Spectroelectrochemistry of Redox Enzymes

Christenson, Andreas

2006

Citation for published version (APA):
Christenson, A. (2006). *Spectroelectrochemistry of Redox Enzymes*. [Doctoral Thesis (compilation), Centre for Analysis and Synthesis]. Department of Analytical Chemistry, Lund University.

Total number of authors:
1

Spectroelectrochemistry of Redox Enzymes

Andreas Christenson

LUND UNIVERSITY

Department of Analytical Chemistry

2006

Academic dissertation for the degree of Doctor in Philosophy, to be publicly defended at the Centre for Chemistry and Chemical Enginering, Sölvegatan 39, Lund, on December 8, 2006 at 13^{15} in Lecture Hall B, by permission of the Faculty of Mathematics and Natural Sciences, Lund University, Sweden.

Faculty opponent: Professor William R. Heineman, Department of Chemistry, University of Cincinnati, OH, USA

The dissertation will be held in English.

Organization
LUND UNIVERSITY

Document name
DOCTORAL DISSERTATION

Date of issue

Sponsoring organization

Author(s)

Title and subtitle

Abstract

Key words:

Classification system and/or index termes (if any):

Supplementary bibliographical information:

Language

ISSN and key title:

ISBN

Recipient’s notes

Number of pages

Price

Security classification

DOKUMENTDATABLAD enl SIS 61 41 21

Distribution by (name and address)
I, the undersigned, being the copyright owner of the abstract of the above-mentioned dissertation, hereby grant to all reference sources permission to publish and disseminate the abstract of the above-mentioned dissertation.

Signature ____ Date__________________________

This thesis include following papers:

I. **Spectroelectrochemistry of cytochrome P450cam.** Nikitas Bistolas, Andreas Christenson, Tautgirdas Ruzgas, Christiane Jung, Frieder W Scheller, Ulla Wollenberger. Biochemical and Biophysical Research Communications (2004), 314(3), 810-816.

II. **Direct heterogeneous electron transfer of theophylline oxidase.** Andreas Christenson, Eva Dock, Lo Gorton, Tautgirdas Ruzgas. Biosensors & Bioelectronics (2004), 20(2), 176-183.

III. **Spectroelectrochemical study of heme- and molybdopterin cofactor-containing chicken liver sulphite oxidase.** Elena Ferapontova, Andreas Christenson, Anja Hellmark, Tautgirdas Ruzgas. Bioelectrochemistry (2004), 63(1-2), 49-53.

IV. **Electrochemical redox transformations of T1 and T2 copper sites in native Trametes hirsuta laccase at gold electrode.** Sergey Shleev, Andreas Christenson, Vladimir Serezhenkov, Dosymzhan Burbaev, Alexander Yaropolov, Lo Gorton, Tautgirdas Ruzgas. Biochemical Journal (2005), 385(3), 745-754.

V. **Characterization of two new multiforms of Trametes pubescens laccase.** Sergey Shleev, Oxana Nikitina, Andreas Christenson, Curt T. Reimann, Alexander Yaropolov, Tautgirdas Ruzgas, Lo Gorton. Bioorganic Chemistry (2006), Article in press.

VI. **Redox potentials of the blue copper sites of bilirubin oxidases.** Andreas Christenson, Sergey Shleev, Nicolas Mano, Adam Heller, Lo Gorton. BBA (Bioenergetics **)** (2006) Article in press.

VII. **Direct and mediated electron transfer between intact succinate:quinone oxidoreductase from *Bacillus subtilis* and a surface modified gold electrode reveals redox state dependent conformational changes.** Andreas Christenson, Tobias Gustavsson, Tautgirdas Ruzgas, Lo Gorton and Cecilia Hägerhäll. In manuscript

Other publications in which I have participated but which are not included in the thesis:

Direct electron transfer between copper-containing proteins and electrodes. Shleev, Sergey; Tkac, Jan; Christenson, Andreas; Ruzgas, Tautgirdas; Yaropolov, Alexander I.; Whittaker, James W.; Gorton, Lo. Biosensors & Bioelectronics (2005), 20(12), 2517-2554.

Direct electron transfer between ligninolytic redox enzymes and electrodes. Christenson, Andreas; Dimcheva, Nina; Ferapontova, Elena E.; Gorton, Lo; Ruzgas, Tautgirdas; Stoica, Leonard; Shleev, Sergey; Yaropolov, Alexander I.; Haltrich, Dietmar; Thorneley, Roger N. F.; Aust, Steven D. Electroanalysis (2004), 16(13-14), 1074-1092.

Spraying Enzymes in Microemulsions of AOT in Nonpolar Organic Solvents for Fabrication of Enzyme Electrodes. Shipovskov, Stepan; Trofimova, Daria; Saprykin, Eduard; Christenson, Andreas; Ruzgas, Tautgirdas; Levashov, Andrey V.; Ferapontova, Elena E. Analytical Chemistry (2005), 77(21), 7074-7079.

A steady-state and flow-through cell for screen-printed eight-electrode arrays. Dock, Eva; Christenson, Andreas; Sapelnikova, Svetlana; Krejci, Jan; Emneus, Jenny; Ruzgas, Tautgirdas. Analytica Chimica Acta (2005), 531(2), 165-172

Amperometric screen-printed biosensor arrays with co-immobilized oxidoreductases and cholinesterases. Solna, R.; Dock, E.; Christenson, A.; Winther-Nielsen, M.; Carlsson, C.; Emneus, J.; Ruzgas, T.; Skladal, P. Analytica Chimica Acta (2005), 528(1), 9-19.

Till
Cecilia, Samuel, Pappa och Mamma

Table of contents

1. Introduction

1.1. From energy production to spectroelectrochemistry

The subject of this work Spectroelectrochemistry of redox enzymes, links three large research fields: spectroscopy, electrochemistry, and biochemistry. One of the motivations for linking these disciplines together comes from a wish to study and understand the transfer of energy from one form to another and from one system to another. An important feature of energy is that whether it is mechanical or electromagnetic, it is interconvertible between different forms. At atomic and molecular levels the interconversions should obey quantum laws.

In biology, energy production starts with the thermonuclear reactions in the sun, where the gravity and temperature create protons with sufficient kinetic energy to overcome the large coulombic barrier that normally bars their interaction. Thereby the two protons can approach each other so closely that the short-range attractive nuclear forces fuse the protons together, and through a chain of fusion reactions the result is the eventual formation of helium. Albert Einstein (in 1905) stated that energy equals to the mass times the square of the speed of light in his famous formula $E=mc^2$. Following this expression and taking into account the difference in mass between four ^{1}H and the mass of the product one ^{4}He a tremendous amount of energy of the order of 10 MeV per reaction event or hundreds of GJ/mol is released in form of radiation. As a reference, chemical binding energies in molecules are in the order of some eV, i.e., hundreds of kJ/mol.

Most of the nuclear energy produced in the sun is emitted as electromagnetic radiation with the photon energy $h\upsilon$ (Max Plank in 1900). This photon radiation covers a 100 octave wide range of wavelengths, ranging from γ rays to long radio waves (Fig. 1).

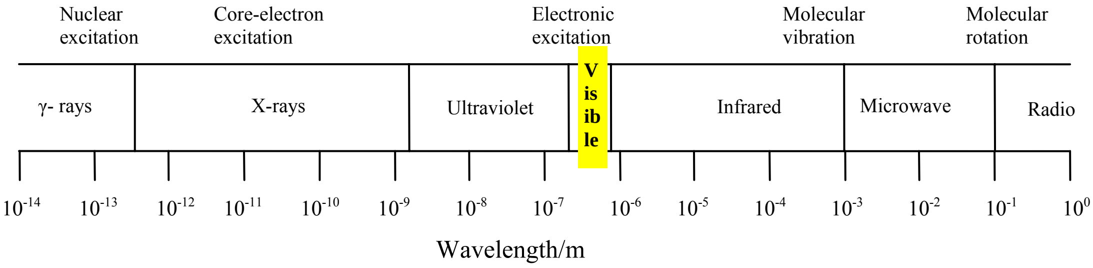


Figure 1. The electromagnetic spectrum. Most of the solar intensity reaching the earth's biosphere is found in the visible and IR region.

The highly energetic radiation from γ rays down to the far UV does not penetrate the earth's magnetic field and atmosphere and thus only a fraction of the electromagnetic spectrum is actually accessible for use by life on earth. Thus, is it not surprising that plants have evolved photon harvesting systems making use of wavelengths at which the largest fraction of solar energy actually reaches the earth's surface. This optimizes the efficiency of photosynthesis as described below.

When the photons from the sunlight, interacts with the thylakoid membranes in plants they give their energy to electrons in redox systems, i.e., electrons are excited to higher energy levels. The photons can only leave their energy to the electron when the excitation energy of the electron and the energy of the photon exactly coincide.

Therefore, in order to utilise more of the solar energy, photo systems I and II (with their specific excitation wavelengths of 680 nm respective 700 nm) utilise photon harvesting complexes with electron excitation energies that cover a somewhat broader spectrum than just the two wavelengths of 680 and 700 nm [1]. In these biological systems, a fraction of the solar radiation energy ends up in electrons of approximately one eV energy. These electrons are incorporated into energy energy-rich compounds like NAD(P)H, ATP and glucose, which can be transferred to a point in the organism where chemical energy is needed.

In order to understand what is going on with the electron on the single atom or molecule level at the moment when the electron accepts the photon energy and becomes excited, it is necessary to invoke quantum physics. The energy of the electrons and the forces involved in atoms can only have quantified discrete energy levels. If taking things a step further, going from atoms and their electron energies to molecules, the concept of describing atomic orbitals by wave functions can be extended to a description of the electronic structures of molecules. In molecular orbital theory, there is a wave function that combines the atom orbitals that extend over all the atoms in a molecule and describe the probability of finding the electron/electrons at different location and the value of their energy. Taking the simple case of a hydrogen molecule, H_2, two 1s atom orbitals combine linearly and the overall wave function is the sum of the two atomic orbitals. The result is that there are two discrete molecular orbital energy levels available for the electrons to occupy (Fig. 2).

