_d) for NADH dissociation from E_2.

4. Discussion

4.1. Consistency of the transient-state kinetic results with a free-diffusion mechanism

Metabolite transfer between enzymes that do not form stable multienzyme complexes is normally assumed to proceed by free diffusion of the metabolite from the donor enzyme to the acceptor enzyme. In the case of NADH transfer among dehydrogenases, this corresponds to the dissociative mechanism in Eq. (1) which prescribes that the transfer of

$$E_1{\cdot}NADH \overset{E_1}{\rightleftharpoons} NADH \overset{E_2}{\rightleftharpoons} E_2{\cdot}NADH \quad (1)$$

NADH to the acceptor enzyme involves an obligatory step of NADH dissociation from the donor enzyme (E_1) to solution. A theoretical analysis has been presented [10] which confirms the intuitive expectation that the transient rate constant for NADH transfer according to Eq. (1) should tend at high concentrations of the acceptor enzyme (E_2) towards a value agreeing with that of NADH dissociation from the donor enzyme.

The present results establish that the transient rate constant for NADH transfer from alcohol dehydroge-

nase to all dehydrogenases tested indeed shows the expected qualitative dependence on the acceptor enzyme concentration (cf. Fig. 1), apparently tending towards a value agreeing with that of the rate constant for NADH dissociation from alcohol dehydrogenase to solution (Table 1). There are no detectable contributions from other processes of coenzyme transfer, so the observed reaction step may be regarded as an obligatory one for coenzyme transfer. These observations are sufficient to establish that the examined kinetics of NADH transfer are fully consistent with the free-diffusion mechanism in the reaction scheme in Eq. (1) and fail to provide evidence for any additional mechanisms of coenzyme transfer.

This conclusion holds true irrespective of the chiral coenzyme specificity of the acceptor enzyme. There are no indications whatsoever that B-side specific acceptor enzymes behave differently from A-side specific enzymes in the process of coenzyme transfer from alcohol dehydrogenase (A-side specific). In particular, the results in Table 1 are inconsistent with the supposition of Srivastava and Bernhard [3] that transient rate constants for the transfer of NADH between dehydrogenases with opposite stereospecificity are generally of the order of 150–250 s^{-1}. Transient rate constants of that order of magnitude have been observed only in cases where NADH dissociates rapidly from the donor enzyme. Computer simulation of two representative systems have established that in such cases, also, the transfer of NADH between dehydrogenases of opposite stereospecificity is fully consistent with the reaction scheme in Eq. (1) [10,13].

4.2. Inconsistency of the transient-state kinetic results with a mechanism of direct NADH transfer

Srivastava and Bernhard [1,2] proposed from steady-state kinetic enzyme buffering experiments that NADH transfer between dehydrogenases with opposite stereospecificity occurs by the associative mechanism in Eq. (2) at high concentrations of the buffering enzyme (E_1).

$$\mathrm{E_1{\cdot}NADH} \overset{\mathrm{E_2}}{\rightleftharpoons} \mathrm{E_1{\cdot}NADH{\cdot}E_2} \overset{\mathrm{E_1}}{\rightleftharpoons} \mathrm{E_2{\cdot}NADH} \qquad (2)$$

Their reported K_m values for the claimed coenzyme activity of the $E_1 \cdot$ NADH complexes ranged from 5 to 50 μM (5–13 μM for reactions involving alcohol dehydrogenase as the donor enzyme). This means that most significant amounts of the postulated $E_1 \cdot$ NADH $\cdot E_2$ complexes should form over the enzyme concentration ranges tested in the transient-state kinetic NADH transfer measurements reported here. It seems relevant, therefore, to discuss whether or not the observations now made can be reconciled with an associative mechanism of coenzyme transfer.

The reaction scheme in Eq. (2) prescribes that NADH transfer from the donor enzyme to the acceptor enzyme involves an obligatory second-order step of acceptor enzyme binding. As intuitively expected and analytically established by Wu et al. [10], this implies that the transient rate constant for NADH transfer should increase monotonically with increasing concentrations of the acceptor enzyme. No such dependence on the acceptor enzyme concentration was observed for anyone of the systems tested in this investigation, but the transient rate of NADH transfer was invariably found to decrease with increasing concentrations of the acceptor enzyme (cf. Fig. 2 and Table 1). This leads us to conclude that the present results cannot be reconciled with the associative mechanism in Eq. (2). Similar results leading to the same conclusion were obtained by Wu et al. [10] as concerns NADH transfer between lactate dehydrogenase and α-glycerolphosphate dehydrogenase.

4.3. Inconsistency of the transient-state kinetic results with proposed extensions of the reaction scheme in Eq. (2)

The apparent lack of effect of varied high concentrations of the acceptor enzyme on the fluorimetrically observed NADH transfer rate led Srivastava and Bernhard [3] to extend the reaction scheme in Eq. (2) to include a step of isomerisation of the two-enzyme complex

$$(E_1 \cdot \mathrm{NADH}) \cdot E_2 \rightleftharpoons E_1 \cdot (E_2 \cdot \mathrm{NADH}) \qquad (3)$$

which was assumed to account for the fluorescence changes observed. The observation of Wu et al. [6] that transient NADH transfer rates are consistent with

Eq. (1) and reported estimates of rate constants in this scheme was taken by Srivastava [7] to indicate simply that the transfer of NADH from E_1 to E_2 according to Eq. (1) occurs at the same rate as the dissociation of NADH from E_1 to solution.

Several objections can be raised towards such an interpretation of the experimental data, particularly as concerns data reported in the present investigation. First, the kinetics and thermodynamics of coenzyme binding to alcohol dehydrogenase are strongly dependent on electrostatic interactions with ionizing groups in vicinity to the coenzyme binding site (including groups at the enzyme surface) and on ligand binding affecting these interactions [14]. The possibility that these interactions (and hence the rate of coenzyme dissociation) might remain essentially unaffected by the binding of a protein in vicinity to and covering the coenzyme binding site of alcohol dehydrogenase [15] would seem to be practically nil.

Second, to be consistent with the present results, the proposed extension of the reaction scheme in Eq. (2) would imply that formation of the postulated $(E_1 \cdot \mathrm{NADH}) \cdot E_2$ complex, in contrast to the subsequent isomerisation of the complex, is not associated with any detectable fluorescence changes. Again, the possibility that such might be the case is practically nil, considering the sensitivity of fluorophores to environmental factors such as hydrophobicity. The environmental changes associated with formation of an $(E_1 \cdot \mathrm{NADH}) \cdot E_2$ complex would certainly be expected to be much more extensive than those associated with a transfer of NADH within a buried region of the complex.

Finally, the extension of the reaction scheme in Eq. (2) to include the isomerisation step in Eq. (1) fails to account for the observations by us (cf. Fig. 1) and Wu et al. [10] that transient NADH transfer rates tend from higher towards lower values with increasing concentration of the acceptor enzyme. The reaction scheme will still include an obligatory forward step of E_2 association, and increasing the rate of this step by increasing the concentration of E_2 can only lead to an increased rate of NADH transfer.

An additional extension of the scheme in Eq. (2) has been considered by Srivastava [7], who argued that formation of the $E_1 \cdot \mathrm{NADH} \cdot E_2$ complex involves only a transiently formed unstable 'open' conformational state of the $E_1 \cdot \mathrm{NADH}$ complex and therefore cannot be detected in equilibrium studies of the interaction of E_1 with E_2. Formation of an $E_1 \cdot \mathrm{NADH} \cdot E_2$ complex by such a mechanism would not escape detection in the transient-state kinetic NADH transfer experiments, however, but remains inconsistent with the observed E_2 concentration dependence of the transient NADH transfer rates.

4.4. Enzyme buffering experiments

In a recent investigation [13], we could confirm the report of Srivastava and Bernhard [1] that the inhibition of alcohol dehydrogenase catalysis (A-side specific) by high concentrations of glyceraldehyde-3-phosphate dehydrogenase (B-side specific) is considerably lower than expected from the coenzyme binding capacity of the latter enzyme, as calculated using the equilibrium constant value $K_d = 1.8$ μM determined by Srivastava and Bernhard for NADH dissociation from the enzyme. The results in Fig. 3 show that there is an analogous lack of consistency between observed and calculated rates as concerns the effect of glyceraldehyde-3-phosphate dehydrogenase on the catalytic action of α-glycerolphosphate dehydrogenase (B-side specific) when calculations are based on the kinetic parameter values and K_d estimate of Srivastava and Bernhard [1,2]. Basing the calculations on a K_d value of 10 μM, the inconsistency is largely eliminated in both of the above two systems (Ref. [13] and Fig. 3).

These observations provide two main inferences. First, they fail to confirm the claim that A-side specific enzymes behave differently from B-side specific enzymes in enzyme-buffering experiments such that deviations between observations and expectations are observed only when the stereospecificity of the buffering enzyme is opposite to that of the catalytic enzyme [2]. Hence, they must lead to a rejection of the proposal that the deviations reflect stereospecificity-dependent mechanistic events.

Second, the finding that observations made in enzyme buffering experiments reported by us (Ref. [13] and Fig. 3) can be accounted for in terms of free diffusion using a K_d value of the order of 10 μM may be taken to indicate that the K_d value of 1.8 μM used by Srivastava and Bernhard [1,2] provides an overestimate of the actual coenzyme-binding capacity of glyceraldehyde-3-phosphate dehydrogenase.

Direct support for this view comes from the available information on the quaternary structure of the enzyme [16,17]. The estimate $K_d = 1.8$ μM was determined from binding studies performed at an enzyme site concentration of 1.3 μM [1], where the enzyme is mainly in the form of monomers and dimers. The enzyme-buffering experiments, however, were performed at site concentrations exceeding 10 μM, where the enzyme is almost exclusively in a tetrameric form. The K_d value reported for NADH dissociation from the tetrameric enzyme form (20 μM [17]) disagrees by one order of magnitude from that used in the calculations of Srivastava and Bernhard [1,2], but is in satisfactory agreement with the best-fit estimate (14 μM) obtained from data in Fig. 3.

The large discrepancies between observed and calculated rates reported by Srivastava and Bernhard [1,2] for experiments involving glyceraldehyde-3-phosphate dehydrogenase as a buffering enzyme, therefore, would seem to derive from their use of an inadequate K_d value for coenzyme dissociation from the enzyme. This conclusion is strongly supported by our previous demonstration [13] that attribution of the discrepancies to a direct transfer of NADH has provided kinetic parameter estimates [1] that fail to account even qualitatively for the observed effect of glyceraldehyde-3-phosphate dehydrogenase on alcohol dehydrogenase catalysis.

