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Mechanism of glyceraldehyde-3-phosphate transfer from aldolase to glyceraldehyde-3-phosphate dehydrogenase

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The catalytic interaction of glyceraldehyde-3-phosphate dehydrogenase with glyceraldehyde 3-phosphate has been examined by transient-state kinetic methods. The results confirm previous reports that the apparent $K_m$ for oxidative phosphorylation of glyceraldehyde 3-phosphate decreases at least 50-fold when the substrate is generated in a coupled reaction system through the action of aldolase on fructose 1,6-bisphosphate. However, the results lend no support to the proposal that glyceraldehyde 3-phosphate is directly transferred between the two enzymes without prior release to the reaction medium. A theoretical analysis is presented which shows that the kinetic behaviour of the coupled two-enzyme system is compatible in all respects tested with a free-diffusion mechanism for the transfer of glyceraldehyde 3-phosphate from the producing enzyme to the consuming one.

Ovádi and Keleti in 1978 reported that the apparent $K_m$ for the catalytic interaction of glyceraldehyde-3-phosphate dehydrogenase with glyceraldehyde 3-phosphate decreases about 50-fold when the substrate is generated in a coupled reaction system through the action of aldolase on fructose 1,6-bisphosphate [1]. This effect was attributed to reversible complex formation between the two enzymes, permitting the substrate to be directly transferred from aldolase to glyceraldehyde-3-phosphate dehydrogenase without prior release to the reaction medium. Kinetic evidence claimed to be indicative of a direct transfer of metabolites has later been presented also for coupled two-enzyme reactions involving aldolase and glyceraldehyde-3-phosphate dehydrogenase [2], as well as glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase [3], alcohol dehydrogenase [4], or various other dehydrogenases [5].

The above reports are of outstanding enzymological interest because they suggest that enzymes in the glycolytic and ancillary pathways might be structurally designed to interact with a protein-substrate complex rather than with a free substrate. They also bring into question inferences provided by such analyses invariably have been based on the tacit assumption that metabolite transfer between consecutive enzymes in the glycolytic pathway occurs via the reaction medium through free diffusion. It seems most important, therefore, to ascertain that unambiguous kinetic evidence has been presented to support the proposed existence of mechanisms for direct metabolite transfer in reaction systems involving glycolytic enzymes.

In this investigation, we have reexamined by stopped-flow techniques the kinetics of fructose-1,6-bisphosphate conversion into 1,3-bisphosphoglycerate in the coupled two-enzyme reaction catalyzed by aldolase and glyceraldehyde-3-phosphate dehydrogenase. The results are largely consistent with those reported by Ovádi and Keleti [1], but the theoretical analysis now presented calls for a reinterpretation of the kinetic effects previously attributed to a direct transfer of the reaction intermediate glyceraldehyde 3-phosphate from the producing enzyme to the consuming one. The latter effects are fully compatible with a free-diffusion mechanism for glyceraldehyde-3-phosphate transfer, but difficult to reconcile with a mechanism of direct metabolite transfer.

**EXPERIMENTAL PROCEDURES**

**Materials**

Rabbit muscle fructose bisphosphate aldolase (specific activity 14.7 IU/mg protein) was obtained from Sigma Chemical Corp. and used without further purification. Rabbit muscle glyceraldehyde-3-phosphate dehydrogenase was purchased from Boehringer (Mannheim). The crystalline suspension of the enzyme was sedimented by centrifugation, dissolved in 50 mM Tris/HCl, 1 mM EDTA, 10 mM Na$_2$HP0$_4$, pH 7.5, and gel-filtered on a Sephadex G-25 column equilibrated with the same solution. Concentrations of the enzymes were determined throughout as protein (tetramer) concentrations and were determined spectrophotometrically at 280 nm using absorption coefficients of 118 and 145 mM$^{-1}$cm$^{-1}$ for, respectively, aldolase and glyceraldehyde-3-phosphate dehydrogenase [1, 8].