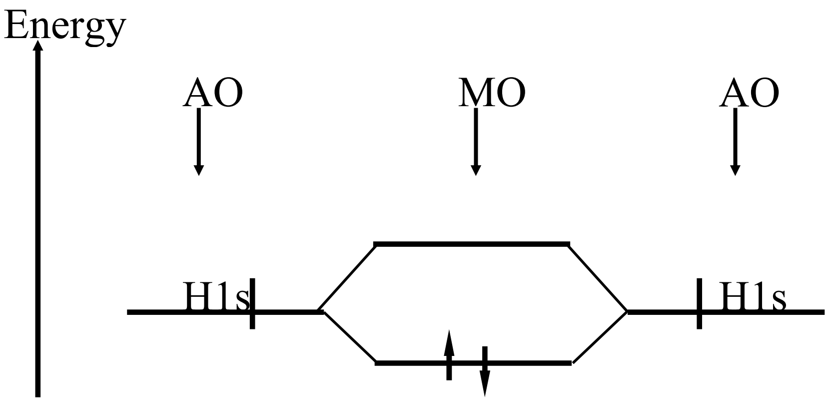
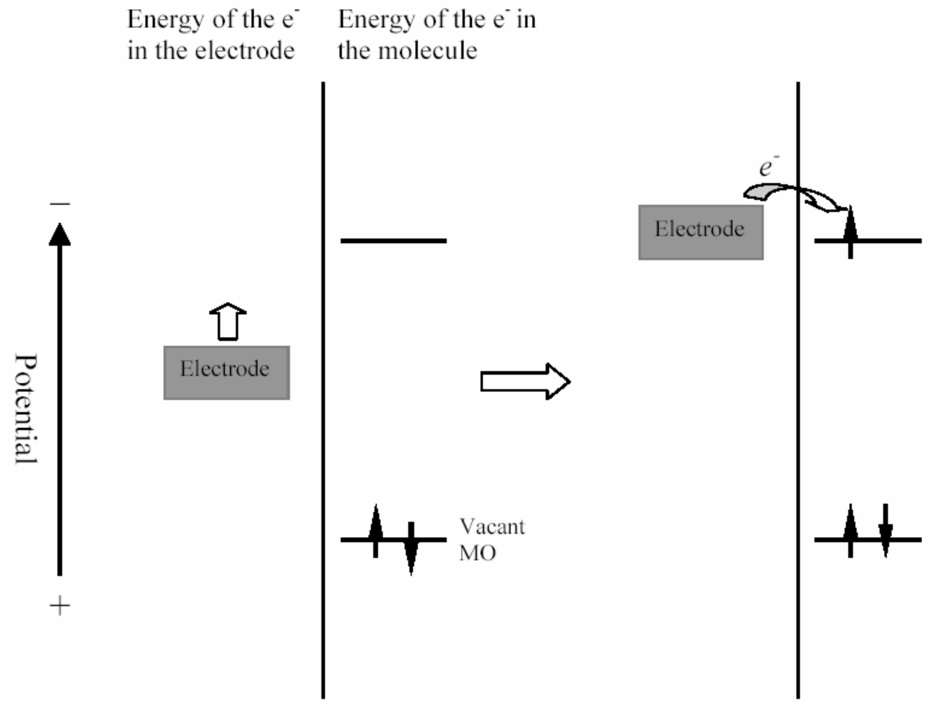


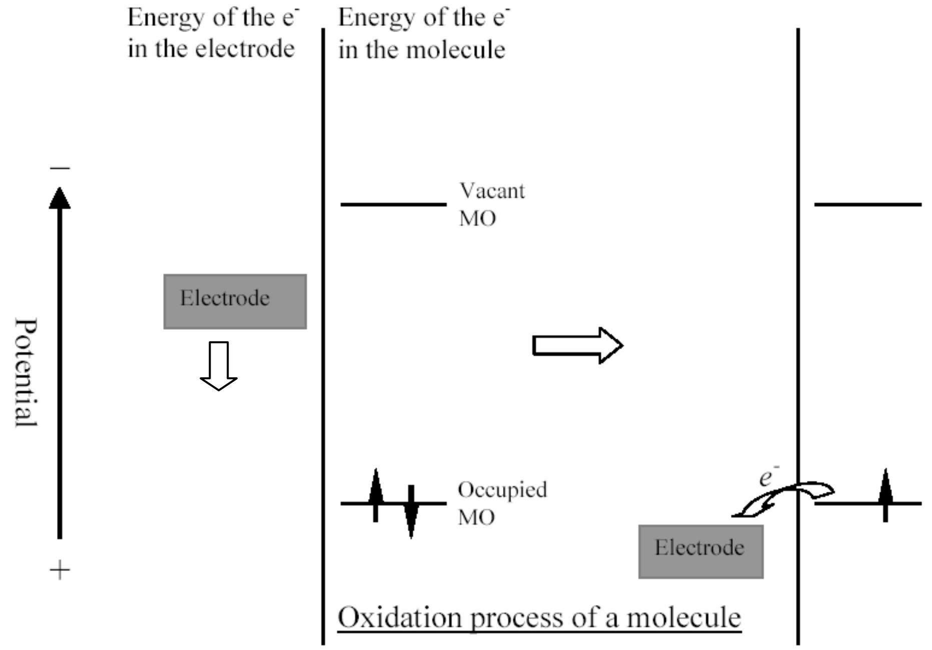
Figure 2. A description of two 1s hydrogen atomic orbitals that combine linearly to one hydrogen molecule H_2 with two levels of energy of the molecular orbitals (MO). The lower molecular orbital energy level corresponds to the bonding MO and the higher level corresponds to the antibonding MO.

These possible quantified excitation energy levels of the molecular orbital result in a specific redox potential for each chemical compound, which in electrochemistry is one of the most fundamental properties of a redox molecule.

In an electrochemistry experiment, as performed in the work represented in this thesis, a chosen potential is applied to an electrode, which renders the possibility to control the energy of the electrons in the electrode. Depending on the energy of the electrons in the electrode, compared with the energy of the highest occupied molecular orbital (HOMO) and lowest unoccupied molecular orbital (LUMO) on the molecule of interest, an oxidation or reduction will occur [2] (Fig. 3). This is the general fundamental view on heterogeneous electron transfer (ET) or, as often simply expressed, the electrochemistry at molecular level.



Reduction process of a molecule



Oxidation process of a molecule

Figure 3. Schematic representation of electrochemical reduction or oxidation at the molecular level. Varying the energy of the electrons by the applied potential on the electrode, relative to the energy of molecular orbitals of the redox molecules, an oxidation or reduction of the molecule can occur.

One can also derive a number of other fundamental physicochemical formulae, which relate to electrochemical characterisation of redox molecules by recording their heterogeneous ET. An important relation is given by Ludwig Boltzmanns theory (in 1868) of the population distribution between different energy states

$$\frac{Ni}{Nj} = e^{-(Ei-Ej)kT} \qquad (1)$$

In this equation k is Boltzmann's constant, T is the temperature, N_i and N_j are the populations of molecules at the different energy levels of E_i and $E_{j,}$ respectively. The consideration of Boltzmann's distribution helps us to understand a fundamental electrochemistry equation, the Nernst equation:

$$E = E^{0'} + \frac{RT}{nF} \ln \frac{Cox}{Cred} \qquad (2)$$

Here E is the applied potential (electrode potential), $E^{0'}$ is the formal redox potential of the molecule, Cox and $Cred$ are the concentrations of the reduced and oxidised forms of the molecules, n is the number electrons involved in a redox process, R is the gas constant, T is the temperature in Kelvin and F is Faraday's constant (the charge of one mole of electrons, 96485 C/mol). The Nernst equation is utilised extensively throughout this thesis in order to determine formal potentials of the redox centres in enzymes.

As a conclusion of this introduction, it is important to point out that the energies of the electronic transitions in biological molecules, often involving energy differences within the order of one volt, are accessible by electrochemical techniques. Moreover, these electronic transition energies often correspond to absorbances at wavelengths in the UV-VIS range of the electromagnetic spectra. This means that UV-Vis range spectroscopy and electrochemistry can be combined into the field ofspectroelectrochemistry, resulting in a versatile tool for studies of biological ET processes.

The studies presented in this thesis addressed only a very minor fraction of the aspects describing biological ET. The focus was on achieving a deeper understanding of the electron transfer between biological redox complexes and the surfaces of conducting materials (gold, carbon). It should be emphasised that the objects of the studies were multicentre redox enzymes or biological electron transfer complexes.

Specifically, the biological objects of this thesis were theophylline oxidase, sulphite oxidase, cytochrome P450, several laccases, bilirubin oxidase and complex II. Measurements of spectral changes of the redox centres as a consequence of electron transfer between the mentioned biological objects and conducting surfaces were used as the main technique (spectroelectrochemistry). The thesis summarises advantages, possibilities and drawbacks of spectroelectrochemical techniques for investigations of complex biological redox systems. The deepest understanding on heterogeneous ET was achieved for multicopper oxidases (several laccases and bilirubin oxidases) as well as for complex II. The results on ET in these systems should be important for the development of biofuel cells as well as for achieving better understanding of the biological role and functions of, especially, laccases and complex II.

1.2. Thermodynamics of biological redox reactions

Reactions in biology, e.g., redox reactions, are considered as usual chemical reactions. They are described by stoichiometric equations like the one below:

$$aA + bB \Leftrightarrow cC + dD \tag{3}$$

The reaction usually proceeds to equilibrium defined by the following equilibrium constant K_{eq}:

$$K_{eq} = \frac{[C]_{eq}^{c}[D]_{eq}^{d}}{[A]_{eq}^{a}[B]_{eq}^{b}} \tag{4}$$

The equilibrium constant is used to calculate standard Gibbs free energy of formation of a compound (formation of 1 mole at 25 C), $\Delta G^{0'}$:

$$\Delta G^{0'} = -RT \ln K_{eq} \tag{5}$$

In any situation when the concentrations of the involved species differ from the equilibrium concentrations, a net driving force (ΔG) will appear and a new reaction constant Γ can be calculated based on the observed concentrations involved in the actual reaction. The new reaction constant Γ is expressed in terms of the observed concentrations as follows:

$$\Gamma = \frac{[C]_{obs}^{c}[D]_{obs}^{d}}{[A]_{obs}^{a}[B]_{obs}^{b}} \tag{6}$$

The effect on the free energy originating from the displacement from equilibrium is shown by the following expression:

$$\Delta G = -2.3RT\log_{10}\frac{K_{eq}}{\Gamma} \tag{7}$$

Equ. 7. can be expressed in another way

$$\Delta G = -2.3RT\log_{10}K_{eq} + 2.3RT\log_{10}\Gamma \tag{8}$$

In this expression it is easier to see the contribution of the displacement concentrations to the free energy. As an example, a 10x lower concentration of the products compared to the equilibrium concentrations yields an extra driving force of -5.7 kJ/mol for the reaction to proceed to the new equilibrium or to do some other work.

A biological example concerning the amount of energy that can be released only due to displacement of concentrations in comparison to equilibrium concentrations, is the example of the ATP/ADP + P reaction [3]. At certain conditions (pH 7, 10 mM Mg^{2+}, 10 mM P_i) the equilibrium constant K of the reaction $\mathrm{ATP} \Leftrightarrow \mathrm{ADP} + \mathrm{P}$ is 10^{5} M, but in the cytoplasm Γ is 10^{-5}, which yields a free energy of -57 kJ/mol, which in biological systems may be considered to be a large amount of energy. It is possible to express the equilibrium part of equation (8) in terms of $\Delta G^{0'}$ values and the observed Γ. Then equation (8) becomes:

$$\Delta G = \Delta G^{0'} + 2.3RT\log_{10}\Gamma \tag{9}$$

As mentioned in the introduction, the energy can be expressed in electron volts. This property is exploited when considering redox reactions, where the driving force is often accounted for by the redox potentials of the reaction components. The relation between Gibbs free energy and potential difference, ΔE, is derived from the following equation,

$$\Delta G = -nF\Delta E \tag{10}$$

Here ΔG is given in J/mol, ΔE is the potential difference (V) and n, F have their usual meaning. As an example, a potential difference of one volt in a one-electron process yields 96.5 kJ/mol free energy. This is also the order of energy involved in the respiratory chain, where the difference between the redox potential of an energy rich redox component, NAD(P)H/NAD(P)$^+$ in the respiratory chain, and the last electron acceptor-molecular oxygen is about 1.1 V. The NADH/NAD$^+$ couple has a formal potential of -320 mV [4] and at pH 7 and 1 atm of oxygen the couple H_2O/O_2 has a formal potential of +820 mV vs. NHE [4]. Thus, the thermodynamics of the respiratory chain spans over a potential difference of the order of one volt, or 100 kJ/mol.