4.5. Concluding remarks

Wu et al. [6] in 1991 concluded that their results, together with those from our laboratory, show that the substrate channelling mechanism in Eq. (2) is not likely to be operative in the glycolytic pathway. This conclusion has not been generally accepted, but many authors have placed greater weight on the observed deviations between measured and calculated rates in enzyme buffering experiments involving dehydrogenases of opposite stereospecificity [7,9,11,12]. The possibility of miscalculations of the NADH binding capacity of the buffering enzyme in such experiments has been taken to be ruled out by the large magnitude of the deviations and by the claimed absence of any corresponding discrepancies between observed and calculated rates in systems involving enzymes with the same stereospecificity.

The present results challenge the latter arguments by failing to confirm that the catalytic activities of A-side and B-side specific dehydrogenases are differently affected by buffering concentrations of glyceraldehyde-3-phosphate dehydrogenase, and by showing that parameter values used in rate calculations involving the latter enzyme actually are likely to have been so gravely biased as to produce the reported large deviations between observations and expectations. As has been previously discussed [8,13], a variety of additional factors could lead to a bias of the calculated coenzyme binding capacity of dehydrogenases at the high protein concentrations used in enzyme buffering experiments.

The consequent weakness of the indirect steady-state kinetic evidence for NADH channelling between dehydrogenases (enzyme buffering experiments) is in striking contrast to the strength of the results obtained in direct transient-state kinetic probes of the NADH transfer mechanism, as reported here and previously [6,13]. Such results provide the unambiguous inference that NADH transfer among the examined dehydrogenases, irrespective of their stereospecificity, occurs by a free-diffusion mechanism without any detectable contributions from a mechanism of direct coenzyme transfer.

Acknowledgements

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II

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Mechanism of metabolite transfer in coupled two-enzyme reactions involving aldolase

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Transient-state kinetic experiments and analyses have been performed to examine the validity of hitherto unchallenged evidence proposed to be indicative of a channelled transfer of triose phosphates from aldolase to glyceraldehyde-3-phosphate dehydrogenase and glycerol-3-phosphate dehydrogenase. The results lend no support to such proposals, but show that the kinetic behaviour of the examined aldolase–dehydrogenase reactions is fully consistent with a free-diffusion mechanism of metabolite transfer.

Keywords: aldolase; channelling; dehydrogenase; glycolysis.

The biological significance of metabolite channelling between consecutive enzymes in metabolic pathways has been a matter of great dispute over the last two decades [1–4]. Particular attention has been paid to systems involving glycolytic enzymes and to the kinetic evidence claimed to be indicative of metabolite channelling in such systems. Much of this evidence has turned out to be untenable [5–9], but there are two outstanding cases where kinetic data interpreted in terms of metabolite channelling remain unchallenged and unexplained in other terms. One of these cases concerns the transient-state rates of dihydroxyacetone phosphate reduction observed in the coupled reaction catalysed by aldolase and glycerol-3-phosphate dehydrogenase [10], and the other concerns the isotope dilution observed for glyceraldehyde 3-phosphate in the coupled reaction catalysed by aldolase and glyceraldehyde-3-phosphate dehydrogenase [11]. These two cases were mentioned in a recent review article [4] as illustrating the occurrence of channelling in glycolysis and methods suitable for the detection of channelling.

The coupled reactions mentioned above have one important mechanistic characteristic in common: the intermediate produced by aldolase (dihydroxyacetone phosphate and glyceraldehyde 3-phosphate, respectively) undergoes reversible nonenzymic hydration in aqueous solution to yield a geminal diol that does not act as a substrate for the subsequent dehydrogenase reaction [12,13]. The present investigation draws attention to the crucial consequences of this nonenzymic hydration step with regard to the interpretation of transient reaction rates and isotope dilution observed in the coupled aldolase–dehydrogenase reactions. Evidence is presented to show that the above, hitherto unchallenged, interpretations of such data in terms of channelling are unjustified and reflect a lack of adequate consideration of the kinetic consequences of the metabolite hydration step.

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Enzymes: fructose-1,6-bisphosphate aldolase (EC 4.1.2.13); glycerol-3-phosphate dehydrogenase (EC 1.1.1.8); glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12).

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THEORY

Basic kinetic relationships for the examined reaction systems

Let us consider the irreversible conversion of a substrate S into a product P through the action of an enzyme operating under pseudo first-order conditions with an apparent first-order rate constant k. The substrate is assumed to be present in two interconvertible forms, one of which (S_{hyd}) does not interact with the enzyme. The catalytically reactive form (S) of the substrate is further assumed to be produced from an external source at a constant rate v, which may or may not equal zero.

The kinetic scheme for such a reaction system may be written as:

$$\begin{array}{ccccc} v & & k & & \\ \rightarrow & S & \rightarrow & P & \\ k_1 & \uparrow\downarrow & k_{-1} & & \\ & S_{hyd} & & & \end{array} \qquad (1)$$

and prescribes that time dependencies of reactant concentrations are given by:

$$\frac{d[S]}{dt} = v - (k + k_{-1})[S] + k_1[S_{hyd}] \qquad (2)$$

$$\frac{d[S_{hyd}]}{dt} = k_{-1}[S] - k_1[S_{hyd}] \qquad (3)$$

$$\frac{d[P]}{dt} = k[S] \qquad (4)$$

This set of linear differential equations can be analytically solved to give:

$$[P] = vt + A_1(1 - e^{-k_1 t}) + A_2(1 - e^{-k_2 t}) \qquad (5)$$

where apparent first-order rate constants λ_1 and λ_2 ($\lambda_1 \geq \lambda_2$) are the two roots of the secular equation:

$$\lambda^2 - (k + k_1 + k_{-1})\lambda + k_1 k = 0. \qquad (6)$$

If $k \gg k_1 + k_{-1}$, then:

$$\lambda_1 \approx k \quad (7)$$

$$\lambda_2 \approx k_1. \quad (8)$$

Amplitudes (A_1 and A_2) of the two transients in Eqn (5) are dependent on initial conditions according to relationships described previously in full [8]. If $v = 0$ and reactions are initiated by mixing enzyme with an equilibrium solution of S and S_{hyd} containing a total substrate concentration of c_s, then Eqn (5) approximately reduces to:

$$[P] = \frac{k_1 c_s}{k_1 + k_{-1}}(1 - e^{kt}) + \frac{k_{-1} c_s}{k_1 + k_{-1}}(1 - e^{k_1 t}) \quad (9)$$

when $k \gg k_1 + k_{-1}$. If substrate is initially absent and reactions are initiated by turning on the supply of S from the external source at a constant rate v, Eqn (5) reduces to:

$$[P] = vt - \frac{v}{k}(1 - e^{kt}) \quad (10)$$

when $k \gg k_1 \gg k_{-1}$.

EXPERIMENTAL PROCEDURES

Materials

Rabbit muscle glycerol-3-phosphate dehydrogenase (type I) and rabbit muscle fructose bisphosphate aldolase (specific activity 13 U·mg^{-1} protein) were purchased from Sigma Chemical Co. Crystals of the enzyme suspensions were collected by centrifugation and dissolved in 50 mM triethanolamine/HCl buffer, pH 8.5, containing 1 mM EDTA and 0.35 mM 2-mercaptoethanesulfonic acid. Immediately before use, enzyme solutions were passed through a HiTrap desalting column, pre-equilibrated with triethanolamine buffer. Concentrations of the enzymes were determined spectrophotometrically as described by Vértessy and Ovádi [10] and are reported throughout as active-site concentrations.

NADH (grade I) was obtained from Boehringer. Dihydroxyacetone phosphate, glyceraldehyde 3-phosphate and fructose 1.6-bisphosphate were from Sigma.

Methods

All experiments were carried out at 25 °C in 50 mM triethanolamine/HCl buffer, pH 8.5, containing 1 mM EDTA and 0.35 mM 2-mercaptoethanesulfonic acid. The activity of aldolase was assayed by the Bergmeyer method [14] using 1.9 mM fructose 1,6-bisphosphate and 0.13 mM NADH. The activity of glycerol-3-phosphate dehydrogenase was determined spectrophotometrically at 340 nm from the rate of enzymic NADH oxidation in the presence of 0.5 mM NADH and 1 mM dihydroxyacetone phosphate.

Transient-state kinetic experiments were performed in a SX-18MV stopped-flow apparatus from Applied Photophysics (UK). Reactions were monitored spectrophotometrically at 340 nm, and estimates of kinetic parameters were determined by nonlinear regression analysis based on the least-squares fitting method.

RESULTS

Kinetics of the glycerol-3-phosphate dehydrogenase reaction

Dihydroxyacetone phosphate exists in aqueous solution mainly as a mixture of the keto form (S) and its geminal-diol hydrate (S_{hyd}) [13]. Previous investigations have established that only the keto form functions as a substrate for glycerol-3-phosphate dehydrogenase [13], such that the corresponding enzymic reduction of dihydroxyacetone phosphate by saturating concentrations of NADH is adequately described by the reaction scheme in Eqn (1) when the enzyme operates under pseudo first-order conditions.

The transient-state kinetics of the above enzymic reaction were examined by stopped-flow techniques in 50 mM triethanolamine/HCl buffer, pH 8.5 (the reaction medium used in experiments reported by Vértessy and Ovádi [10]). Reactions were performed using various enzyme concentrations (0.1–25 μM) and a dihydroxyacetone concentration (5 μM) much lower than the K_m for its enzymic reduction (to ensure that the enzymic activity can be described by an apparent first-order rate constant k; [10]). As illustrated by the typical trace in Fig. 1, substrate reduction under such conditions is governed by two exponential transients that become well-separated at enzyme concentrations above 5 μM. The amplitude (≈40% of the total absorbance change) and rate constant ($\lambda_2 \approx 0.7$ s^{-1}) of the slower transient showed no major dependence on the enzyme concentration over the range 5–25 μM. The rate constant (λ_1) of the rapid transient was found to be proportional to the enzyme concentration (Fig. 2).

These observations are analogous to those made previously in transient-state kinetic studies of glycerol-3-phosphate dehydrogenase catalysis [13] and can be readily interpreted. The rapid transient reflects the action of the enzyme on substrate that is initially present in the catalytically active keto form (S). According to Eqn (9), the apparent first-order rate constant of this transient should be given by the rate constant k in Eqn (1) at high enzyme concentrations (i.e. at high values of k, which are proportional to the enzyme concentration). The data given in Fig. 2 confirm that this is the case, therefore λ_1 can be taken to provide a measure of the true catalytic reactivity of the enzyme.