NAD$^+$ and d-fructose 1,6-bisphosphate were from Boehringer. d-Glyceraldehyde 3-phosphate was obtained from Sigma as the dimethylacetal; it was prepared and assayed as specified by the company. Other chemicals were commercial preparations of reagent grade.

**Kinetic measurements**

All experiments were performed at 25°C in 50 mM Tris/HCl buffer solutions, pH 7.5, containing 1 mM EDTA.
Aldolase activity was assayed by the method of Jagannathan et al. [9] in the presence of 3 mM fructose 1,6-bisphosphate and 10 mM hydrazine sulphate.

The kinetics of oxidative phosphorylation of glyceraldehyde 3-phosphate (10-1000 μM) were examined in the presence of catalytic amounts of glyceraldehyde-3-phosphate dehydrogenase (0.1-0.8 nM and 0.2-1.0 nM for, respectively, steady-state and transient-state kinetic measurements) and saturating concentrations of NAD+ (2 mM) and inorganic orthophosphate (10 mM). NADH production during the reactions was monitored spectrophotometrically at 340 nm by stopped-flow techniques, using the rapid-reaction equipment described previously [10]. NADH concentrations were estimated using an absorption coefficient of 6200 M⁻¹·cm⁻¹ at this wavelength [11].

NADH production resulting from the catalytic interaction of glyceraldehyde-3-phosphate dehydrogenase (0.2-1.0 nM) with glyceraldehyde 3-phosphate formed through the action of aldolase (60-300 nM) on 3 mM fructose 1,6-bisphosphate in the presence of 2 mM NAD+ and 10 mM orthophosphate was monitored by stopped-flow techniques at 340 nm.

**Data processing**

All kinetic parameter values reported were determined statistically by computer-programmed non-linear regression analysis [12], using algorithms providing least-squares fits of the respective regression functions to the experimental observations.

**THEORY**

Scheme 1 shows the free-diffusion mechanism for glyceraldehyde-3-phosphate transfer discussed by Ovádi and Keleti in their kinetic study of the coupled reaction catalyzed by aldolase and glyceraldehyde-3-phosphate dehydrogenase [1]. The action of aldolase (E₁) on fructose 1,6-bisphosphate is envisaged to yield the aldehyde form (Sₐld) of glyceraldehyde 3-phosphate as a primary reaction product [13] at a rate v₁ which can be considered as constant when nearly saturating concentrations of substrate are used. The subsequent interaction of Sₐld with NAD⁺ and orthophosphate, catalyzed by glyceraldehyde-3-phosphate dehydrogenase (E₂), is assumed to result in NADH production at a rate v₂ conforming [1, 14] to the Michaelis-Menten relationship

\[
    v₂ = \frac{k_{\text{cat}E₂}[S_{\text{ald}}]}{K_m + [S_{\text{ald}}]}
\]

where \(c_{E₂}\) denotes the total concentration of glyceraldehyde-3-phosphate dehydrogenase. Since the aldehyde form of glyceraldehyde 3-phosphate undergoes reversible non-enzymatic hydration with formation of a geminal diol (Sₐdol) in aqueous solution [15], the corresponding reaction steps are included in Scheme 1.

The kinetics of the reaction system in Scheme 1 are governed by the differential equations

\[
\frac{d[S_{\text{ald}}]}{dt} = v₁ - v₂ - k_{-1}[S_{\text{ald}}] + k₁[S_{\text{dol}}]
\]

\[
\frac{d[S_{\text{dol}}]}{dt} = k₁[S_{\text{ald}}] - k₁[S_{\text{dol}}]
\]

\[
\frac{d[NADH]}{dt} = v₂.
\]

If the products glyceraldehyde 3-phosphate and NADH are absent at the start of the reaction, the relationship

\[
k_{-1}[S_{\text{ald}}] \gg k₁[S_{\text{dol}}]
\]

will hold true in the initial reaction phase. Restricting the present analysis to that phase, and assuming that \([S_{\text{ald}}] \ll K_m\), Eqns (1, 2) reduce to

\[
    v₂ = \frac{k_{\text{cat}E₂}[S_{\text{ald}}]}{K_m}
\]

\[
\frac{d[S_{\text{ald}}]}{dt} = v₁ - λ[S_{\text{ald}}]
\]

where

\[
    λ = k₁ + \frac{k_{\text{cat}E₂}}{K_m}
\]