It seems natural at this stage to invoke redox potentials [4], their relation to Gibb´s free energy and to the fundamental Nernst equation. As was mentioned above, equation (9) expresses the dependence of ΔG on the concentration of the involved substances. ΔG can be replaced in equation (9) with $-nFE$ and $\Delta G^{0'}$ with $-nFE^{0'}$, which than yields the following equation:

$$E = E^{0'} - 2.3\frac{RT}{nF}\log_{10}\Gamma \tag{11}$$

In a redox reaction of the following type

$$Ox^{+} + e^{-} \Leftrightarrow Red \tag{12}$$

Γ is expressed in the ratio of the concentrations (more correctly in activities) between the oxidised form and the reduced form of the participating redox couple:

$$\Gamma = \frac{[Red]}{[Ox]} \tag{13}$$

When equation (9) is expressed as the natural logarithm the more common expression of the Nernst equation is obtained [5]:

$$E = E^{0'} + \frac{RT}{nF} ln \frac{[Ox]}{[Red]} \quad \text{or} \quad E = E^{0'} - 2.3 \frac{RT}{nF} log_{10} \frac{[Red]}{[Ox]} \tag{14}$$

This equation gives the relation between the electrode potential (E) and the equilibrium concentrations of the redox components in the solution. The factor $2.3\frac{RT}{nF}$ becomes 59.2 mV in a one electron process, 29.6 mV for a two electron process and so on. Under standard conditions, the redox potential $E^{o'}$ is called the formal redox potential. The classical way to determine redox potentials of redox compounds is by potentiometric titrations [6] [7]. From eq. (14) it follows that if a platinum electrode is dipped into a solution containing a 1:1 ratio of the oxidised vs. reduced forms of the redox components, the electrode potential will become equal to the formal redox potential $E^{o'}$.

Below follows some examples of common biological redox reactions, which can be seen as typical redox reactions, Some of them involve only the transfer of electrons and others have the transfer of electrons coupled with the transfer of protons. The involvement of the proton transfer in these reactions results in redox potentials being dependent on the solution pH.

The small heme-containing redox protein cytochrome *c* undergoes a 1 e^- redox reaction :

$$Cytochrome\ c \cdot \left(Fe^{2+}\right) + 1e^- \Leftrightarrow Cytochrome.c \cdot \left(Fe^{2+}\right) \tag{15}$$

The corresponding redox potential dependence on concentrations of the two redox forms of cytochrome c will become:

$$E = E^{0'}_{cytc} - \frac{2.3RT}{1F} log_{10} \frac{[\mathrm{Cytc{\cdot}Fe^{2+}}]}{[Cytc \cdot Fe^{3+}]} \tag{16}$$

Another example is cofactor nicotinamide adenine dinucleotide, which is the soluble redox cofactor for some 300 dehydrogenases. NAD^+ undergoes a 2 e^- reaction and involves a transfer of one proton.

$$NAD^+ + 2e^- + H^+ \Leftrightarrow NADH \tag{17}$$

Here the dependence of the redox potential is as follows:

$$E = E^{0'}_{NADH/NAD^+} - \frac{2.3RT}{2F} \log_{10} \frac{[NADH]}{[NAD^+][H^+]} \tag{18}$$

Furthermore, the biological redox molecule ubiquinone is a partly hydrophobic redox molecule that carries electrons in the respiratory chain inside the bacterial or mitochondrial membranes. It undergoes a 2 e^- reaction and involves at the same time a transfer of 2 H^+ (reaction 19)

$$UQ + 2e^- + 2H^+ \Leftrightarrow UQH_2 \tag{19}$$

which results in the dependence of the redox potential reflected by equation (20)

$$E = E^{0'}_{ubiquinone} - 2.3\frac{RT}{2F} \log_{10} \frac{[UQH_2]}{[UQ][H^+]^2} \tag{20}$$

The same reaction scheme holds true for flavin adenine dinucleotide (FAD), which is a common prosthetic group in many redox enzymes, e.g., complex II (see paper 7). It undergoes a 2 e^- reaction (21) and involves at the same time a transfer of 2 H^+.

$$FAD + 2e^- + 2H^+ \Leftrightarrow FADH_2 \tag{21}$$

This results in the dependence of the redox potential seen as follows:

$$E = E^{0'}_{FADH/FAD^+} - \frac{2.3RT}{2F}\log_{10}\frac{[FADH_2]}{[FAD][H^+]^2} \tag{22}$$

It is important to be aware of how strongly $[H^+]$ affects the redox potential of biological compounds especially in reactions involving two protons.

In conclusion, it could be summarised that the thermodynamics of redox reactions can be studied by simple measurements of electrode potentials. From this chapter it follows that the electrode potential will be dependent on the concentrations of the redox components in the electrochemical cell and in certain cases on the pH of the solution.

1.3. Kinetics of ET in proteins: Marcus Theory

Biological systems, e.g., living cells, contain a mixture of different redox components. There is no doubt that there are considerable driving forces to bring all redox components into thermodynamic equilibrium (death). Some of the redox processes are very rapid in biological systems, while some are so slow that they are never even considered. In other words biological life is a struggle against the ultimate driving force of entropy, and there is crucial need to harness energy for this purpose. Among other things, electron transfer has to be controlled and directed. In redox proteins the redox sites are usually electrically shielded from their surrounding by a low conductivity protein matrix. This keeps the electrons from escaping, threreby preventing undesirable electron flow. To direct the electron flow within protein matrices, distinct electron pathways within and along the proteins exist. Such a conducting structure can be exemplified by NADH dehydrogenase [8,9] and in the respiratory chain, where seven Fe-S redox clusters are distributed evenly within the enzyme with approximately 10-15 Å separation between them. These distances between the redox centres fit well with the distances, which are found sufficient to realise biologically relevant electron transfer rates [10]. Complex II (paper 7) is another example of an enzyme enclosing conducting electron pathways with three Fe-S clusters and two hemes yielding extra conductivity.

Theoretically, electron transfer in proteins is described as a quantum mechanical tunnelling effect [11,12], where the intervening organic medium [13,14] is highly important in its enhancement of the electron tunnelling, enabling a relevant physiological electron transfer rate within the proteins [15]. The dominant coupling and electron transfer are via covalent bonds, hydrogen bonds, and van der Waals contacts. The Marcus theory [16,17] provides a simple expression for the ET rate in biological redox processes as seen in the following equation: (23).

$$k_{ET} = 10^{13} e^{-\beta(d-d_0)} e^{-(\Delta G-\lambda)^2/4\lambda RT} \tag{23}$$

Here the electron transfer rate (k_{ET}) is dependent on β, a decay constant reflecting the conducting property of the medium, which connects the electron donor and the acceptor; λ is the reorganisation energy for both the reacting species and the solvent surrounding the reaction centres; d_0 and d are distances measured as van der Waals distance and the actual distance between the redox centres, respectively; and finally ΔG is the thermodynamic driving force behind the ET transition transfer. An important prediction of the equation is that k_{ET} decreases exponentially with increasing distance between the redox centres.

ET processes in living systems present a colourful picture with respect to the Marcus theory, and this theory is frequently applied for ET reactions concerning redox proteins. There are a number of other redox components in cells, e.g., NAD(P)H/NAD(P)$^+$, glutathione, NO, oxygen radicals and more.. In the phospholipid bilayers of different cell membranes, hydrophobic quinones are the common electron carriers. Their function is to diffuse inside the membranes, and to carry electrons between different membrane-bound enzymes. The respiratory chain in the mitochondria is composed of many different redox components and is a good illustration of biological "electronic circuits" (Fig. 4).

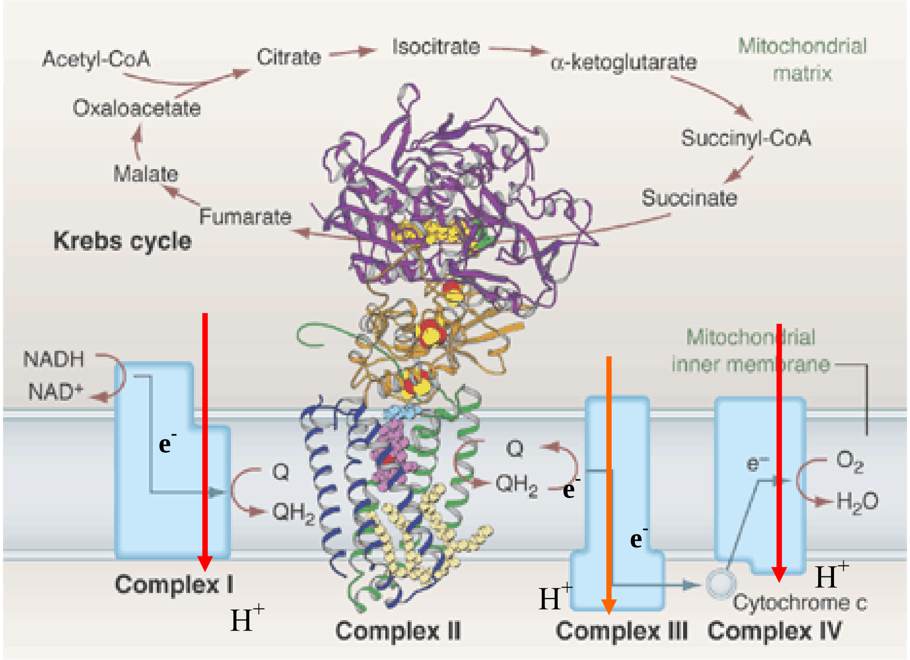


Figure 4. A schematic representation of electron transfer in the respiratory chain of mitochondria.

The mitochondrial respiratory ET chain comprises a set of different redox reaction centres. In a two- electron reaction, which also involves the transfer of proton(s), the electrons from NADH (originating, e.g., from the glycolysis) enter the NADH dehydrogenase (FMN protein of 850 kDa) [8,9,18]. The NADH/NAD$^+$ couple has a redox potential as low as -320 mV [4]. From FMN and through seven Fe-S clusters, in NADH dehydrogenase, the electrons tunnel to the final quinone binding site, where the reduction of a quinone takes place. Complex II (SQR) [19,20] is involved in the citric acid cycle in which succinate is oxidised to fumarate, while at the same time the electrons from succinate are transferred first to FAD and then via the three Fe-S clusters through the enzyme to reduce the hydrophobic quinone, contributing to the overall quinone pool. The reduced hydrophobic quinones diffuse within the mitochondrial membrane further on towards complex III (ubiquinone-cytochrome *c* oxidoreductase), where the quinone leaves its electrons to cytchrome *c* accompanied by the transfer of a proton. The reduced cytochrome *c* diffuses inside the intermembrane space and encounters to complex IV, where the final electron acceptor molecular oxygen becomes reduced to water ($E^{0'}$ is 820 mV for this reaction). The overall reaction in the respiratory chain releases – 220 kJ/mol ($\Delta G = -nF\Delta E$). A study of heterogeneous ET of complex II was one of the objectives of this thesis.