The slow transient reflects that the substrate is partly present in a hydrated form (S_{hyd}), which has to be dehydrated before it can be catalytically reduced by NADH. According to Eqn (9), the approximate magnitude of the rate constants for the hydration/dehydration step can be estimated from the rate and amplitude of the slow transient. Estimates thus obtained were refined by due consideration of the exact solution to Eqn (6),

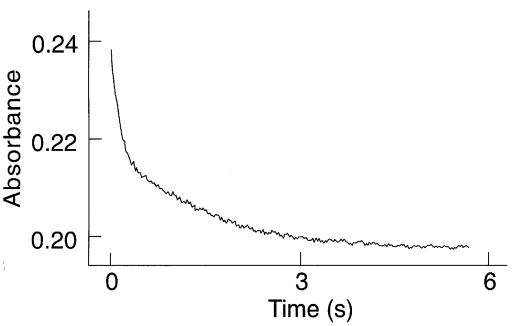

Fig. 1. Rapid and slow transients governing the interaction of glycerol-3-phosphate dehydrogenase with dihydroxyacetone phosphate in solution. Absorbance changes recorded at 340 nm and 25 °C for the reduction of 5 μM dihydroxyacetone phosphate by 50 μM NADH in 50 mM triethanolamine/HCl buffer, pH 8.5, containing 25 μM glycerol-3-phosphate dehydrogenase.

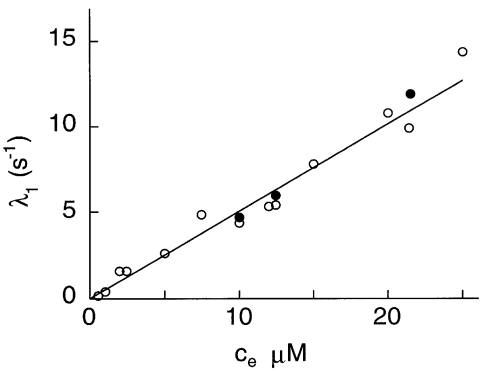

Fig. 2. Enzyme concentration dependence of the rate constant for the rapid transient. Apparent first-order rate constants (λ_1) determined from data such as those in Fig. 1 (○) or Fig. 4 (●) at different concentrations of glycerol-3-phosphate dehydrogenase.

which gave $k_1 = 0.79$ (± 0.08) s^{-1} and $k_{-1} = 0.44$ (± 0.06) s^{-1}. These values do not differ much from those determined previously under somewhat different conditions [13].

At enzyme concentrations below 5 μM, the two transients showed extensive overlap and their individual rates and amplitudes could not be determined reliably. Under the conditions of the transient-state kinetic experiments reported by Vértessy and Ovádi [10], i.e. at an enzyme concentration of 0.15 μM), reaction traces conformed satisfactorily to a single-exponential transient (Fig. 3, trace A) with an apparent first-order rate constant of 0.10 (± 0.03) s^{-1}. This agrees closely with the estimate (0.11 s^{-1}) obtained by Vértessy and Ovádi [10].

Kinetics of the coupled reaction catalysed by aldolase and glycerol-3-phosphate dehydrogenase

In a second series of experiments, the kinetics of glycerol-3-phosphate dehydrogenase catalysed reduction of dihydroxyacetone phosphate by NADH were examined in a coupled reaction where the substrate was produced at a constant rate through the action of aldolase (2 μM) on fructose 1,6-bisphosphate (1 mM). The typical trace in Fig. 4 confirms that product formation under such conditions exhibits a transient lag phase preceding the steady-state reaction phase [10]. Fitting Eqn (5) to traces obtained with 10–25 μM glycerol-3-phosphate dehydrogenase gave reliable estimates of the rate constant (λ_1) for the rapid transient governing the kinetics of the lag phase. Values thus obtained are included in Fig. 2 and do not differ significantly from the λ_1 values determined for the dehydrogenase reaction alone.

Trace B in Fig. 3 shows the time course of the coupled reaction under the corresponding experimental conditions reported by Vértessy and Ovádi [10]. During the first 25 s, the reaction rate increases steadily due to the slowness of the transient, reflecting the catalytic action of the dehydrogenase (cf. trace A). After 25 s, the reaction rate starts to decrease and eventually reaches zero, which obviously reflects consumption and final depletion of the coenzyme NADH during the catalytic reaction. In other words, NADH consumption rapidly becomes of kinetic significance and the assumption that the dehydrogenase exhibits a constant activity (expressed by the rate constant k in the scheme in Eqn 1) is certainly invalid after 25 s. This means that the reaction never reaches a steady-state of sufficient duration to permit reliable calculation of the apparent first-order rate constant for the initial transient.

Assuming that the tangent to trace B in Fig. 3 at the inflexion point at 25 s represents the steady-state rate expected for a constant value of k, we obtained an apparent first-order rate constant estimate agreeing with that (0.18 s^{-1}) reported by Vértessy and Ovádi [10]. Because this tangent must represent a underestimation of the actual steady-state rate that would have been obtained for a constant value of k, it can be assumed to lead to an overestimation of the actual value of the rate constant of the transient. The data given in Fig. 3 confirm the observations made by Vértessy and Ovádi [10], but cannot be taken as evidence for the existence of a transient-state kinetic rate discrepancy reflecting a difference in dehydrogenase activity depending on whether dihydroxyacetone phosphate is added to solution or enzymically produced through the action of aldolase on fructose 1,6-bisphosphate.

Isotope dilution in the coupled reaction catalysed by aldolase and glyceraldehyde-3-phosphate dehydrogenase

The isotope dilution experiment leading Orosz and Ovádi [11] to conclude that there is a channelled transfer of glyceraldehyde-3-phosphate from aldolase to glyceraldehyde-3-phosphate dehydrogenase was performed under such conditions that the reaction scheme in Eqn (1) applies. Reactions

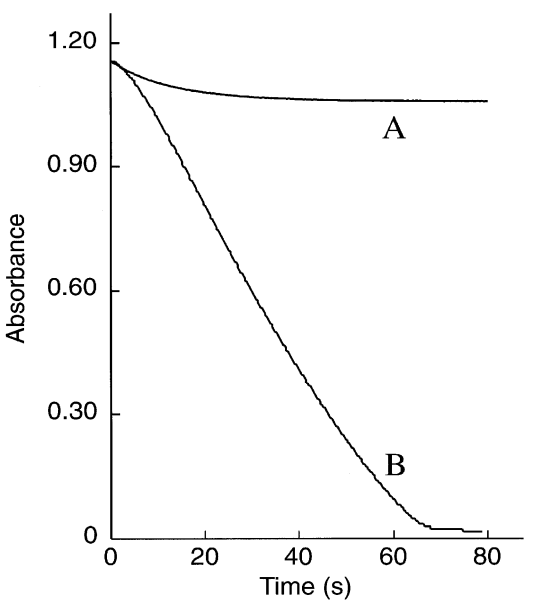

Fig. 3. Time-course of reactions catalysed by glycerol-3-phosphate dehydrogenase in experiments reported by Vértessy and Ovádi. Absorbance changes recorded at 340 nm for the interaction of 0.3 μM glycerol-3-phosphate dehydrogenase with dihydroxyacetone phosphate added to solution (16 μM; trace A) or produced through the action of 8 μM aldolase on 1 mM fructose 1,6-bisphosphate (trace B). Reactions were performed at 25 °C in 50 mM triethanolamine/HCl buffer, pH 8.5.

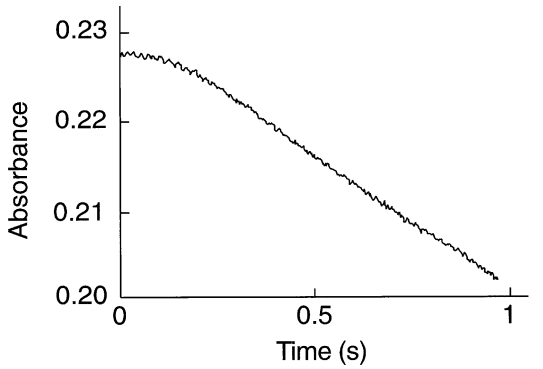

Fig. 4. Rapid transient governing the interaction of glycerol-3-phosphate dehydrogenase with dihydroxyacetone phosphate produced through the action of aldolase on fructose 1,6-bisphosphate. Conditions are as described in the legend of Fig. 1, except that 10 μM glycerol-3-phosphate dehydrogenase was used and dihydroxyacetone phosphate was produced through the action of 2 μM aldolase on 1 mM fructose 1,6-bisphosphate.

were initiated by turning on a supply of isotopically labelled substrate at a constant rate (v), and were carried out for a certain period (t) in the presence of a known total concentration (c_s) of unlabelled substrate. Reaction parameters relevant for the evaluation of their experiment are listed in Table 1.

The amount of labelled product (P*) formed during the reaction was calculated from Eqn (9) and the isotope dilution factor r was defined as:

$$r = \frac{[P*]}{[P*] + [P]} \quad (11)$$

where [P] denotes the amount of unlabelled product formed. The latter was calculated from the relationship:

$$[P] = c_s(1 - e^{-kt}) \quad (12)$$

which was derived without considering that glyceraldehyde 3-phosphate added to solution participates in a hydration/dehydration step. For reported values of rate constants of the latter step (Table 1), Eqn (9) may be written as:

$$[P] = 0.027\,c_s(1 - e^{-kt}) + 0.973\,c_s(1 - e^{-k_1 t}) \quad (13)$$

illustrating the nonapplicability of Eqn (12) and indicating that product formation will actually be governed mainly by the slower of the two transients according to the approximate relationship:

$$[P] = c_s(1 - e^{-k_1 t}). \quad (14)$$

Table 1. Reaction parameters relevant for evaluation of the isotope dilution experiment of Orosz and Ovádi [11].

Parameter	Value	Unit	Reference
t	22	s	[11]
c_s	59	μM	[11]
v	6.3	μM·s^{-1}	[11]
k	0.20	s^{-1}	[11]
k'	0.043	s^{-1}	[11]
k_1	0.039	s^{-1}	[8]
k_{-1}	1.2	s^{-1}	[8]

Using Eqn (13) or (14) and the data given in Table 1 to calculate the amount of unlabelled product formed in the experiment of Orosz and Ovádi [11], one obtains $r = 0.76$ as the theoretical value of the isotope dilution factor expected for the free-diffusion mechanism in Eqn (1). Substituting the k_1 estimate determined in our laboratories with the slightly larger value reported by Orosz and Ovádi for the rate constant of the slow transient (k' in Table 1), one obtains $r = 0.75$. These calculated values of the isotope dilution factor are in excellent agreement with the value ($r = 0.75$) determined experimentally [11].

DISCUSSION

Metabolite transfer from aldolase to glyceraldehyde-3-phosphate dehydrogenase

In 1978 Ovádi and Keleti [15] proposed that there is a channelled transfer of glyceraldehyde 3-phosphate from aldolase to glyceraldehyde-3-phosphate dehydrogenase. Their proposal was based on the observation that K_m for glyceraldehyde 3-phosphate oxidation (as indicated, for instance, by the magnitude of the transient lag-time) in the coupled reaction catalysed by these enzymes corresponds to that of the aldehyde form of the substrate in spite of the existence of the aldehyde–diol interconversion. This proposal was challenged by Kvassman *et al.* [5], who presented a theoretical analysis leading them to conclude that the transient-state kinetic observations made by Ovádi and Keleti [15] are fully consistent with the free-diffusion mechanism of metabolite transfer in Eqn (1). Because the conclusions drawn by Kvassman *et al.* were recently stated to be incorrect [4], there is reason to briefly recapitulate the grounds for them in terms of the more complete rate equations now presented for the reaction scheme in Eqn (1).