Eqn (7) has the solution

\[
[S_{\text{ald}}] = \frac{v₁}{λ} \left(1 - e^{-λt}\right).
\]

Insertion of this into Eqns (4) and (6) followed by integration gives

\[
[NADH] = v_m \cdot t - \frac{v_m}{λ} \left(1 - e^{-λt}\right)
\]

where \(v_m\) represents a steady-state velocity given by

\[
v_m = \frac{v₁(λ - k_{-1})}{λ}.
\]

**RESULTS**

**Kinetics of the glyceraldehyde-3-phosphate dehydrogenase reaction**

Aqueous solutions of glyceraldehyde 3-phosphate are an equilibrium mixture of the free aldehyde and the corresponding geminal diol, the former representing the true substrate for glyceraldehyde-3-phosphate dehydrogenase [14, 15]. Michaelis-Menten parameters for the catalytic interaction of the enzyme with glyceraldehyde 3-phosphate in solution were determined by standard steady-state kinetic methods, using enzyme concentrations below 1 nM to ensure that the aldehyde and diol forms of the substrate equilibrate rapidly in
3-phosphate dehydrogenase.

Hydrogenase

containing 10 mM orthophosphate in 50 mM Tris/HCl buffer, pH 7.5, containing 1 mM EDTA and 1.1 μM glyceraldehyde-3-phosphate dehydrogenase

Comparison to the rate of the enzymic reaction [15]. The parameter values thus obtained in the presence of saturating concentrations of NAD⁺ (2 mM) and orthophosphate (10 mM) were k_cat = 120 (± 20) s⁻¹ and K_m,app = 160 (± 30) μM; the latter parameter is denoted as apparent because the value given refers to the total concentration of glyceraldehyde-3-phosphate and not to the concentration of the true substrate form. Variation of the enzyme concentration over 0.1–0.8 nM had no detectable effect on the magnitude of the kinetic parameter values.

In a second series of experiments, the oxidative phosphorylation of glyceraldehyde-3-phosphate was carried out in the presence of about 1 μM enzyme such that the non-enzymic dehydrogenation of the diol form of the substrate becomes rate-limiting [14, 15] for the steady-state reaction (cf. Scheme 1). The formation of NADH during the transient-state and steady-state phases of the reaction was monitored by stopped-flow techniques, and a typical trace is shown in Fig. 1. Rate constants (defined as in Scheme 1) for the interconversion of free and hydrated glyceraldehyde-3-phosphate were calculated from such data as described by Trentham et al. [15], which gave k₁ = 0.014 (± 0.002) s⁻¹ and k₋₁ = 1.0 (± 0.1) s⁻¹.

The latter estimates, which agree well with those reported by Ovádi and Keleti (k₁ = 0.012 s⁻¹ and k₋₁ = 0.72 s⁻¹ [1]), can be used to calculate the true K_m for the aldehyde form of glyceraldehyde-3-phosphate from the relationship

\[
K_m = \frac{K_{m,app}}{1 + \frac{k₋₁}{k₁}}
\]

The value thus obtained from the above estimate of K_m,app was K_m = 2.3 μM.

We have not been able to confirm the reports of Keleti and coworkers [1, 16] that the molar activity of glyceraldehyde-3-phosphate dehydrogenase decreases with increasing high concentrations of the enzyme. Their conclusion was based on rate determinations relating to an assumed pseudo-steady state following the burst of NADH production and preceding the true steady state that is rate-limited by the dehydration of the diol form of the substrate. Over the enzyme concentration range (0.4–4 μM) where the decrease in molar activity was claimed to be pronounced, we have found it virtually impossible to detect, define, or characterize such a distinct pseudo-steady state. The reported evidence for an enzyme concentration dependence of the molar activity of glyceraldehyde-3-phosphate dehydrogenase, therefore, would not seem to be reliable.