1.4. Heterogeneous ET of biomolecules: direct and mediated ET

In the case of protein electrochemistry the Marcus theory of ET is also valid. In this sense, when attempting a man-made connection to an enzyme, the distance and coupling (β constant) between the proteins/enzymes and electrodes should be optimised. To decrease the distance between the redox site in proteins and the electrode surface, different strategies of electrode surface modifications are used. E.g., surface modifiers such as thiols [21-23], bearing different heads groups, which can direct the enzyme into the most proper orientation to the electrode, thus minimising the distance between the redox active site of the protein and the electrode. Such thiol modifiers have been frequently utilised during the spectroelectrochemical studies presented in Papers 1, 2, 3, and 7, to enhance the direct electron transfer rate between the electrode and the redox active site of the enzyme. In proteins with deeply buried redox sites, the direct electron transfer distances might be too large; hence, small redox mediators, able to penetrate the protein structure, should be utilised to facilitate the electron communication between the electrode and the redox site. Small redox mediators, were used in the spectroelectrochemical studies summarised in Papers 4-6, as well as in Manuscript 1 of this thesis.

There has for a long time been a great interest in studying redox enzymes by electrochemical methods [24-26]. The study of heterogeneous electron transfer between enzymes and the electrodes contributes to the understanding of the function of enzymes and, in addition, promotes development of their practical applications, e.g., in biofuel cells [27], and biosensors for medical [28-30] and industrial use [31]. Heterogeneous electron transfer mechanisms are usually divided into two classes, specifically, direct and mediated ET. as displayed in Fig. 6. From Fig. 6 it can be seen that mediated ET can be organised in three different sub-classes.

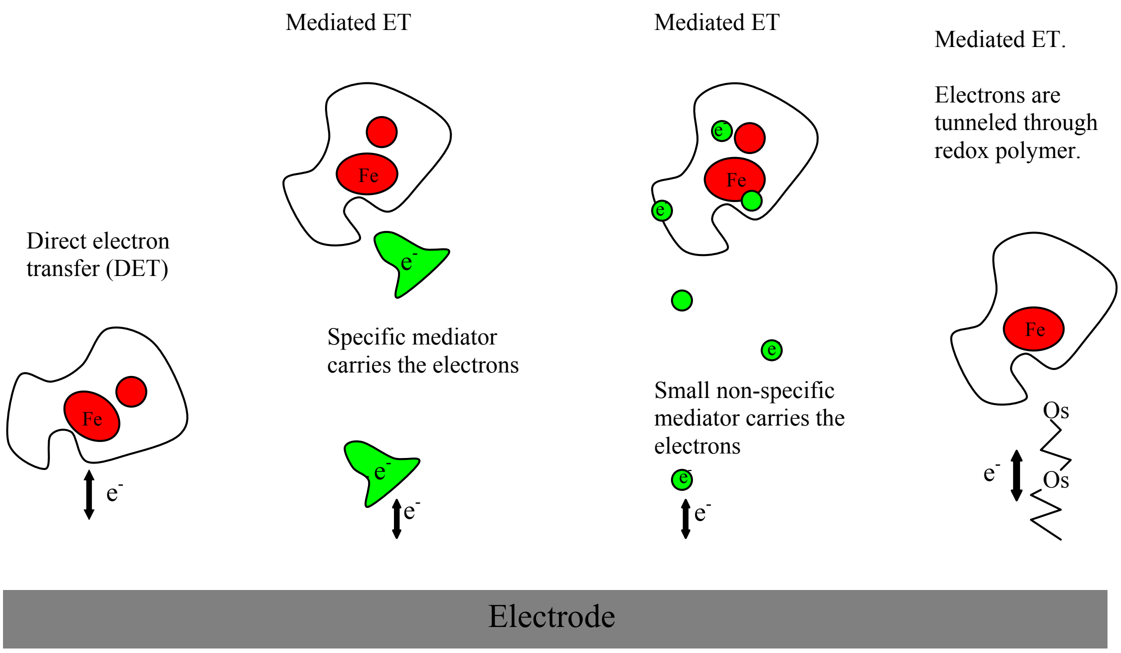


Figure 6. Schematic representation of different ET mechanisms for enzymes at electrodes.

1.5. Direct ET (DET) of proteins and enzymes

The direct electronic coupling between the redox cofactor of a protein and an electrode is highly interesting, especially in the field of biosensors. The realisation of this mechanism enables the construction of reagentless and highly selective biosensors. In this thesis, a variety of spectroelectrochemical studies have been performed in order to better understand direct ET of proteins and enzymes, as presented specifically, in Papers 1-4, 6, and Manuscript 1.

The first reports on DET with a redox-active protein were published in 1977 when Eddowes and Hill (1977) and Yeh and Kuwana (1977) independently confirmed that cytochrome *c* adsorbed on bipyridyl-modified gold and tin doped indium oxide electrodes, respectively, showed virtually reversible electrochemistry as revealed by cyclic voltammetry [32,33]. In 1987 spectroelectrochemistry of cytochrome *c* was demonstrated [34]. Cyt *c*, being an electron carrier protein in the respiratory chain, possesses no catalytic activity.

The next important step in the history of DET studies of proteins could be counted in 1978/1979, when reports from the Berezin group in Moscow provided indirect evidence of DET bioelectrocatalysis for large redox proteins with enzyme activity (oxidoreductases). They showed that laccase-modified [35,36] and peroxidase-modified [37] carbon electrodes exhibited DET in the presence of the substrates dioxygen and hydrogen peroxide, respectively. Nowadays, even large complex enzymes have been shown to be able to directly communicate electronically with metal surfaces, e.g., cytochrome *c* oxidase [38], mitochondria succinate dehydrogenase [39,40], fumarate reductase [41], Complex I [8], and Complex II (Manuscript 1) though at a low heterogeneous DET rate. The question seems not to be whether DET is possible for electron transfer chain complexes, but rather whether the rate of DET is sufficient to realise practical applications, and how DET for membrane biological ET complexes at electrodes can be

Armstrong et al. [42] made a distinction between intrinsic and extrinsic redox enzymes, depending on their biological roles, which could have impact on the possibility to achieve DET. An intrinsic enzyme was denoted as one where the ET between the enzyme and the substrate occurs directly on the spot of the prosthetic group. In such a case there is no natural reason for an ET pathway to exist within the enzyme. An extrinsic enzyme, on the other hand, has a redox protein partner that docks at the surface of the enzyme, but somewhat apart from the redox site. Hence a natural ET pathway needs to exist between the docking site and the redox site. For direct heterogeneous electron transfer, the extrinsic type of enzyme should possess faster rates of ET. As an example of an extrinsic enzyme complex IV (cytochrome *c* oxidase) in the respiratory chain could be mentioned. This enzyme it accepts its electrons from the docking of the small electron carrier protein cyt *c*. Other examples of extrinsic enzymes that have shown DET include cyt *c* peroxidase [43-48] fructose dehydrogenase [49,50], and more. Up to now, numerous different redox enzymes and proteins have shown direct heterogeneous electron transfer between proteins and electrodes, and heme and copper-containing proteins are frequently mentioned in the literature, e.g., peroxidase [24,51,52], laccase (e.g., [53-55], Paper 4), theophylline oxidase [56], bilirubin oxidase [57,58], cellobiose dehydrogenase [59,60], sulfite oxidase [61]. Several reviews have appeared on this subject [24,62-65]

As a final remark about heterogeneous direct electron transfer it should be mentioned that, as was done already in 1977 [32,33], by attaching thiols (which chemisorb to many metal surfaces) one can greatly improve direct heterogeneous electron transfer rates for many enzymes. The adsorption of thiols to the electrode surfaces, the so-called self-assembled monolayers (SAM) [21], isused to orient the redox proteins for the most facile communication between the redox site and the electrode. SAMs of different types have been extensively studied in this respect [21-23,66].

1.6. Mediated ET

Historically, mediated ET is defined as the ET mechanism which exploits small protein-soluble electron carriers. The most common field of utilization of soluble mediators is in the "second generation" biosensors, where small redox mediators are used to enhance the communication between the catalytic enzyme and the electrodes [67-70]). Another common use of small redox mediators is in potentiometric redox titrations for evaluation of redox potentials of redox enzymes [71,72]. The development of this type of ET brought a few new possibilities as presented in Fig. 6, e.g., wiring of enzymes into redox hydrogels [73]. Redox polymer mediated electrochemistry is another way of establishing more efficient enzyme-electrode coupling [28,73-75], as well as even efficient ET between cells and electrodes [76].There is wide diversity of both one- and two-electron mediators, where both hydrophilic and hydrophobic ones are utilised [7,68,69,72,77].

Ferrocyanide and molybdenium cyanide are examples of such non-specific artificial 1 e^- redox mediators that have been utilised for determination of the redox potentials of the enzymes studied during some of the spectroelectrochemical studies (Papers 4-6) involved in this thesis.

There are also possibilities to utilise biologically specific natural redox mediators such as different types of quinones for the electron transfer specifically between an electrode and the quinone sites of, e.g., complex II in the respiratory chain. Such experiments were conducted in the spectroelectrochemical study presented in Manuscript 1, which enabled the directed control of the electron flow by specific quinones, while at the same time, achieving a facile communication between the enzyme and the electrode. As was seen in equation (19), the redox mediator quinone undergoes a 2 e^- transition, involving two protons in the reaction scheme.

From above it is clear that direct and mediated ET can be established for a vast number of redox protein and enzymes. However, in many cases the mechanisms of ET is not obvious. In such cases additional studies need to be performed with refined tools. In the work presented in this thesis, it has been demonstrated that spectroelectrochemistry is a very useful tool.

2. Methods for heterogeneous ET studies

Among the methods for studies of heterogeneous ET transfer we found a number of different techniques with a dominant theme of electrochemistry. The combination of electrochemistry with different kinds of spectroscopy has been of interest. In the following paragraphs these techniques are briefly described, as it is mainly these that have been used to carry out the work summarized in this thesis.

2.1. Electrochemistry

As follows from its name, electrochemistry considers the chemistry of electrode reactions with a particular focus on charge transfer at the electrode-solution interface. The technique might probe thermodynamics of redox reactions and their kinetics; it can be purely analytical as in the case of glucose sensors monitoring human blood sugar levels; or it can provide the basis for the development of more efficient batteries or biofuel cells. Whatever the use of electrochemistry, the main point for the technique can be easily understood if considering the applied potential (or current) as an experimental means to provide a certain driving force for a heterogeneous reaction. The electrode where the extent of the heterogeneous reaction is experimentally controlled is usually called the working electrode. Depending on how the potential or the current is manipulated at the working electrode, we can list a number of different electrochemical techniques, e.g., including amperometry; chronoamperometry; linear sweep voltammetry; cyclic voltammetry; etc. Below, only cyclic voltammetry is described as this was the main technique used in this thesis.

Cyclic voltammetry (CV) is one of the most popular electrochemical techniques. It has proven to be highly useful for studies of rather complicated electrode reactions [2]. It provides qualitative information about the redox process including kinetics and thermodynamics, as exemplified by, the evaluation of redox potentials of redox molecules. Obtaining diffusion coefficients and following the course of events regarding absorption/desorption processes are other abilities of CV. In CV, a potential is applied on the working electrode and is linearly altered at a defined speed (forwards and backwards) between two extreme values usually arranged to cause both a reduction (at electrode potentials lower than the $E^{0'}$ of the molecule) and oxidation (at electrode potentials higher than the $E^{0'}$ of the molecule).