Under the experimental conditions reported by Ovádi and Keleti [15], the kinetics of the dehydrogenase-catalysed oxidation of glyceraldehyde 3-phosphate are governed by two exponential transients (Eqn 5). The faster reflects the interaction of the dehydrogenase with the aldehyde form of the substrate, and the slower the nonenzymic dehydration of the diol form of the substrate. The rapid transient will predominate in the coupled two-enzyme reaction because the substrate is produced by aldolase in the aldehyde form; this is why the lag-time constant observed by Ovádi and Keleti [15] corresponds to the K_m of the aldehyde form. The aldehyde–diol interconversion will have no readily detectable effect on the transient-state kinetics of the coupled reaction because of the low rate of the diol dehydration step ($k_1 \approx 0.01$ s^{-1}) compared with that of the rapid transient (20–100 s^{-1}).

The converse situation applies when the dehydrogenase acts upon glyceraldehyde 3-phosphate added to (and equilibrated with) solution. As noted by Orosz and Ovádi [11], the main transient governing the latter reaction is much slower than the transient observed in the coupled reaction. This was considered unexpected and was taken to provide additional evidence for a channelled transfer of glyceraldehyde 3-phosphate in the coupled reaction. In reality, the rate discrepancy observed by Orosz and Ovádi is precisely what one would expect to find for the free-diffusion mechanism in Eqn (1). According to rate constant estimates given in Table 1 for the hydration/dehydration of glyceraldehyde 3-phosphate, an equilibrium solution of the substrate will contain $< 3\%$ of the catalytically active aldehyde form. The rapid transient reflecting direct enzymic oxidation of the substrate will then show a correspondingly low amplitude (cf. Eqn 13). The predominant transient will be the slow one, which reflects that initially the substrate is

predominantly in a diol form that has to undergo dehydration before it can be enzymically dehydrogenated. Both transients have been detected by the application of adequate techniques [8] and were found to exhibit characteristics consistent with Eqn (13).

Against the above background, it becomes obvious that Eqn (12) should not be used to calculate the effect expected to be caused by nonlabelled glyceraldehyde 3-phosphate added to solution in the isotope dilution experiment of Orosz and Ovádi [11]; the assumption that substrate equilibrated with solution is dehydrogenated at the same rate as substrate produced by aldolase is neither consistent with the predictions of Eqn (1), nor supported by the results of experimental rate determinations [8,11]. If the calculations are based on Eqns (13) or (14), however, the kinetic effects of the aldehyde–diol interconversion will be adequately accounted for.

The present report on the outcome of such calculations lends no support to the conclusion of Orosz and Ovádi [11] that the experimentally observed isotope dilution is lower than expected for a free-diffusion mechanism and indicative of the presence of a leaky channel for glyceraldehyde 3-phosphate transfer. On the contrary, our demonstration that the observed isotope dilution factor is in excellent agreement with that expected for the free-diffusion mechanism in Eqn (1) must lead to the conclusion that the isotope dilution experiment of Orosz and Ovádi [11] provides exceptionally convincing evidence for the absence of significant contributions from channelling. With this observation, we consider the long-standing controversy regarding the mechanism of glyceraldehyde-3-phosphate transfer from aldolase to glyceraldehyde-3-phosphate dehydrogenase to have been definitely resolved.

Metabolite transfer from aldolase to glycerol-3-phosphate dehydrogenase

Vértessy and Ovádi [10] reported that the catalytic action of glycerol-3-phosphate dehydrogenase on dihydroxyacetone phosphate is associated with a higher transient rate constant in the coupled reaction involving aldolase than in the reaction with dehydrogenase alone. Hence they concluded that the dehydrogenase exhibits a higher activity towards aldolase-produced substrate than towards substrate added to the solution, which led them to propose that there is a channelled transfer of the substrate from aldolase to the dehydrogenase.

Because dihydroxyacetone phosphate undergoes reversible hydration in solution, the transient-state kinetics of its enzymic pseudo first-order reduction should conform to the reaction scheme in Eqn (1) and hence be governed by two exponential transients. The transient-state kinetic experiments of Vértessy and Ovádi [10] were performed at such low dehydrogenase concentrations that the pseudo first-order rate constant k for the catalytic action of the enzyme is of the same order of magnitude as the rate constants for hydration and dehydration of the substrate. Under such conditions, neither of the two transients is likely to be governed mainly by a single reaction step, and the actual relationship between the rates of observed transients and the dehydrogenase activity becomes far from obvious. In the case of the coupled two-enzyme reaction, there is even strong reason to question that reliable estimates of transient rate constants may be obtained from data such as those in Fig. 3 (trace B). Additional evidence would certainly be required to establish the existence of the claimed transient rate discrepancy [10], and to justify the proposal that such a rate discrepancy reflects a difference in dehydrogenase activity attributable to substrate channelling.

As shown previously [13] and corroborated by the results given here (Fig. 1), conditions can be found where the two transients governing dihydroxyacetone phosphate reduction by the dehydrogenase are well separated and where one of them becomes directly related to the dehydrogenase activity. The results in Fig. 2 were obtained under such conditions and provide clear evidence that the dehydrogenase exhibits the same activity towards aldolase-produced substrate as towards substrate added to the solution. Consequently, the proposal [10] that there is a channelled transfer of dihydroxyacetone phosphate from aldolase to glycerol-3-phosphate dehydrogenase has failed the tests now performed. We cannot confirm that the dehydrogenase exhibits different activity depending on whether the substrate is enzymically produced. We cannot even confirm the existence of the transient-state kinetic rate discrepancy on which this claim was based.

In 1991 Gutfreund and Chock [16] concluded that proposals of substrate channelling had failed tests in systems involving glycolytic enzymes [16]. In view of some crucial subsequent reports [7–9] and the results obtained in this investigation, we now consider such a conclusion to be fully justified.

ACKNOWLEDGEMENT

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III

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Kinetics of the coupled reaction catalysed by a fusion protein of yeast mitochondrial malate dehydrogenase and citrate synthase

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The mechanistic implications of the kinetic behaviour of a fusion protein of mitochondrial malate dehydrogenase and citrate synthase have been reanalysed in view of predictions based on experimentally determined kinetic parameter values for the dehydrogenase and synthase activities of the protein. The results show that the time-course of citrate formation from malate in the coupled reaction catalysed by the fusion protein can be most satisfactorily accounted for in terms of a free-diffusion mechanism when consideration is taken to the inhibitory effects of NADH and oxaloacetate on the malate dehydrogenase activity. The effect of aspartate aminotransferase on the coupled reaction is likewise fully consistent with that expected for a free-diffusion mechanism. It is concluded that no tenable kinetic evidence is available to support the proposal that the fusion protein catalyses citrate formation from malate by a mechanism involving channelling of the intermediate oxaloacetate.

Keywords: fusion protein; channelling; malate dehydrogenase; citrate synthase.

The biological significance of a channelled transfer of metabolites between sequentially operating enzymes in metabolic pathways has been a matter of great dispute [1–3]. Several authors have argued that metabolite channelling is likely to occur due to 'proximity effects' once two sequential enzymes are brought close together, e.g. through the dynamic or static formation of a bienzyme complex. Such complex formation can be anticipated to decrease the average distance between the metabolite-producing and metabolite-consuming enzymic sites, and this has been envisaged to offer catalytic advantages in the form of enhanced steady-state and/or transient reaction rates [4–6].

The significance of proximity effects in enzymic metabolite transfer processes has been experimentally probed by kinetic studies of sequential enzymes that have been covalently fused to each others by chemical modification or genetic engineering [7–10]. Particular attention has been paid to fusion proteins containing the sequential enzymes malate dehydrogenase and citrate synthase in the metabolically central citric acid cycle. A fusion protein of the yeast form of these enzymes was biosynthesized and examined by Lindbladh *et al.* [11], who found that the transient lag time for product formation in the coupled reaction catalysed by the two enzymes was shorter for the fusion protein than for the free enzymes. This was taken to indicate that there is a channelled transfer of the intermediate oxaloacetate in the fusion protein, an interpretation that received support by the observation that inhibition of the coupled reaction by aspartate aminotransferase (which competes with citrate synthase for oxaloacetate) was less with the fusion protein than with the free enzymes. Similar results have later been obtained with a fusion protein of the porcine form of the enzymes [12].

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Abbreviations: OAA, oxaloacetate; MDH, malate dehydrogenase; CS, citrate synthase; AAT, aspartate aminotransferase.

Enzymes: malate dehydrogenase (EC 1.1.1.37); citrate synthase (EC 4.1.3.7); aspartate aminotransferase (EC 2.6.1.1).

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Unfortunately, kinetic parameter values for the malate dehydrogenase and citrate synthase activities of the fusion proteins differ somewhat from those of the free enzymes [11,12]. This leaves some uncertainty as to whether the different kinetic behaviour of the fusion protein and the free enzymes may reflect the observed differences in kinetic parameter values rather than substrate channelling. To eliminate this uncertainty, we have now performed a more detailed kinetic study of the coupled reaction catalysed by the fusion protein containing the yeast form of the enzymes. The results do not support the previous interpretation of the reaction kinetics in terms of substrate channelling, but provide clear evidence that the kinetic behaviour of the fusion protein is fully consistent with a free-diffusion mechanism of oxaloacetate transfer between the malate dehydrogenase and citrate synthase moieties of the protein.

EXPERIMENTAL PROCEDURES

Materials

Porcine heart mitochondrial malate dehydrogenase (MDH) and aspartate aminotransferase (AAT) were purchased from Sigma Chemicals Co. Crystals of the enzyme suspensions were collected by centrifugation and dissolved in 40 mM potassium phosphate buffer, pH 8.1, containing 0.1 mM EDTA and 0.35 mM 2-mercaptoethanesulfonic acid. Immediately before use, enzyme solutions were passed through a HiTrap desalting column, pre-equilibrated with the above phosphate buffer. Active-site concentrations of the enzymes were determined spectrophotometrically at 280 nm using an absorption coefficient of 19.8 $mM^{-1} \cdot cm^{-1}$ for MDH [13] and 65.8 $mM^{-1} \cdot cm^{-1}$ for AAT [14].