Kinetics of the coupled two-enzyme reaction

The catalytic interaction of glyceraldehyde-3-phosphate dehydrogenase with glyceraldehyde 3-phosphate, produced through the action of aldolase on fructose 1,6-bisphosphate, was examined by stopped-flow techniques under experimental conditions similar to those described by Ovádi and Keleti [1], i.e. using relatively high enzyme concentrations and saturating concentrations of the substrates fructose 1,6-bisphosphate, NAD⁺, and inorganic phosphate. As illustrated by the typical result in Fig. 2, NADH production in the coupled two-enzyme reaction exhibits a transient lag prior to the attainment of steady-state conditions. Since these experiments were carried out using glyceraldehyde-3-phosphate dehydrogenase in large excess to aldolase, the concentration of the aldehyde form of glyceraldehyde-3-phosphate would be expected to be low such that the first-order approximation [S₀] ≪ K_m should hold true. Confirmatively, the kinetic transient was found to be governed by a single exponential and estimates of the corresponding apparent first-order rate constant λ were determined by least-squares fits of Eqn (10) to the experimental data.

Fig. 3 shows that the λ values thus obtained vary linearly with the concentration of glyceraldehyde-3-phosphate dehydrogenase, the regression line exhibiting a slope of 55 (± 10) s⁻¹ μM⁻¹ and an intercept differing insignificantly from zero (± 3 s⁻¹). This is consistent with the predictions of Scheme 1 and Eqn (8), according to which the regression line should have an intercept equal to k₋₁ (i.e. about 1 s⁻¹) and a slope equal to k_cat/K_m for the interaction of glyceraldehyde-3-phosphate dehydrogenase with the aldehyde form of fructose 1,6-bisphosphate, with 2 mM NAD⁺ and 10 mM orthophosphate in 50 mM Tris/HCl buffer, pH 7.5, containing 1 mM EDTA and 0.2 μM glyceraldehyde-3-phosphate dehydrogenase.
glyceraldehyde 3-phosphate. The linearity of the plot in Fig. 3 provides evidence that the quotient $k_{cat}/K_m$ to (2.3 pM) calculated from the steady-state kinetic data reported in the preceding section. This is consistent with Eqn (11) that observed values are considerably higher than the estimated magnitude (1 s$^{-1}$) of the rate constant for non-enzymic hydration of the aldehyde form of glyceraldehyde 3-phosphate. According to Eqn (9), the lag of NADH production reflects the transient approach of the concentration of the aldehyde form of glyceraldehyde 3-phosphate towards a steady-state value given by $v_1/\lambda$, i.e. ranging over 0.04 – 0.25 $\mu$M in the above kinetic experiments. These concentrations are well below the $K_m$ (about 2 $\mu$M) for oxidative phosphorylation of the substrate. It can be similarly shown from Eqs (3) and (9) that the maximum amounts of substrate which may accumulate during the first few seconds of the reaction are well below the limit put by Eqn (5). Consequently, the experimental results are consistent also with the main assumptions made in the derivation of Eqs (8 – 11).

DISCUSSION

The present results are consistent in all essential respects with the experimental observations made by Ovadi and Keleti in their kinetic study of the coupled two-enzyme system involving aldolase and glyceraldehyde-3-phosphate dehydrogenase [1, 17]. In particular, application of the transient-state kinetic relationships now derived and tested confirms that the $K_m$ for oxidative phosphorylation of glyceraldehyde 3-phosphate in the coupled reaction (where the substrate is generated through the action of aldolase on fructose 1,6-bisphosphate) is more than 50-fold lower than the apparent $K_m$ value determined by standard steady-state kinetic methods for glyceraldehyde 3-phosphate added to the reaction medium. Our estimates of rate constants for the reversible non-enzymic hydration of glyceraldehyde 3-phosphate corroborate also the observation that the low $K_m$ value governing glyceraldehyde-3-phosphate consumption in the coupled reaction agrees closely with that expected for the aldehyde form of the substrate. As pointed out by Ovadi and Keleti [1], the latter observation provides clear evidence that catalytic turnover of glyceraldehyde 3-phosphate in the coupled reaction involves primarily the aldehyde (as opposed to the diol) form of the substrate.