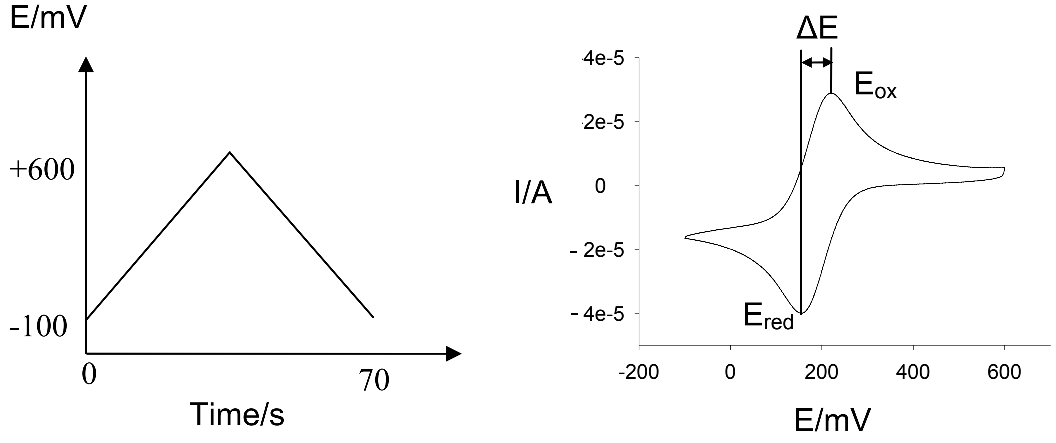
In a given experiment, the potential sweep starts at one of the endpoints, i.e., at a potential lower than the $E^{0'}$ of the redox couple (Fig. 7a). In due course the potential is increased with the speed of the scan rate, passing the $E^{0'}$ of the redox couple and progressing further to the highest potential (endpoint) of the sweep and back again to the low potential endpoint. A typical current response from such a triangular sweep is shown in Fig. 7b.

In CV experiments the electrochemical process (or heterogeneous electron transfer) is differentiated/defined as reversible, quasi reversible or irreversible. In CV the degree of reversibility can be defined as the measure of the difference in potential between the anodic and cathodic peak current ($\Delta E_p = E_{p,a} - E_{p,c}$). In general this is related to the shift from Nernstian equilibrium in comparison to the applied potential, i.e, a redox process is electrochemically reversible when the ratio between [*Ox*] and [*Red*] defined by the Nernst equation is reached at the outer Helmholz plane of the electrode surface at any applied potential and at any time during the CV. In terms of CV data ΔE_p is 59 mV/n (at 25°C and in case of electrochemistry of soluble compounds) and the process is defined as a fast electron transfer process in comparison to the sweep rate. A fully irreversible process is one, in which only one direction of the oxidation-reduction process appears to proceed at an applied potential. Finally, a quasireversible process lies in between these two extremes.

For any degree of reversibility, often, symmetric voltammograms will follow allowing an estimation of the formal redox potential of the process according to the following expression $E^{0'}=(E_{ox}+E_{red})/2$. In the kinetically limited process, during CV measurements the concentration of the redox compound that undergoes heterogeneous ET reaction is not in equilibrium with the applied potential. Thus, the current is dependent on the rate of the heterogeneous reaction. This allows the calculation of different kinetic ET parameters for electrode reactions.

In reversible and irreversible electrode reactions with diffusing (soluble) redox molecules, the current is proportional to the square root $v^{1/2}$of the scan rate, while for species attached to or on purpose immobilised to the the electrode,the peak current will be proportional to the scan rate v. The scan rate v is the rate at which the potential is scanned and is given in V/s. The above mentioned dependences generate the possibility to follow adsorption-desorption processes, e.g., self assembling of thiol monolayers (SAM) [78], which are highly important in establishing facile heterogeneous electron transfer between the biomolecules and the electrode.

In bioelectrochemistry CV is a frequently used technique to follow the redox transitions in redox proteins including multi-redox centre enzymes [59,79,80]. CV was utilised in Papers 3, 4, 5, and Manuscript 1. Such electrochemical studies can generate important information about intrinsic thermodynamic, kinetic and mechanistic properties of the proteins. Furthermore structural characteristics, binding characteristics, and orientation of the redox proteins on different electrode surfaces can be revealed [81].



(a) (b)

Figure 7. a) A typical triangular potential sweep in cyclic voltammetry at 20 mV/s; b) registered cyclic voltammogram for the $FeCN_6^{3-}/FeCN_6^{4-}$ *redox couple.*

2.2. EPR-spectroelectrochemistry

Electron paramagnetic resonance (EPR) or electron spin resonance (ESR) is an attractive technique for the identification and study of species containing an odd number of electrons [82], e.g., radicals, radical ions, and certain transition metal species. The signal in an EPR experiment relies on the spin possessed by an electron. This is accounted for by the electron spin angular momentum (*S*) that results in a magnetic moment $\boldsymbol{\mu_s}$ as can be seen as follows:

$$\mu_s = -g\beta S \qquad (23)$$

Here β refers to the electronic Bohr magneton (equal to $e\hbar/(2mc)$), g is the *g*-factor (which is dimensionless) and *e* and *m* refer to the charge and the mass of the electron, respectively. This magnetic moment can be affected by an external magnetic field, i.e. as provided by the EPR instrument.

A high information content of EPR spectroscopy [82] together with an acceptable sensitivity of the technique (from 10^{-8} M, [83]) made EPR the method of choice for investigating complex redox reactions which proceed via radical intermediates or formation of EPR-visible cofactors, and metal-containing redox-active sites of proteins and enzymes. Thus, EPR spectroelectrochemistry is a very powerful tool to study the formation of free radicals during the catalytic turnover of redox enzymes [84], as well as to monitor the redox states of iron-sulfur clusters [85,86], some other metal-containing sites (e.g., [87,88], Paper 4), and FAD [85]. It should be emphasised that in some cases EPR spectroscopy is an irreplaceable technique to follow the redox states of many cofactors and enzyme centres, e.g., in the case of iron-sulfur clusters and T2 copper sites, which are silent in many others spectroscopic measurements.

In 1958, Ingram and coworkers became the first researchers to combine dynamic electrochemistry with EPR [89]. They collected radical anions of compounds like anthracene, benzophenone, and anthraquinone, produced by electrolysis. After the electrode reactions had proceeded they filled the EPR tubes with the sample and chilled them in liquid nitrogen for EPR measurements. A similar technique was utilised to probe redox titrations of the T2 copper redox centre of some blue multicopper oxidases [87,88]. In Paper 4 almost the same technique was used to study the redox transformations of the T2 site in *Trametes hirsuta* laccase.

2.3. UV-Vis spectroelectrochemistry

The UV-Vis region of the electromagnetic spectrum spans the wavelength range between approximately 200 nm (ultraviolet radiation) and 700 nm [90] and corresponds to energies between 6.2 eV (UV) to 1.8 eV for the red visible light. This is consistent with the energies of electron excitation and changes in the electronic distribution in molecules [90]. As was mentioned in the Introduction, electron excitation energies are quantised and photons can only deposit their energy when this energy level exactly coincides with the energy difference between the ground and electronically excited electronic orbitals. This should theoretically result in line spectra corresponding to these excitation energies. Such spectra are, e.g., observed for low-pressure atomic gases. But in the case of molecule UV-Vis spectra, several other energies contribute, like vibrational and rotational energies. Even though they have relatively low energy, they distort the electronic orbitals, which results in a smoothing and broadening of the spectra. Though the spectra of the molecules are broad, they can still be assigned to certain structures e.g., in, the redox site in the biomolecule. This feature is extremely useful in combination with electrochemistry for identifying compounds, which undergo heterogeneous redox conversion. Thus, in spectroelectrochemistrical techniques, the great advantage lies in the combination of the two techniques, spectroscopy and electrochemistry, enabling one to follow a specific molecular response as a consequence of a chosen electrode potential or current. Many of the redox proteins that exist exhibit absorbances in the UV-Vis region due to the occurring electronic energy transitions. Hence, UV-Vis spectroelectrochemistry is highly suitable for studies of electron transfer of these redox proteins. Many redox proteins, such as cytochrome *c* [91-97], soluble spinach ferredoxin [98], blue copper proteins [95,99], myoglobin [97,100], and hemoglobin [101] have been studied with UV-Vis spectroelectrochemistry.

Spectroelectrochemical results are usually evaluated by fitting the spectroelectrochemical titration data to the following equation, which is a combination of Lawbert-Beer´s law and the Nernst equation.

$$A = \varepsilon_{red} \cdot l \cdot C_t + l(\varepsilon_{ox} + \varepsilon_{red}) \frac{C_t \cdot exp\left[\frac{(E - E^{\circ'})nF}{RT}\right]}{1 + exp\left[\frac{(E - E^{\circ'})nF}{RT}\right]} \tag{24}$$

Here l is the length of the optical path of the cell, ε_{ox} and ε_{red} are the values of the specific absorption coefficients of the oxidised and reduced form of the redox couple and C_t is the total concentration of the enzyme. Fortunately it is not necessary to know the values of ε_{ox} , ε_{red} *and* C_t, in order to estimate the number of electrons involved n and to obtain the formal potential $E^{\circ\prime}$.

Such a processing of data gives the $E^{\circ\prime}$ of the redox couple and also n. In Fig. 8 several theoretical Nernstian plots are shown, all of them having the same formal potential but different numbers of electrons n.

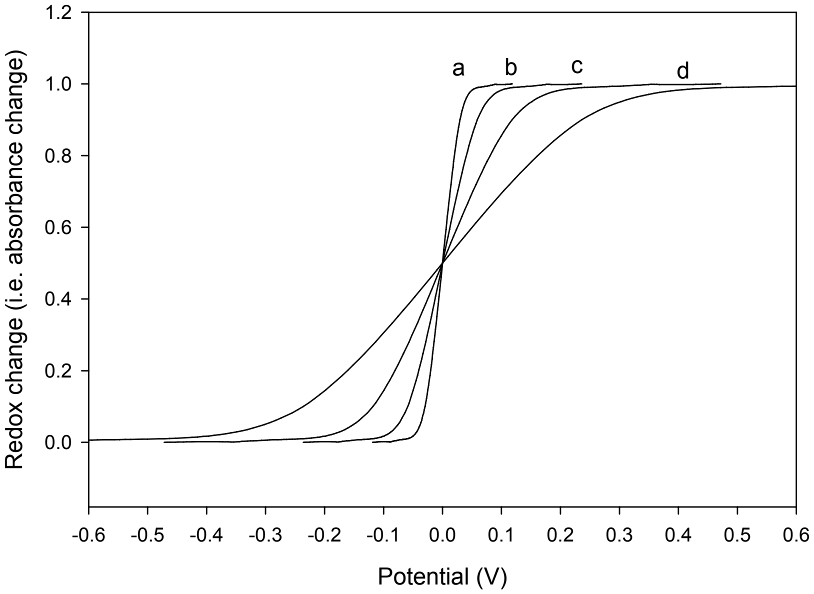


Figure 8. Different theoretical Nernstian redox slopes (redox titrations) corresponding to (a) 2 e$^-$; (b) 1 e$^-$; (c) 0.5 e$^-$ and (d)0.25 e$^-$ transitions.