The fusion protein of yeast citrate synthase and yeast mitochondrial malate dehydrogenase was prepared according to the protocol elaborated by Srere and coworkers, as detailed for their construction and purification of the fusion protein of the porcine enzymes [12]. *Escherichia coli* strain BL21, harbouring plasmid pODC29-Cit1–3-Mdh1 containing the gene for the fusion protein, was kindly provided by them. The purified

fusion protein obtained in the final gel filtration step was precipitated with 70% ammonium sulfate and stored at −20 °C. Protein concentrations during purification were determined by the Bradford method [15]. The concentration of the fusion protein was estimated from its steady-state MDH activity in the direction of malate formation, as determined in 50 mM Tris/HCl buffer (pH 7.5) using 0.1 mM oxaloacetate and 0.1 mM NADH. 1 unit of the fusion protein is defined as the amount of protein liberating 1 μmol NAD^+ per min under such conditions.

NAD^+ (grade I) was obtained from Boehringer Mannheim. Malate, oxaloacetate, acetyl-CoA, glutamate, aspartate, and dithionitrobenzoate were from Sigma Chemical Co.

Methods

All kinetic experiments were carried out at 25 °C in 40 mM potassium phosphate buffer, pH 8.1. The equilibrium constant for the oxidation of malate by NAD^+ was determined by reacting 10 mM malate with 4 mM NAD^+ in the presence of about 0.6 μM malate dehydrogenase. The amount of NADH formed after equilibration of the reaction solution was calculated from the 340 nm absorbance changes observed, using an absorption coefficient of 6200 $M^{-1}\cdot cm^{-1}$. The MDH activity of the fusion protein was similarly determined at 340 nm in reaction solutions containing 10 mM malate, 4 mM NAD^+, and 0–40 μM oxaloacetate or 0–50 μM NADH.

The citrate synthase (CS) activity of the fusion protein was determined using 0.1 mM acetyl-CoA, 1 mM dithionitrobenzoate, and varied concentrations of oxaloacetate (0–1 mM). Reactions were monitored at 412 nm, where the reaction product of CoA and dithionitrobenzoate shows maximum absorption (ε = 13 600 $M^{-1}\cdot cm^{-1}$). The conversion of malate into citrate in the coupled reaction catalysed by the MDH and CS activities of the fusion protein (about 90 $U\cdot L^{-1}$) was similarly determined at 412 nm, using 10 mM malate, 4 mM NAD^+, 0.1 mM acetyl-CoA, and 0.4 mM dithionitrobenzoate. In experiments where the effect of AAT on the coupled reaction was examined, 4 mM glutamate and 10–1000 nM AAT were added to the reaction solution.

Kinetic parameter values were determined by nonlinear regression analysis based on the least-squares fitting method. Numerical solutions of kinetic differential equations were obtained using integration procedures of the commercial computer program MATHEMATICA.

RESULTS

Preparation and general properties of the fusion protein

The fusion protein of the yeast forms of malate dehydrogenase (MDH) and citrate synthase (CS) was prepared according to the protocol elaborated by Srere and coworkers [12]. The protein was purified from 30 g wet *E. coli*, and only MDH activity was measured in the purification steps. Recovered amounts (in MDH units) of the fusion protein during purification are given in Table 1.

As previously described [11,12], the fusion protein retains malate dehydrogenase (MDH) and citrate synthase (CS) activities. These can be assayed separately, or in the coupled reaction converting malate into citrate with intermediate formation of oxaloacetate (OAA) according to the reaction scheme in Fig. 1. The concentration of free OAA in the coupled reaction can be estimated, in principle, from the inhibition of citrate formation caused by aspartate production from OAA in the presence of glutamate and aspartate aminotransferase (AAT). The corresponding reaction step is included in Fig. 1.

In this investigation, the rate behaviour of the reaction system in Fig. 1 was examined under the same conditions as those used in the kinetic experiments reported by Lindbladh *et al.* [11], i.e. at 25 °C in 40 mM phosphate buffer (pH 8.1) containing 10 mM malate, 4 mM NAD^+, 0.1 mM acetyl-CoA and, when applicable, 4 mM glutamate.

Malate dehydrogenase activity of the fusion protein

MDH catalysis of the reversible oxidation of malate by NAD^+ is known to be governed by Michaelis–Menten kinetics [16] and, at fixed concentrations of the two substrates, should conform to the steady-state rate equation

$$v_1 = \frac{\alpha(1 - q[\mathrm{OAA}][\mathrm{NADH}])}{1 + \beta_1[\mathrm{OAA}] + \beta_2[\mathrm{NADH}] + \beta_3[\mathrm{OAA}][\mathrm{NADH}]} \quad (1)$$

$$q = \frac{1}{[\mathrm{malate}][\mathrm{NAD^+}]K_{\mathrm{eq}}} \quad (2)$$

where α stands for the reaction velocity in the absence of products. β_1, β_2, and β_3 represent kinetic parameters for inhibition of the reaction by the products NADH and OAA.

Figure 2 shows initial steady-state velocities (v_1) recorded for the MDH activity of the fusion protein in the presence of 10 mM malate, 4 mM NAD^+, and varied concentrations of OAA. A fit of Eqn (1) to these data for [NADH] = 0 gave α = 87 (± 3) $nmol\cdot min^{-1}\cdot U^{-1}$and β_1 = 0.135 (± 0.011) μM^{-1}. The parameter β_2 was similarly determined from initial velocity measurements performed in

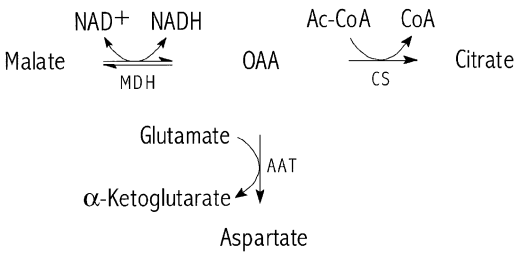

Fig. 1. Reaction system considered.

Table 1. Purification of the MDH/CS fusion protein from *E. coli* cells.

Purification step	Protein (mg)	MDH activity (U)	Specific activity ($U\cdot mg^{-1}$)
Sonication	1600	19 400	12
Nickel–agarose chromatography	17.2	1750	102
Gel filtration sephacryl S200	7.3	1550	213

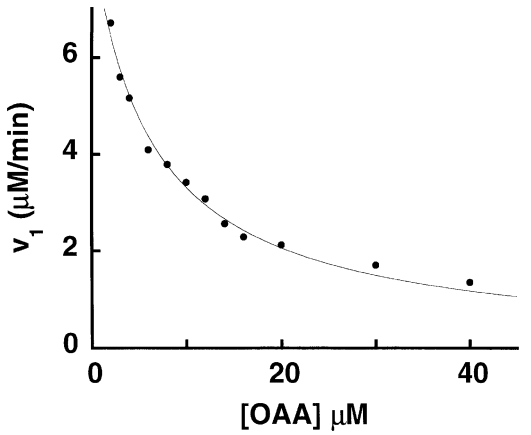

Fig. 2. MDH activity of the fusion protein. Effect of OAA on the steady-state rate (v_1) of oxidation of malate (10 mM) by NAD (4 mM) in 40 mM phosphate buffer, pH 8.1, catalysed by the fusion protein (92 U·L^{-1}).

the absence of OAA and presence of varied concentrations of NADH. This gave $\beta_2 = 0.0112$ ($\pm$ 0.0005) μM^{-1}. The parameter β_3 was found to be too small (less than 0.001 μM^{-2}) to have any detectable effect on the reaction kinetics at OAA and NADH concentrations below 50 μM; it was put equal to zero in all simulations reported below.

The denominator term containing q in Eqns (1) and (3) reflects the product inhibition contributed by the approach to equilibrium conditions. The equilibrium constant K_{eq} for the oxidation of malate by NAD^+ under the present experimental conditions was determined by measurement of the amount of NADH formed after catalytic equilibration of a reaction mixture containing high known concentrations of malate and NAD^+. This gave $K_{eq} = 9.9 \times 10^{-6}$, corresponding to $q = 2.5 \times 10^9$ M^{-2} for 10 mM malate and 4 mM NAD^+.

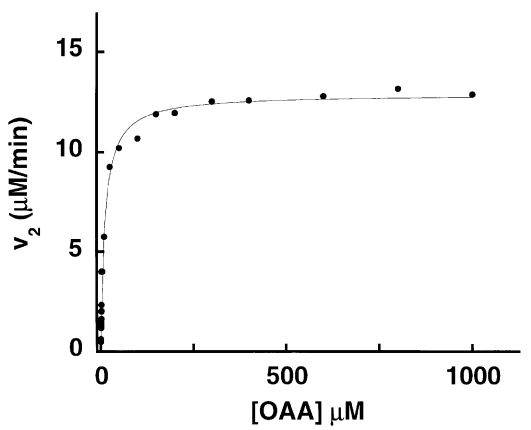

Fig. 3. CS activity of the fusion protein. Steady-state rate (v_2) of the reaction of varied concentrations of OAA with acetyl-CoA (0.1 mM) in 40 mM phosphate buffer, pH 8.1, catalysed by the fusion protein (92 U·L^{-1}).

Michaelian parameters for reactions involving OAA

Figure 3 shows initial steady-state velocities (v_2) recorded for the CS activity of the fusion protein in the presence of 0.1 mM acetyl-CoA and varied concentrations of OAA. As would be expected from previous reports [17], the Michaelis–Menten equation

$$v_2 = \frac{V[\mathrm{OAA}]}{K_m + [\mathrm{OAA}]} \quad (3)$$

could be well fitted to the experimental data, giving the best-fit estimates $V = 140$ ($\pm$ 8) nmol·min^{-1}·U^{-1} and $K_m = 11.9$ ($\pm$ 0.8) μM.

Parameters defined by the steady-state rate equation

$$v_3 = \frac{k'_{cat}[\mathrm{OAA}]c_e}{K_m + [\mathrm{OAA}]} \quad (4)$$

for the action of AAT (active site concentration c_e) on OAA in the presence of 4 mM glutamate were similarly determined. This gave $k'_{cat} = 69$ ($\pm$ 1) s^{-1} and $K'_m = 133$ ($\pm$ 6) μM.

Rate behaviour predicted by a free-diffusion mechanism

As substrates in the reactions now considered are present in large excess to the proteins, enzymic species in Fig. 1 can be assumed to be in a steady state over the time-hierarchy where the main changes in concentration of nonenzymic reactants occur. Provided that reactions are carried out for such a short period of time that the high initial concentrations of the substrates malate, NAD^+, acetyl-CoA, and glutamate remain essentially unchanged, one has to consider only the changes in concentration of the reactants OAA and NADH to account for the time-course of formation of the products citrate and CoA. The time-dependence of the latter four concentration variables can be readily expressed if the transfer of OAA occurs by a free-diffusion mechanism, and is governed by the differential equations

$$\frac{d[\mathrm{NADH}]}{dt} = v_1 \quad (5)$$

$$\frac{d[\mathrm{OAA}]}{dt} = v_1 - v_2 - v_3 \quad (6)$$

$$\frac{d[\mathrm{citrate}]}{dt} = \frac{d[\mathrm{CoA}]}{dt} = v_2 \quad (7)$$

where v_3, v_1, and v_2 denote the steady-state rates of the reactions catalysed by, respectively, AAT and the MDH and CS moieties of the fusion protein.