Experiments of Ovadi and Keleti [1] were performed under such conditions that the aldehyde form of glyceraldehyde 3-phosphate, if released to the reaction medium when produced through the action of aldolase on fructose 1,6-bisphosphate, should undergo non-enzymic hydration at a rate comparable to that of enzymatic oxidative phosphorylation of the substrate. The authors assumed on this ground that the $K_m$ value for glyceraldehyde 3-phosphate in the latter process should approach the apparent one determined for glyceraldehyde 3-phosphate in solution. Hence they arrived at the conclusion that the low $K_m$ value actually observed for the oxidative phosphorylation of glyceraldehyde 3-phosphate in the coupled reaction reflects a direct transfer of the aldehyde form of the substrate from the producing enzyme to the consuming one without prior release of the substrate to solution. There would seem to be no sound rationale for such an assumption and conclusion, however. Our studies of the coupled two-enzyme system, as well as those of Ovadi and Keleti [1], have been performed under conditions where the non-enzymic dehydration of the diol form of glyceraldehyde 3-phosphate is so slow ($k_d = 0.014 s^{-1}$, corresponding to a half-time of 50 s) that this reaction step may be completely neglected over the short periods of time (usually less than 1 s) considered in the kinetic experiments. Under such conditions, the predictions of Scheme 1 can be readily established and the theoretical analysis now presented shows (Eqs 8 – 11) that the kinetics of the coupled reaction must be governed by the true $K_m$ for the catalytically involved aldehyde form of the substrate irrespective of the rate at which the substrate undergoes non-enzymic hydration. The kinetics of oxidative phosphorylation of glyceraldehyde 3-phosphate in the absence of aldolase are analogously governed basically by the low $K_m$ value relating to the aldehyde form of the substrate; the high apparent $K_m$ value usually reported for the latter reaction obtains merely because, for practical reasons, one prefers to relate the kinetic parameter to the total concentration of glyceraldehyde 3-phosphate added to the reaction solution. There is no reason under any experimental conditions to believe that the true kinetic parameter values determined for the coupled reaction system by the evaluation methods used by us or by Ovadi and Keleti [1] should approach or provide measures of such an apparent $K_m$ relating to the total concentration of glyceraldehyde 3-phosphate.

Consequently, the observation that the kinetics of the coupled two-enzyme reaction are governed by a $K_m$ agreeing with that determined for the aldehyde form of glyceraldehyde 3-phosphate cannot possibly be taken as evidence that glyceraldehyde 3-phosphate is transferred from aldolase to glyceraldehyde-3-phosphate dehydrogenase without prior release to solution (this conclusion holds true whether or not non-enzymic hydration of the substrate occurs at a significant rate). The present experimental results, as well as those of
Ovádi and Keleti [1], are fully compatible with the reaction mechanism in Scheme 1 and corroborate previous conclusions that the aldehyde form of glyceraldehyde 3-phosphate represents the immediate product of the aldolase reaction [13] and the true substrate for glyceraldehyde-3-phosphate dehydrogenase [14, 15]. In particular, the results are consistent with the assumption in Scheme 1 that glyceraldehyde-3-phosphate transfer from the producing enzyme to the consuming one occurs by a mechanism of free diffusion. Since it seems unreasonable to believe that the interaction of glyceraldehyde-3-phosphate dehydrogenase with an aldolase-glyceraldehyde-3-phosphate complex might be characterized by the same $K_m$ value as the one determined for the interaction of the enzyme with free glyceraldehyde 3-phosphate, reported kinetic data for the coupled reaction must actually be taken to provide clear evidence against a mechanism of direct metabolite transfer between the two enzymes.

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