A linear dependence of the applied potential vs. ln([Ox]/[Red]) is a very useful measure since and the horizontal axis intercept will correspond to $E^{0'}$ and the slope will give the number of electrons involved in the process (Fig. 9).

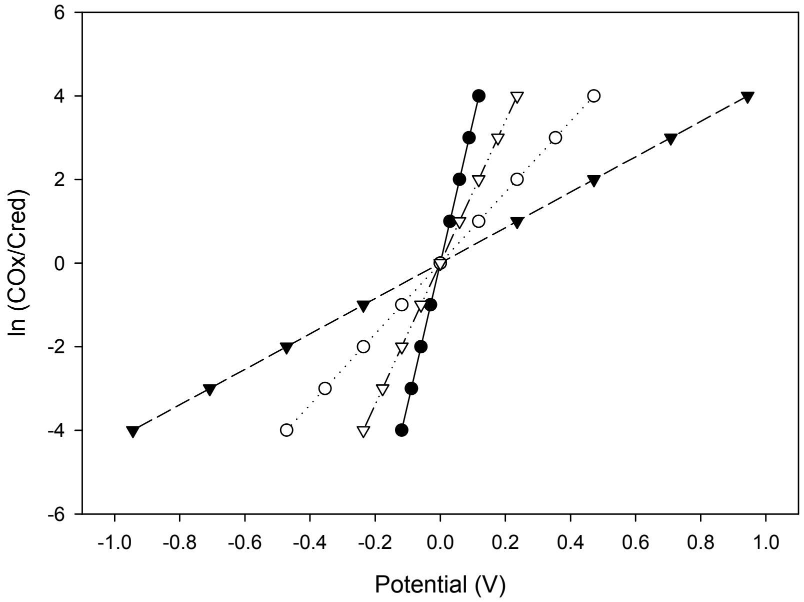


Figure 9. Different theoretical linear Nernstian redox dependencies corresponding to a 2 e$^-$ (▼), 1 e$^-$ (○), 0.5 e$^-$ (Δ) and 0.25e$^-$ (●), electron processes, respectively.

The spectroelectrochemical method described above has been employed in all work reported included in this thesis and attached publications. The reason for this is that a majority of redox proteins and enzymes exhibit absorbances in the UV-Vis region (besides the normal 280 nm absorbance), which change during the redox transformation due to the presence in their structures of different cofactors and metal centres, such as FAD, heme, some copper-containing centres (a T1 and T3 copper site), and so forth.

To succeed with spectroelectrochemical measurements, several conditions should be fulfilled. Some of these have been suggested in [102] and some of them follow below from the experience gained during the work presented in this thesis:

(i) All the redox material of interest has to be fully converted electrochemically to the extent described by the Nernst equation, i.e., at a certain applied, potential equilibrium between the electrode potential and the redox compound(s) in the bulk should be reached. This implies that the electrolysis time for each potential step in the redox titration has to be long in comparison with the rates of heterogeneous ET or mass transfer (diffusion) to achieve full equilibrium.

(ii) In order to achieve Nernstian equilibrium rapidly, the diffusion distances and cell resistance should be kept at a minimum. The use of small redox mediators usually helps in faster achieving Nernstian equilibrium, but with the disadvantage that they often exhibit redox-state-dependent UV-Vis spectra which interfere with spectrum of the the molecule of interest.

(iii) The concentration of the redox protein in the spectroelectrochemical cell should be constant. Especially in low volume cells, adsorption of proteins in the system should be completed prior to performing the spectroelectrochemical experiments. A decreasing concentration due to ongoing surface adsorption could wrongly be interpreted as a redox change.

(iv) In any electrode-driven redox titration experiment, both a reduction and an oxidation experiment should be carried out. This is of extra importance in experiments concerning multi redox site enzymes and in situations in which the obtained electron stoichiometry is less then the type of the reaction of interest. A redox transition giving $n = 0.5$ in a reaction known to be 1 e^- reaction indicates a non-equilibrium situation and hence a distorsion of the true values of the thermodynamic parameters. These values can in the best case, be averaged with data from the reverse experiment.

In order to easily pursue spectroelectrochemical measurements, a number of different spectroelectrochemical cell constructions have been fabricated and tested over the years. The most commonly used scheme is, however, the OTTLE (optically transparent thin layer electrochemical cell), which was developed in 1967 by Murray and co-workers [103]. Numerous constructions of OTTLE cells [100,104-109] have since then been constructed based on the same idea. The great advantage they possess is the rapid electrolysis rate due to small diffusion distances for the redox species to react at the electrode, as well as, the need for only a tiny amount of sample due to the small cell volume (μl). Unfortunatelly the short optical path in these OTTLE cells results in a low optical sensitivity. Increased sensitivity could be obtained with OTTLE cells featuring a long optical pathway [110-112]. Recently an alternative spectroelectrochemical cell was constructed from an ordinary quartz cuvette utilises stirring in order to facilitate mass transfer and efficient electrolysis [113].

3. Gold capillary spectroelectrochemical cell

In order to drastically increase the sensitivity of the spectroelectrochemical experiments, a long-optical-pathway thin capillary-type spectroelectrochemical cell was constructed. It was based on the previous idea of a gold capillary utilised as a working electrode which was constructed by Larsson et al. [114]. The previous construction was not stable and thus it only enabled short time studies (fast ET experiments); furthermore it was difficult to operate. The new construction has proven to be stable in 24 h experiments and is also reasonably easy to handle.

In the spectroelectrochemic cell constructed in our lab, a combined deuterium and halogen lamp was utilised for the emission of light in the UV-Vis region of approximately 310 - 800 nm. The light was directed through an optical fiber to the gold capillary working electrode containing the sample solution. The light that passed the gold capillary cell was then directed to the spectrometer by an optical fiber. A three-electrode system in combination with a potentiostat was employed to control the potential of the gold capillary electrode. The complete spectroelectrochemical system is shown schematically in Fig. 10.

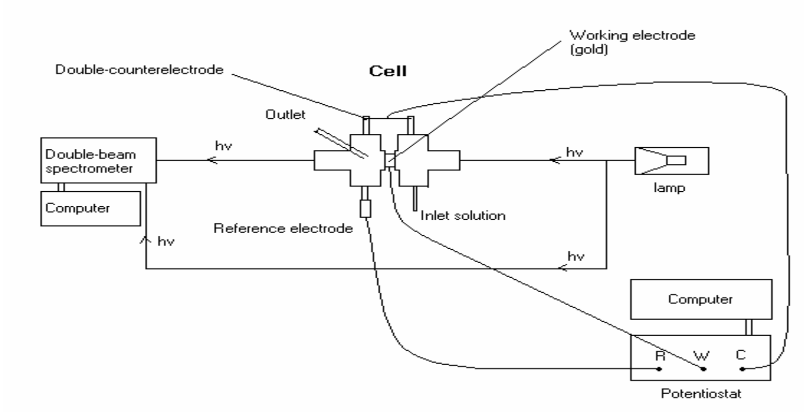


Figure 10. A schematic representation of the full spectroelectrochemical system

Cell material

The gold capillary electrode was constructed from a 3-mm-diameter gold rod purchased from Goodfellow (Cambridge, UK). The counterelectrode made of platinum was also from Goodfellow. The $Ag|AgCI|KCl_{sat}$ reference electrode was purchased from Cypress Systems Inc. (Lawrence, KS, USA). The PEEK materials (P-777) and micro cross assembly, F-132 ferrule 1/16 inch inner diameter, P-416 nut female5/16-24, 1/16 and 360 μm OD tubings) were from Upchurch Scientific (Oak Harbor, WA, USA). The adhesives were epoxy 2-Ton Clear Epoxy (Devcon, MA, USA) but any kind of soft silicon can be used insead. The ceramic frits, Teflon, PVC and polyethene tubings were of various kinds.

Equipment and instrumentation

The optical fibers FCB-UV 400/050-2, FC-UV 200, the light source DH-2000 and the computer software Spectra Win version 4.2 were all from TOP Sensor Systems (Eerbreek, Netherlands). The potentiostat was a CV-50 W instrument from BAS (West Lafayette, IN, USA). The spectrometer SD 2000 and the analog-to-digital conversion board ADC-500 were from Ocean Optics (Dunedin, FL, USA).

Cell construction

The motivation for constructing a new spectroelectrochemical cell was based on a number of considerations. The cell has to be stable and it should have a relatively high sensitivity. Furthermore, it should have a small volume for low consumption of sample and minimal diffusion distances for the molecules to be redox- transformed by electrolysis. Finally, the gold electrode has to be easily cleanable, i.e., with pirahna solution. The gold capillary (Fig. 11) appeared to be robust, provided a relatively long optical pathway (1 cm), and thus, yielded good sensitivity. A small inner diameter (300 μm) of the gold capillary was chosen to limit the diffusion path of the redox proteins or enzymes during electrolysis.

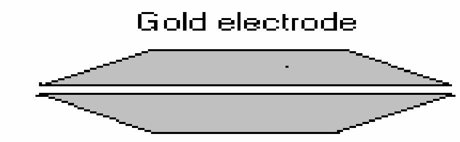


Figure 11. The gold capillary working electrode of the spectroelectrochemical set-up.

In order for light to pass through the system, the two optical fibers and the thin hole in the gold capillary had to be aligned. The easiest way to achieve this was to construct a self-centreing system. This was done by turning both ends of the gold rod capillary into conical shapes that fit the inner side of the PEEK crossings where the optical fibers were attached. In Fig. 12 one can see the alignment of the electrode and the crossings with its optical fibers.

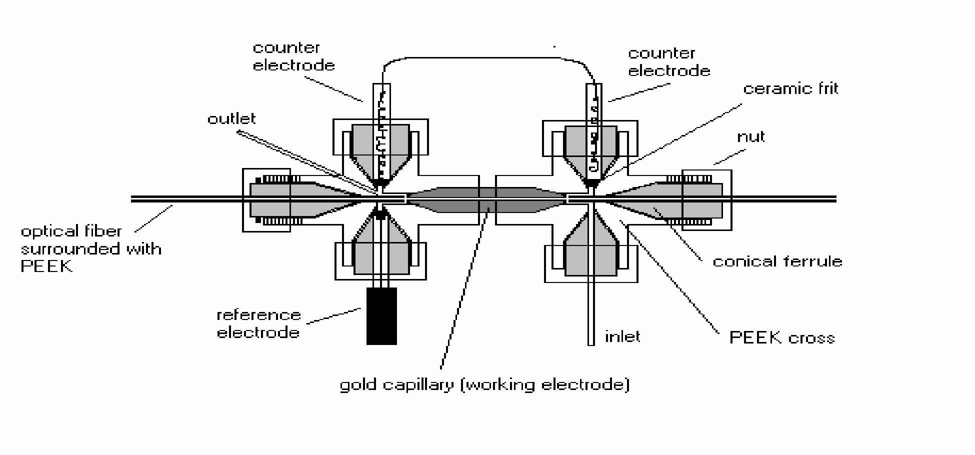


Figure 12. A schematic representation of the spectroelectrochemical cell.

By pressing the two PEEK crossings together with a nut on a Teflon holder before the experiment, a sealed and centreed system was achieved (Fig. 13). This simple construction was also easy to disassemble, hence enabling easy cleaning of the gold electrode after the experiments.