Kinetics of the coupled reaction

Figure 4 shows the time-course of CoA formation in the coupled reaction initiated by the addition of catalytic amounts of the fusion protein (92 U·L^{-1}) to a 40-mM phosphate buffer solution (pH 8.1) containing fixed high concentrations of the substrates malate (10 mM), NAD^+ (4 mM), and acetyl-CoA (0.1 mM). The time-dependence expected for concentration variables in this reaction (if it proceeds by free diffusion) was calculated by numerical solution of Eqns (5–7) for $v_3 = 0$ with the assumption that Eqns (1–3) apply, and using the kinetic parameter values reported in the previous sections. The results are given in Fig. 5. The calculated trajectory for the concentration of CoA, multiplied with the absorption coefficient ($\varepsilon = 13\,600$ M^{-1}·cm^{-1}) for the chromophore used to

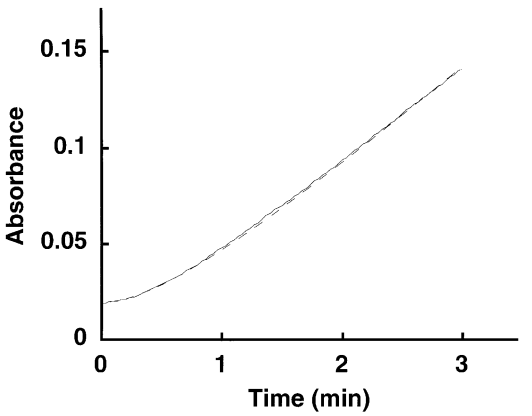

Fig. 4. Time-course of the fusion-protein-catalysed coupled reaction. Absorbance changes recorded at 412 nm for the conversion of malate into citrate and CoA. Conditions as in Fig. 2, except that reactions were performed in the presence of 0.1 mM acetyl-CoA and 0.4 mM dithionitrobenzoate. The dashed curve indicates the absorbance changes expected for a free-diffusion mechanism.

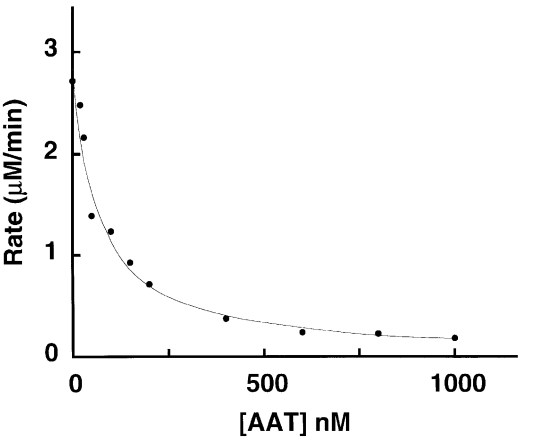

Fig. 6. Effect of AAT on the steady-state rate of the fusion-protein-catalysed coupled reaction. Conditions as in Fig. 4, except that reactions were performed in the presence of varied concentrations of AAT and that the concentration of fusion protein was 82 $U \cdot L^{-1}$. The curve drawn represents reaction rates expected for a free-diffusion mechanism.

monitor the formation of CoA experimentally, is included in Fig. 4 for comparison with the experimentally recorded trace. It accounts within experimental precision for the observed time-course of CoA formation over the entire reaction time interval examined (0–3 min).

Effect of aspartate aminotransferase

Figure 6 shows the inhibitory effect of varied concentrations of aspartate aminotransferase on the pseudo steady-state rate of the coupled reaction catalysed by the fusion protein (82 $U \cdot L^{-1}$) under the conditions of the experiment in Fig. 4. The effect expected for a free-diffusion mechanism was estimated from reaction trajectories calculated by numerical solution of Eqns (5–7) with the assumption that Eqns (1–4) apply and using the kinetic parameter values reported in the previous sections. The results are included in Fig. 6 and show that there is an excellent agreement between the calculated steady-state rates and those observed experimentally over the entire range of AAT concentrations tested (10–1000 nM).

Furthermore, the transient approach of the inhibited reactions towards the pseudo steady-state invariably could be satisfactorily accounted for in terms of a free-diffusion mechanism. This is illustrated in Fig. 7 by example of the results obtained for reactions carried out in the presence of 10, 50, and 600 nM AAT. Inspection of Fig. 7 shows that the transient lag time decreases with increasing concentrations of AAT and eventually

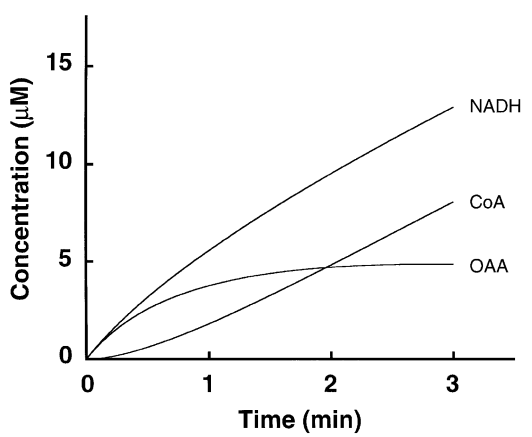

Fig. 5. Calculated time-course of reactant concentration changes in the fusion-protein-catalysed coupled reaction. Trajectories obtained by numerical solution of Eqns (1–7) using parameter values reported in the text.

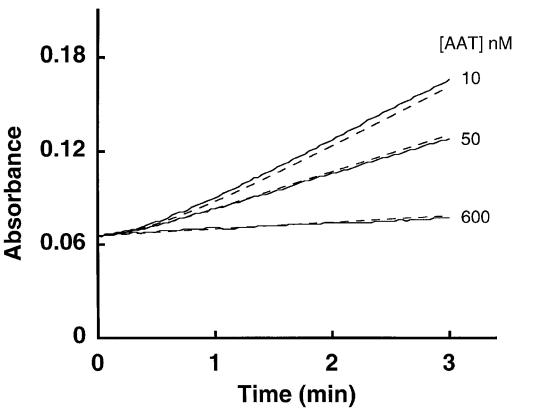

Fig. 7. Effect of AAT on the transient phase of the fusion-protein-catalysed coupled reaction. Time-course of absorbance changes recorded at 412 nm for reactions performed in the presence of 10, 50, and 600 nM AAT under the conditions in Fig. 6. Dashed curves show the absorbance changes expected for a free-diffusion mechanism, as calculated by numerical solution of Eqns (2–8).

becomes immeasurably short. This effect was not detected by Lindbladh *et al.* [11] over the more limited range of AAT concentrations tested by them.

DISCUSSION

Consistency of the kinetic data with a free-diffusion mechanism

The fusion protein of malate dehydrogenase and citrate synthase examined in this investigation is identical with that described by Lindbladh *et al.* [11], except that a His-tag was introduced at the N-terminus to facilitate purification of the protein [12]. This His-tag modification has been found not to affect the kinetic properties of the porcine variant of the fusion protein [12] and the kinetic results now obtained with the yeast variant do not differ in any essential respect from those previously reported. The main new information provided here does not concern the actual rate behaviour of the fusion protein, but the mechanistic interpretation of the rate behaviour.

Lindbladh *et al.* [11], in their analysis of the kinetics of the coupled reaction catalysed by the fusion protein, assumed that the expected time-course for a free-diffusion reaction should agree with that observed for an adequately composed mixture of the free enzymes; the differences in kinetic properties of the free enzymes and the corresponding moieties of the fusion protein were considered to be small enough to justify such an approach. In this investigation, we have taken the more direct approach of basing all predictions of the rate behaviour of the fusion protein on the kinetic properties of the fusion protein itself. This means that the expected kinetics of the reaction system in Fig. 1 can be unambiguously established by solution of Eqns (5–7) in case a free-diffusion mechanism applies.

The results in Figs 4 and 5 therefore provide the indisputable inference that the first 3 min of the observed time-course of the fusion-protein-catalysed malate to citrate conversion can be excellently accounted for with the assumption that the reaction conforms to the scheme in Fig. 1 with OAA transfer by free diffusion. This observation has direct bearing on the mechanistic problem now considered; conclusions drawn by Lindbladh *et al.* [11] were based on the behaviour of the fusion protein in the reaction phase reflecting the transient approach to steady-state conditions, and this reaction phase is well covered by the data in Fig. 4.

Arguments previously presented in support of channelling

Lindbladh *et al.* [11] argued that transient lag times for the coupled reaction catalysed by the fusion protein are indicative of OAA channelling because they are much shorter than expected for a free-diffusion mechanism. The results in Fig. 4 invalidate this argument by showing that the experimental trace during the first 3 min of the reaction differs insignificantly from that calculated for a free-diffusion mechanism. This means that the observed transient time, as well as the observed steady-state rate of the reaction, agrees with that predicted by the kinetic properties of the MDH and CS moieties of the fusion protein. As the transient time and steady-state rate both reflect the steady-state level of OAA that is reached after the transient reaction phase, data in Fig. 4 provide clear evidence that OAA attains a steady-state concentration agreeing with that expected for a free-diffusion mechanism. The data lend no support to the idea that the fusion protein acts upon a catalytically effective concentration of the intermediate OAA that is higher than the concentration of OAA in bulk solution or otherwise leading to a more rapid transfer of OAA than that obtained by free diffusion.

In view of these results, the observation [11] that the transient time for the coupled reaction is longer with the free enzymes than with the fusion protein looses relevance with regard to the mechanism of action of the fusion protein and merely indicates that a higher steady-state level of OAA is reached with the free enzymes. Such a difference in behaviour of the two systems seems to be fully consistent with the reported differences in K_m(OAA) values for the MDH and CS activities of the free and fused enzymes [11]. The net balance of these K_m value differences is in the direction that would be expected to favour a lower steady-state level of OAA in the fusion protein.

Similar considerations apply for the effect of AAT on the fusion-protein-catalysed coupled reaction. Data in Figs 6,7 establish that the experimentally determined effects of AAT on the steady-state and presteady-state time-course of the reaction are in satisfactory agreement with those expected for a free-diffusion mechanism according to the rate parameter estimates obtained for the enzymic activities involved. Consequently, there is no longer any reason to believe that the fusion protein sequesters the intermediate OAA and prevents it from being fully accessible to AAT. The observation that the coupled reaction is more strongly inhibited by AAT when catalysed by the free enzymes is rendered irrelevant by the results in Figs 6,7 and has to be explained in terms of the existing differences in kinetic parameter values for the free and fused enzymes.