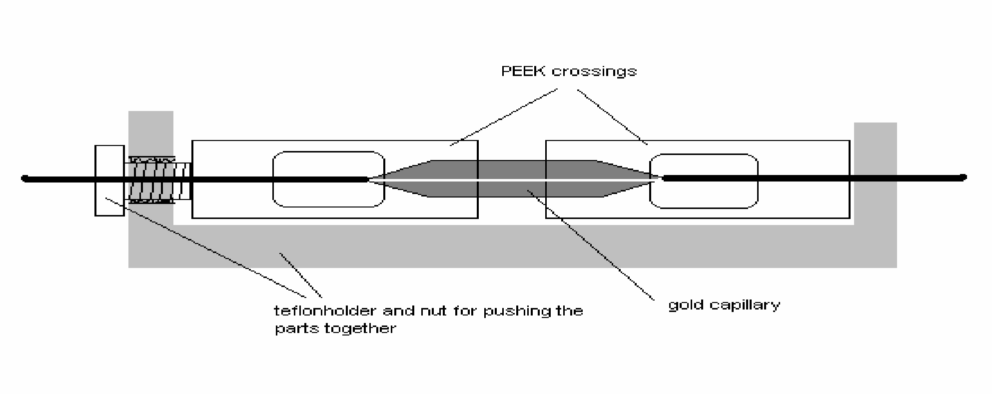


Figure 13. Side view of the Teflon holder and nut for pushing the two PEEK crossings together, enabling a simultaneous sealing and centreing of the gold capillary.

Out of the two PEEK crossings, four ends remain after using two ends for the optical fibers and two ends for the working electrode. This allowed two of the remaining ends to be used for the two counter-electrodes, one for the reference electrode, and one for the inlet. The outlet for the solution was drilled in the centre on the upper side of one of the PEEK crossings.

By using the well known PEEK low pressure tubing system with its cone-shaped connecting surface system in the extensions (except for the glued ceramic fritts in the counter and the reference compartments) reliable connections were obtained, while it was still possible to disconnect the different parts for cleaning. It should be mentioned that the integrity of the system was frequently tested by applying a water pressure (syringe) to all the connections of the PEEK crossings and the whole system in order to verify a non-leaking system. This is obviously the first crucial factor, which has to be observed. Were leakage to occur, various problems would arise air bubles could lead to unstable spectra hampering the interpretation. A schematic representation of a ferrule holding the optical fiber (surrounded and strengthened by a PEEK tubing) is shown in Fig. 14. The optical fibers had a slightly larger diameter (400 µm) than the capillary thus allowing some mis-centreing for the capillary-optical fiber assembly.

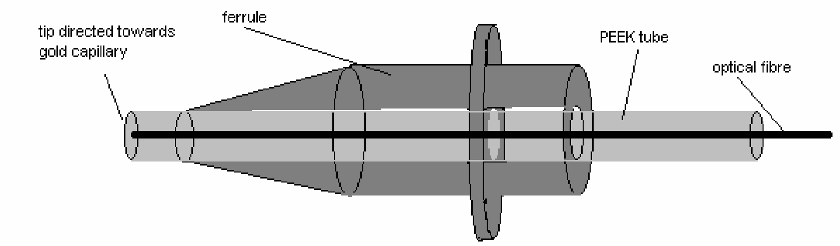


Figure 14. A ferrule with an inserted PEEK tube and the accompanied optical fiber.

Since it is crucial that there will be no enzyme solution which is not accessible for electrolysis in the optical pathway, the optical fiber was pushed all the way to the gold electrode. To ensure that the optical fiber was not blocking the capillary, but was still residing very close in order to ensure the same protein diffusion distances as in the inside of the capillary (half 300 µm), a slit was made in the tip of the PEEK tubing surrounding the optical fiber.

One problem with the ferrule surrounding the optical fiber was that when tightening the nut and the conical tightening system, the ferrule exerteda high pressure that could crush the glass optical fiber. To avoid this, a PEEK tube with a larger inner diameter than that of the outer diameter of the optical fiber was chosen. To be able to still obtain good centreing of the optical fiber in the PEEK tube, a smaller PEEK tube was inserted at the tip between the outer PEEK tube and the optical fiber. This smaller

PEEK tube had a perfect fit between both the optical fiber and the outer PEEK tube (Fig. 15).

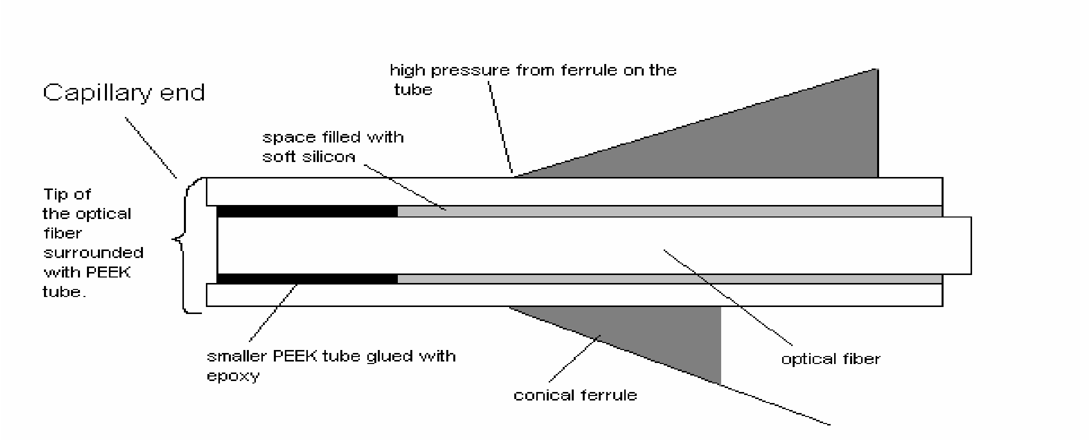


Figure 15. The optical fiber with surrounding PEEK tubes.

The connections for the counter electrodes and the reference electrode were easier to construct since their compartments were all separated with glued ceramic frits and hence no ferrules were involved. The ceramic frits located between the enzyme solution and the counter electrode solution kept the enzyme molecules in the cell apart from the buffer counter solution and while still allowing a current to flow between the working and the two platinum counter electrodes. Thus, when choosing the ceramic frits, the pore size is important to retain the enzyme molecules in the cell while and still having a moderate resistance. Too high a resistance would decrease the current between the working and the counter electrode due to the ohmic drop. The frits appeared to function well, although they were somewhat fragile.

A reference electrode was connected to the compartment for the reference electrode. This compartment was separated from the sample solution with a very thin ceramic frit. No potential drop was observed during slow scans (2 mV/s) in cyclic voltammetric experiments on ferrycyanide (data not shown). Additionally, the redox behavior of cytochrome *c* in spectroelectrochemical titrations showed perfect reversibility, revealing no unwanted ohmic resistances in the cell.

Electrode preparation

To achieve and facilitate sufficient electronic communication between the electrode and the redox proteins/enzymes the gold capillary was often pretreated with a layer of thiols (Fig. 16) [64], i.e., SAM (self assembled monolayer). The purpose of this was to orient the enzyme during interactions with the electrode surface to obtain the most advanteous position of the redox centre with respect to the surface and through this increasing the heterogeneous electron transfer rate as well as minimising the irreversible adsorption of the enzyme at the gold electrode. In order to achieve a well-defined immobilisation of the SAM-layer, the gold capillary electrode was cleaned in piranha solution for approximately 1 h. After rinsing thoroughly with Millipore water the cell was dipped for about 15 min in thiol solution. The cell was then thoroughly rinsed with buffer to eliminate residual thiol solution.

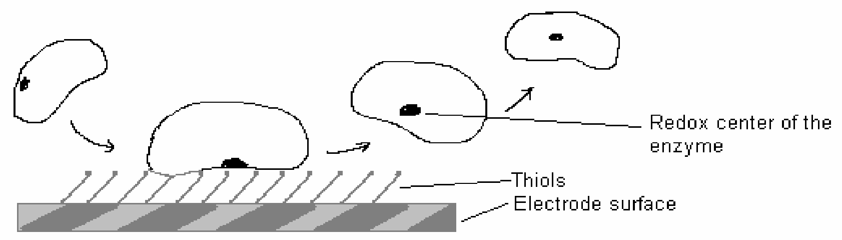


Figure 16. Schematic representation of a SAM-layer of thiols on the surface of the gold electrode orienting the redox site of the enzyme closer to the electrode surface during diffusing onto the electrode surface and away from it.

4. Results and discussion

4.1. Spectroelectrochemistry of proteins, enzymes, and biological complexes with single and multi redox centres

Several redox proteins, enzymes and complex II have been probed by the described spectroelectrochemical technique. As described above, an original cell construction has been fabricated and characterised. Spectroelectrochemical studies of cytochrome *c* were undertaken as a first step to evaluate the suitability of the spectroelectrochemical system for the investigation of DET and MET of redox enzymes. In later studies of theophylline oxidase, cytochrome P450 cam, and sulphite oxidase the objective was to spectroelectrochemically interrogate the existence or the absence of a direct electronic communication between these enzymes and a gold electrode. Spectroelectrochemical DET and MET measurements of azurin, laccases, and bilirubin oxidases were carried out in order to understand the mechanisms of heterogeneous ET of copper containing active sites in these redox proteins at gold electrodes. In the investigation of complex II the spectroelectrochemical measurements addressed the possibility of DET between the complex and a gold surface as well as the possible existence of an unknown quinone binding site in the structure of this complex. The following presentation of the main results is summarised in the context of known achievements from other research groups.

4.2. Cytochrome *c*: testing the spectroelectrochemical system

Horse heart cytochrome *c* was used as a simple, and well-known model heme-containing redox protein for testing our spectroelectrochemical system as well as for testing a procedure for titration. The protein (Fig. 17) contains a *c*-type heme prosthetic group located near the N-terminus [78]. The heme group is linked by two cysteins to the protein and the iron ion in the centre of the heme is complexed by histidine and methionine [115-117]. The iron is six-coordinated with two characteristic redox states, Fe^{2+} and Fe^{3+}. The natural function of cytochrome *c* is to serve as an electron carrier between redox enzymes in the respiratory chain [117]. The protein exhibits a spectrum that is dominated by the absorption of the heme prosthetic group. Two porphyrin $\pi \rightarrow \pi^*$ transitions give rise to the intense Soret band signal at 408 nm. One broad signal is found at 530 nm in protein's oxidised state. In its reduced state, the α-band and the β-band show sharp absorption bands at 550 nm and 521 nm, respectively. The $E^{0'}$ values of cytochrome *c* at pH 7.0 from different sources range from 260 to 280 mV vs. NHE [118,119].