General conclusions

Summing up the above discussion, the present results invalidate the main kinetic arguments that led Lindbladh *et al.* [11] to propose that there is a channelled transfer of the intermediate OAA in the coupled reaction catalysed by the fusion protein. It may now be concluded that the rate behaviour of the fusion protein of yeast MDH and CS is fully consistent with that expected for a free-diffusion mechanism of OAA transfer. This finding provides some additional inferences of general interest.

First, ambiguities obviously arise when the kinetics of coupled reactions catalysed by fusion proteins are interpreted using the rate behaviour of the free enzymes as a predictive model of the expected free-diffusion behaviour of the fused enzymes. All claims so far made for the kinetic detection of metabolite channelling in fusion proteins would seem to have been based on such an interpretational approach. There is reason to consider these claims as tentative until they have been confirmed by less ambiguous methods such as the one described in this investigation.

Second, bringing two sequential enzymes together in a fusion protein (or, by inference, in a bienzyme complex or through adjacent attachment to cell structures) is obviously not sufficient to cause any kinetically significant metabolite channelling through proximity effects. This is consistent with the conclusion drawn by Elcock & McCammon [18] from Brownian dynamics simulation studies of a tentative model structure of the fusion protein of MDH and CS, which indicated that less than 1% of the OAA molecules produced at the MDH site would be directly (i.e. without prior release to solution) transferred to the CS site through Brownian motion alone.

However, Elcock & McCammon [18] also found that a positive electrostatic surface exists between the two enzymic sites, and that the inclusion of electrostatic interactions in the Brownian dynamics simulations increased the estimated direct transfer efficiency to around 45%. This lead them to propose that electrostatic surface diffusion accounts for a highly

efficient channelling of OAA in the fusion protein, and subsequent theoretical work [19] indicated that this proposal could be reconciled with the kinetic effects that Lindbladh *et al.* [11] attributed to channelling. The present results call for a revaluation of the calculations performed by Elcock and collaborators [18,19] and lend no support to their proposal. The data in Figs 4–7 establish that if surface diffusion contributes to the transfer of OAA, then these contributions are too small to bring the MDH-produced OAA out of apparent equilibrium with bulk solution and to cause any kinetically significant deviations from the rate behaviour predicted by a free-diffusion mechanism.

Finally, it should be mentioned that Eqns (5–7) were derived with the tacit assumption that the MDH and CS moieties of the fusion protein operate independently of each other, such that kinetic parameters for the MDH activity remain unaffected by catalytic events at the CS site (and vice versa). The validity of this noncooperativity assumption is corroborated by the agreement between calculated and observed data in Figs 4–7. The only detectable kinetic consequence of the fusion of the two enzymes therefore is the previously reported modification of the kinetic parameter values for the MDH and CS activities from those of the free enzymes to those of the fusion protein [11]. These parameter modifications (and the underlying rate constant changes) could be of biological interest as an illustration of effects that might be caused by complex formation between consecutive enzymes in metabolic pathways. For example, and by analogy to what seems to be the case for the fusion protein of MDH and CS, changes in the kinetic parameter values could result in lower steady-state levels of accumulating intermediates and hence in shorter transient times for the response of the pathway to changes in the environmental conditions. Such parameter-related effects have nothing to do with metabolite channelling, however, but can be adequately described and understood in terms of metabolite transfer by free diffusion.

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IV

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Kinetics of the coupled reaction catalysed by a fusion protein of β-galactosidase and galactose dehydrogenase

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Abstract

The mechanistic implications of the kinetic behaviour of a fusion protein of β-galactosidase and galactose dehydrogenase have been analysed in view of predictions based on experimentally determined kinetic parameter values for the galactosidase and dehydrogenase activities of the protein. The results show that the time course of galactonolactone formation from lactose in the coupled reaction catalysed by the fusion protein can be most satisfactorily accounted for in terms of a free-diffusion mechanism when consideration is given to the mutarotation of the reaction intermediate galactose. It is concluded that no tenable kinetic evidence is available to support the proposal that the fusion protein catalyses galactonolactone formation from lactose by a mechanism involving channelling of galactose. © 2001 Published by Elsevier Science B.V.

Keywords: Fusion protein; Channeling; β-Galactosidase; Galactose dehydrogenase

1. Introduction

The view has been frequently expressed that the transfer of metabolites between sequentially operating enzymes in metabolic pathways is likely to be channelled due to 'proximity effects' if the sequential enzymes form a static or dynamic bienzyme complex [1–6]. Experimental evidence claimed to demonstrate the significance of proximity effects comes from kinetic studies of sequential enzymes that have been covalently fused to each other by chemical modification or genetic engineering [7–10]. The transient lag time for product formation in the coupled reactions catalysed by the sequential enzymes was found to be shorter for the fusion proteins than for the free enzymes. This was taken to indicate that there is a channelled transfer of metabolites in the fusion proteins.

In a recent reinvestigation of the kinetics of a fusion protein of citrate synthase and malate dehydrogenase [11], attention was drawn to the methodological inadequacy of basing interpretations of the rate behaviour of fusion proteins on a comparison with the free enzymes. An alternative and more reliable approach was described, according to which inferences about the rate behaviour of a fusion protein may be drawn from the kinetic properties of the fusion protein itself. Taking such an approach, it was found that previously presented kinetic evidence for a channelled transfer of oxaloacetate from malate dehydrogenase to citrate synthase is untenable.

We have now used the same new approach to test the proposal that there is a channelled transfer of galactose in the coupled reaction catalysed by a fu-

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sion protein of β-galactosidase and galactose dehydrogenase [9]. This system is of particular interest because the proposed channelling of galactose was reported to manifest itself not only in a decreased transient time, but also in an increased steady-state rate of the coupled reaction. The results now presented lend no support to such an interpretation of the experimental data, but show that the rate behaviour of the fusion protein is fully consistent with a free-diffusion mechanism of galactose transfer.

2. Materials and methods

2.1. Materials

A fusion protein of β-galactosidase and galactose dehydrogenase was prepared as detailed by Ljungcrantz et al. [9], using their *Escherichia coli* strain harbouring the plasmid expressing the protein. The purified protein was precipitated with ammonium sulphate and stored at 8°C. Protein concentrations during the purification procedures were determined by the Bradford method [12]. Concentrations of the fusion protein in the kinetic experiments were estimated from the steady-state galactose dehydrogenase activity of the protein.

NAD^+ (grade I) and galactose dehydrogenase (from *Pseudomonas fluorescens*) were obtained from Boehringer Mannheim. D(+)-Galactose (consisting mainly of α-D-galactopyranose) and lactose were from Sigma.

2.2. Methods

All kinetic experiments were carried out at 25°C in 90 mM Tris–HCl buffer (pH 7.5 or 8.5), containing 59 mM $MgCl_2$. The steady-state galactose dehydrogenase activity of the fusion protein was determined using 0.5 or 4 mM NAD^+ and various concentrations of galactose, reactions being monitored spectrophotometrically at 340 nm where the reaction product NADH shows maximum absorption ($\varepsilon = 6\ 200\ M^{-1}\ cm^{-1}$). One unit of the fusion protein is defined as the amount of protein liberating 1 μmol of NADH per minute in the above buffer (pH 8.5) with close to saturating concentrations of the two substrates (4 mM NAD^+ and 16.6 mM galactose).

The galactosidase activity of the fusion protein was similarly determined at 340 nm using 0.5 or 4 mM NAD^+, 2 or 20 mM lactose, and a large excess of galactose dehydrogenase [9]. The conversion of lactose (2 or 20 mM) to galactonolactone in the coupled reaction catalysed by the fusion protein was monitored at 340 nm, reactions being performed in the presence of 0.5 or 4 mM NAD^+.

The mutarotation of galactose was examined using an AA-10 Automatic Polarimeter from Optical Activity. The sum of rate constants k_a and k_b in Fig. 1 was assumed to equal the apparent first-order rate constant for relaxation of a freshly prepared solution of α-D-galactopyranose, as determined from the time dependence of the change in optical rotation of the solution [13]. The quotient of rate constants k_a and k_b was determined from the equilibrium value of the optical rotation, as calculated with the assumption that the specific optical rotations of pure α-galactose and β-galactose are, respectively, +151° and +53° [13].

Kinetic parameter values were determined by non-linear regression analysis based on the least-squares fitting method. Numerical integrations of differential equations were carried out using the commercial computer program MATHEMATICA.

3. Results

3.1. Reaction system considered

A fusion protein of β-galactosidase and galactose dehydrogenase was prepared according to the protocol elaborated by Ljungcrantz et al. [9]. As reported by them, the fusion protein retains β-galactosidase and galactose dehydrogenase activities. These can be assayed separately, or in the coupled reaction con-

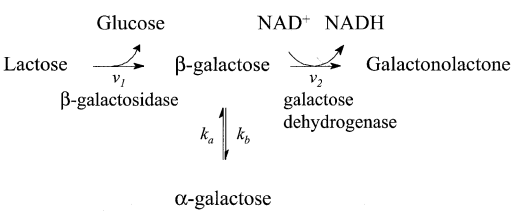

Fig. 1. Reaction system considered.

Table 1
Experimental estimates of rate constants for the mutarotation of galactose

pH	k_a (min^{-1})	k_b (min^{-1})
7.5	0.036 (±0.001)	0.010 (±0.0003)
8.5	0.125 (±0.005)	0.032 (±0.001)

verting lactose into galactonolactone with intermediate formation of galactose according to the reaction scheme in Fig. 1. Since the fusion protein is likely to show the same stereospecificity as the free enzymes, β-galactose (i.e. β-D-galactopyranose) is depicted as the immediate product of the β-galactosidase reaction [14] and as the actual substrate for the galactose dehydrogenase reaction [15]. In aqueous solution, β-galactose undergoes reversible mutarotation to form α-galactose (i.e. α-D-galactopyranose) at an appreciable rate. This reaction, therefore, is included in Fig. 1. Additional species of galactose (e.g. furanose and open-chain forms) have been detected in aqueous solution [13], but are present in too small amounts to require consideration here.

The present study of the kinetic properties of the fusion protein was carried out under the conditions of the experiments reported by Ljungcrantz et al. [9]. All reactions were performed in 90 mM Tris–HCl buffer, pH 7.5 or 8.5, containing 59 mM $MgCl_2$ and, when applicable, fixed concentrations of the substrates lactose (2 or 20 mM) and NAD^+ (0.5 or 4 mM).

3.2. Mutarotation of galactose

Rate constants for the interconversion of α- and β-galactose were determined by standard polarimetric methods as detailed in Section 2. The results are given in Table 1 and imply that an equilibrium solution of galactose in 90 mM Tris–HCl buffer contains about 80% of the β-form at pH 7.5 as well as at pH 8.5.

Control experiments established that the rates of mutarotation of galactose were not significantly affected by the presence of the fusion protein at a concentration agreeing with that used in the kinetic experiments reported below.

3.3. Galactosidase activity of the fusion protein

The steady-state galactosidase activity (v_1) of the fusion protein with 2 mM lactose was determined from the rate of NADH formation observed in the presence of 4 mM NAD^+ and a large excess of free galactose dehydrogenase [9]. The results obtained at pH 7.5 and 8.5 are given in Table 2. Control experiments established that the activity was not significantly affected by the presence of 0.1 mM glucose, 0.1 mM galactonolactone, or 2 mM galactose (as an equilibrium solution of the α- and β-forms).

The production of NADH in the above assays was linearly dependent on time and showed no detectable initial lag phase. This confirms the expectation that β-galactose is the immediate product of the galactosidase activity of the fusion protein.

3.4. Dehydrogenase activity of the fusion protein

The dehydrogenase activity of the fusion protein (v_2) was assayed at pH 7.5 and 8.5 in the presence of 4 mM NAD^+, using various concentrations of an equilibrium solution of the α- and β-forms of galactose. The reaction was found to conform to Michaelis–Menten kinetics with regard to the total galactose concentration, and the estimates of V and K_m obtained by regression analysis of the data are given in Table 2. Reaction rates observed with 50 μM galactose were not significantly affected by the presence of 2 mM lactose, 0.1 mM glucose, or 0.1 mM galactonolactone.

Table 2
Experimental estimates of kinetic parameters for the fusion protein

pH	v_1 (μM/min)	V (μM/min)	K_m (μM)	$K_{m,\beta}$ (μM)
7.5	4.52 (±0.22)	10.6 (±0.2)	180 (±14)	140
8.5	4.03 (±0.21)	14.5 (±0.5)	480 (±20)	380

Values of v_1 and V refer to a fusion protein concentration of 5.8 and 14.5 units/l at, respectively, pH 7.5 and 8.5.

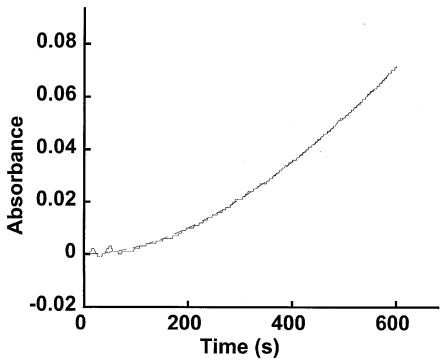

Fig. 2. Time course of the coupled reaction catalysed by the fusion protein at pH 7.5. Absorbance changes recorded at 340 nm for the production of NADH during the catalytic conversion of 2 mM lactose into galactonolactone in the presence of 4 mM NAD^+ at a fusion protein concentration of 5.8 units/l. The dashed curve shows the time course expected for a free-diffusion mechanism, as calculated from Eqs. 1–4 using data in Tables 1 and 2.

Galactose dehydrogenase from *Pseudomonas* has been reported to act exclusively on β-galactose [15]. Control experiments performed using substrate solutions initially containing about 90% of α-galactose confirmed that this is also the case with the dehydrogenase activity of the fusion protein, and failed to provide evidence for any significant inhibitory effect of the α-form of the substrate. This means that Michaelis–Menten constants ($K_{m,\beta}$) referring to the catalytically active β-form of the substrate may be calculated from the K_m values listed in Table 2 by due consideration of the actual equilibrium concentration of β-galactose, i.e. as $K_m k_a/(k_a+k_b)$. The $K_{m,\beta}$ values thus obtained from the equilibrium data in Table 1 are included in Table 2.

3.5. The coupled reaction catalysed by the fusion protein

The coupled reaction catalysed by the fusion protein was examined at pH 7.5 and 8.5 using 2 mM lactose in the presence of 4 mM NAD^+. Records of the absorbance changes reflecting formation of the end product NADH (and hence of galactonolactone) are given in Figs. 2 and 3.

3.6. Rate behaviour predicted by a free-diffusion mechanism

Since substrates in the coupled reactions now considered are present in large excess to the catalysts, the enzymically catalysed steps in Fig. 1 can be assumed to be in a steady state over the time hierarchy where the main changes in concentration of non-enzymic reactants occur. Provided that reactions are carried out for such short periods of time that the high initial concentrations of the substrates lactose and NAD^+ remain essentially unchanged, one has to consider only the changes in concentration of the reactants α-galactose (αGal) and β-galactose (βGal) to account for the time course of formation of the product NADH. The time dependence of the latter three concentration variables can be readily expressed if the transfer of β-galactose occurs by a free-diffusion mechanism and, according to Fig. 1, is governed by the differential equations

$$\frac{d[NADH]}{dt} = v_2 \quad (1)$$

$$\frac{d[\alpha Gal]}{dt} = k_b[\beta Gal] - k_a[\alpha Gal] \quad (2)$$

$$\frac{d[\beta Gal]}{dt} = v_1 - v_2 + k_a[\alpha Gal] - k_b[\beta Gal] \quad (3)$$

where v_1 and v_2 denote the steady-state rates of the reactions catalysed by, respectively, the galactosidase

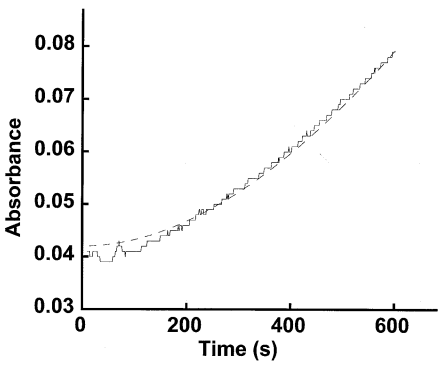

Fig. 3. Time course of the coupled reaction catalysed by the fusion protein at pH 8.5. Other conditions as in Fig. 2, except that the fusion protein concentration was 14.5 units/l.

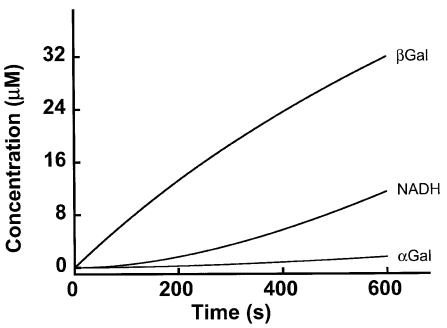

Fig. 4. Simulated trajectories for concentration variables in the coupled reaction catalysed by the fusion protein at pH 7.5. Simulations performed by numerical integration of Eqs. 1–4 using data in Tables 1 and 2 with the assumption that the initial concentrations of α-galactose, β-galactose, and NADH are zero.

and dehydrogenase moieties of the fusion protein. Since no evidence for product inhibition of the latter reactions was observed in the control experiments described above, one may assume that v_1 is constant and that v_2 is given by

$$v_2 = \frac{V[\beta\text{Gal}]}{K_{m,\beta} + [\beta\text{Gal}]} \quad (4)$$

Using the experimentally determined estimates (Tables 1 and 2) of kinetic constants in Eqs. 1–4, these equations were integrated numerically for simulation of the reactions performed at pH 7.5 and 8.5. Typical trajectories thus obtained for the concentration variables are shown in Fig. 4 by example of the reaction performed at pH 7.5. The corresponding absorbance change trajectories at the wavelength used to monitor the formation of NADH are included in Figs. 2 and 3 for comparison with the experimentally recorded traces. The results show that the calculated time course accounts within experimental precision for the experimentally observed one over the entire reaction time interval examined (0–10 min), at pH 7.5 as well as at pH 8.5.

Entirely analogous results were obtained in experiments where 20 mM lactose or a non-saturating concentration of NAD^+ (0.5 mM) was used in the coupled reaction and for determination of kinetic parameters for the enzymic activities of the fusion protein.

4. Discussion

The fusion protein of β-galactosidase and galactose dehydrogenase now examined has been prepared as described by Ljungcrantz et al. [9], using their *E. coli* strain harbouring the plasmid expressing the protein. Activity data for our fusion protein preparation agree well with those reported by Ljungcrantz et al. [9], and our kinetic studies of the protein have been performed under the experimental conditions described by them using similar concentrations of the fusion protein. Consequently, the kinetic data in Figs. 2 and 3 should be representative for those leading Ljungcrantz et al. [9] to propose that there is a channelled transfer of galactose in the coupled reaction catalysed by the fusion protein.

The latter proposal was based on the observed differences between the fusion protein and an adequately composed mixture of the free enzymes as concerns the estimated transient and steady-state rates of their catalytic conversion of lactose to galactonolactone. Such an approach for testing the mechanism of galactose transfer between the two enzymic moieties of the fusion protein seems rather problematic. Apart from the difficulties to obtain reliable experimental estimates of steady-state rates and transient times from reaction traces such as those in Figs. 1 and 2, there is strong reason to question [11] that the rate behaviour of the free enzymes serves as a useful model for the expected behaviour of the fusion protein in case a free-diffusion mechanism applies. This predictive approach becomes ambiguous as soon as the kinetic properties of the fusion protein differ from those of the free enzymes, which seems to be the case for all fusion proteins so far prepared.

In the present investigation, the more direct approach has been taken of basing all predictions as to the rate behaviour of the fusion protein on the kinetic properties of the fusion protein itself. In case a free-diffusion mechanism applies, the expected time course of the coupled reaction catalysed by the fusion protein may be calculated from Eqs. 1–4 using the kinetic data in Tables 1 and 2. The results in

Figs. 2 and 3 establish that progress curves thus calculated account satisfactorily for the experimentally observed ones. This means that the rate behaviour of the fusion protein is fully consistent with the free-diffusion mechanism in Fig. 1 and with the kinetic parameter values determined individually for the two enzymic activities of the fusion protein.

Three main conclusions can be drawn from this observation. First, the kinetics of the coupled reaction catalysed by the fusion protein of β-galactosidase and galactose dehydrogenase fail to provide evidence for any significant channelling of the reaction intermediate β-galactose. Proximity effects attributable to formation of the fusion protein are either absent or of such low strength that they cannot be detected at the level of precision of the present standard kinetic experiments.

Second, there is not even any kinetically significant cooperativity in the catalytic operation of the two enzymic moieties of the fusion protein. This follows from the agreement of the experimental progress curves in Figs. 2 and 3 with those calculated for the coupled reaction using the kinetic parameter values (Table 2) determined for the uncoupled activities of the two enzymic moieties.

Finally, this is the second time that the application of strict kinetic tests has failed to confirm claims for the detection of a channelled transfer of intermediates in coupled reactions catalysed by fusion proteins. Since such claims invariably have been based on the approach of using the free enzymes as a model for the expected rate behaviour of the fusion protein, it seems reasonable to conclude that inferences drawn on the basis of the latter approach cannot be trusted. In other words, no trustworthy evidence is presently available for a channelled transfer of intermediates in coupled reactions catalysed by fusion proteins.

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