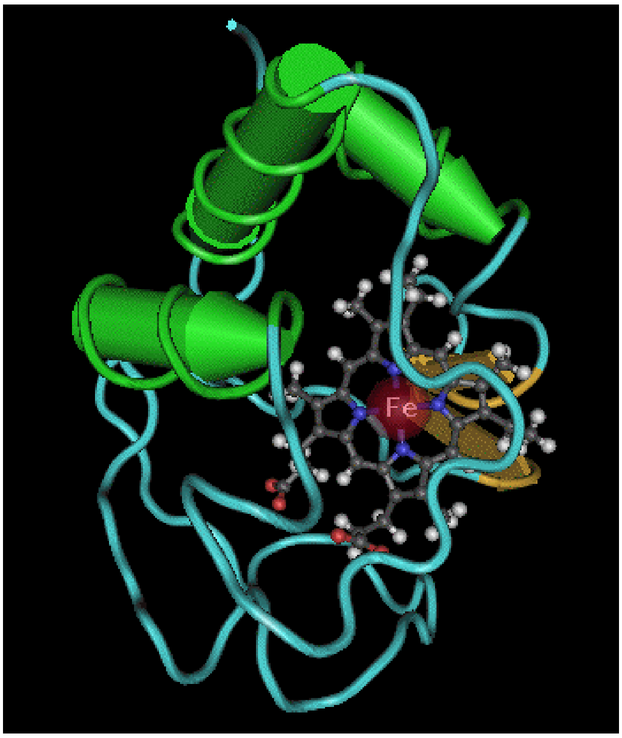


Figure 17. The structure of horse heart cytochrome c.

Detailed spectroelectrochemical studies of cytochrome *c* have previously been performed including both DET- and MET-based spectroelectrochemical investigations of the protein in solution [34,120-122] and in a surface confined or adsorbed state [123,124]. Two sources of cytochrome *c* are mostly used, namely horse heart and yeast. In these studies different spectral monitorings of the proteins were exploited, such as UV-Vis [34,125,126], Raman [121,124], FTIR [120], and X-ray spectroscopy [127], as well as their different combinations [122,123].

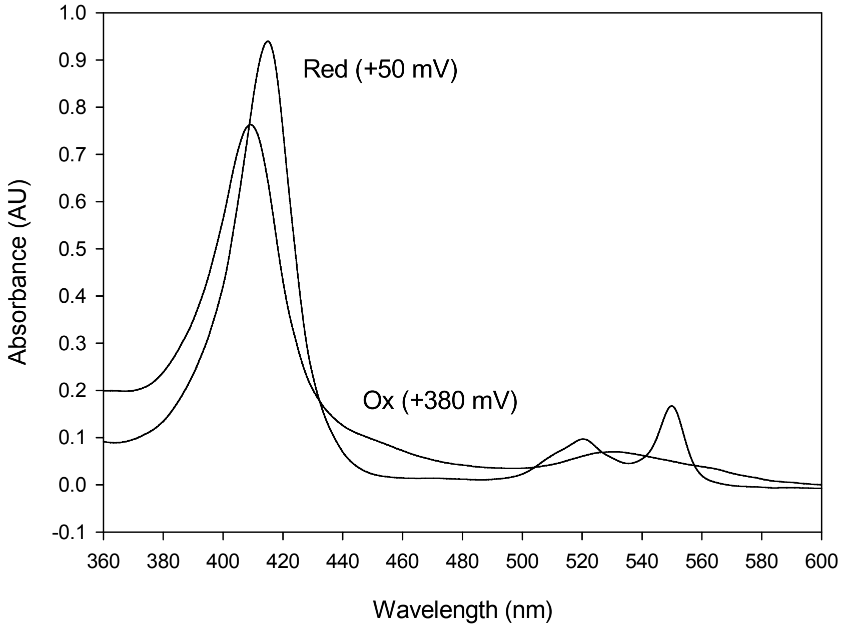


Figure 18. Spectra of oxidised and reduced (bovine heart) cytochrome c obtained by direct electron transfer between cytochrome c *and the gold capillary electrode (aldrithiol-modified capillary gold electrode, pH 7.0).*

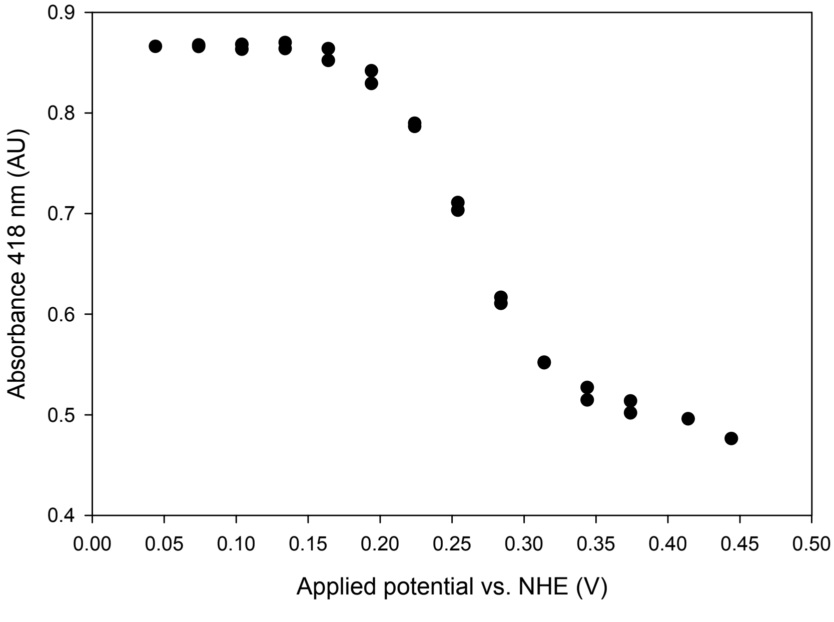


Figure 19. Absorbance vs. applied potential of the redox conversion of (bovine heart) cytochrome c based on direct ET at an aldrithiol-modified capillary gold electrode, pH 7.0.

The spectroelectrochemical techniques employed were used for measuring the redox potentials of nature and site-directed mutants of cytochrome *c* (e.g., [94,125,126]), for assessing electron transfer stoichiometry (*n*-values, i.e., number of electrons in DET or MET) and kinetics [96,124,128], for studies of the conformational changes of the proteins, e.g. [120,122,124], and even for structural analysis [127].

In the present work horse heart cytochrome *c* was used as a simple heme-containing redox protein in DET-based spectroelectrochemical studies. Reversible, fast, and reproducible DET-based electrochemistry of the protein inside an aldrithiol-modified capillary gold electrode was observed in our studies (Figs. 18 and 19) and the following parameters could be extracted from the titration curves, midpoint potential 256 mV and $n = 0.95$. The most important conclusion from these experiments is that the spectroelectrochemical system as described in the method for heterogeneous ET studies of this thesis is sufficiently good to conduct DET studies of proteins and enzymes.

4.3. Spectroelectrochemical evidence of DET for redox enzymes

In many cases the results from CV or amperometric measurements are presented as evidence for DET between redox proteins and electrodes. This is not a problem, when the DET process is well known or is observed at potentials which are in agreement with the formal potentials of the redox sites in the proteins of interest. However, if for example the redox potentials calculated from CV data deviate from known formal potential values for the proteins of interest, spectroelectrochemical measurements are needed for confirming the redox site which is in electron contact with the surface of the electrode. As an example redox conversion of a disulfide bond in the proteins can give CV data that are similar to a heterogeneous redox transformation of e.g. a heme site, as was found for horseradish peroxidise [35]. The following spectroelectrochemical studies of three redox proteins and enzymes were undertaken to prove the existence of DET between their active sites and electrodes.

4.3.1. Cytochrome P450 cam

Cytochrome P450 (EC 1.14.15.1) is a heme-thiolate containing monooxygenase, which can be found in all living organisms, including bacteria, fungi, plants, insects, and vertebrates [129]. In humans, many toxic hydrophobic compounds are usually metabolised by cyt P450. In addition, cyt P450 catalyses endogenous substances such as steroids, lipids, and cholesterols. The cyt P450cam studied in our experiments was first discovered in 1959 by Bradshaw and co-authors [130] and is an oxidoreductase isolated from the bacterium *Psoudomonas putida*. The enzyme was later crystallised and the 3-D structure obtained by Poulos [131]. The structure of cyt P450cam used in the present study is shown in Fig. 20. The protein consists of 414 amino acids with a molecular weight of 46.6 kDa.

Figure 20. The structure of cytochrome P450cam.

The redox potential of cyt P450cam is a characteristic parameter, which significantly influences the catalytic turnover [132]. Thus, a number of publications describe determination of the redox potentials of the wild type and mutant forms of cyt P450cam by common mediated redox titration followed by optical spectroscopy to determine oxidation of the ferrous heme to ferric heme during the titration, e.g. [133]. Moreover, MET-based spectroelectrochemistry of the enzymes using a thin-layer spectroelectrochemical technique was also performed, e.g. [134]. Despite this, there is a large variation in the reported values of the redox potentials. For substrate-free cyt P450cam, the values have been reported to range from –270 mV to –330 mV vs. NHE [134-138]. Upon saturation with camphor, it shifts to more positive values ranging from –140 to –210 mV [134-137]. Experimental uncertainties in the methods used to determine potentials have been implicated for such a wide variation [139]. From the above it is obvious that interpretation of DET measurements is not simple and spectroelectrochemical studies should follow to explain electrochemical measurements involving redox conversion of cyt P450.

The major aim of our spectroelectrochemical investigation was to find out whether a DET reaction between a gold electrode and an active protein can be established. This property is, e.g., important in the development of biosensors for environmental hazards. We found that cyt P450 cam is catalytically active after DET is established with an aldrithiol-coated gold electrode. An estimation of the formal potential for the enzyme was determined to be equal to -130 mV vs. NHE. Detailed information about this study is presented in Paper 1.

4.3.2. Theophylline oxidase

The major aim of our spectroelectrochemical investigation of theophylline oxidase (ThOx) was to find out whether this enzyme could establish DET with electrodes for later development of biosensors based on the DET principal [140]. As ThOx is a multicofactor redox enzyme containing one catalytic domain with an unknown cofactor as well as heme; it was believed that the heme would serve as a “built in mediator” facilitating DET characteristics with electrode in accordance with what Ikeda et al. described in 1993 for alcohol PQQ-dehydrogenase [141]. Cytochrome *c* or ferrocene mediators were exploited in previously published ThOx-based amperometric biosensors for detection of theophylline [142,143]. Such studies were motivated because of the importance of this compound as a drug that is widely used in the treatment of asthmatic and other bronchospastic diseases [144,145]. ThOx is a heme-containing complex redox metalloenzyme that catalyses the metabolic oxidation of theophylline to 1,3-dimethyluricacid. In the catalytic oxidation, the presence of oxygen is not needed and the enzyme can be reoxidised by its natural redox partner, cytochrome *c*. Microbial ThOx was isolated by GDS Technology

[146]; however, no detailed kinetic characterisation of ThOx, as well as no data on the crystal structure of ThOx and the number and nature of the redox sites or domains were ever presented.

DET between ThOx and the surface of an aldrithiol-modified gold electrode was confirmed without doubt using spectroelectrochemistry (Paper 2). The spectral recordings demonstrated that DET redox conversion of the heme prosthetic group of the enzyme had taken place (Fig. 5, Paper 2). When several sets of redox titrations were plotted in accordance with Nernst equation, a scattered picture of the redox titrations was observed, and the formal redox potential was roughly estimated to be -75 vs. NHE at pH 7.0. A very slow heterogeneous electron transfer resulted in the fact that no Nernstian equilibrium was reached during the measurements. This limitation is reflected in the number of electrons equal to only 0.53 and obviously contributing to the error in the estimation of the value of the formal potential. It was suggested that the reason for the non linearity (a tendency of two slopes) of the data in Fig. 6 (Paper 2) could be due to a second redox site yielding all together a double redox process. Future studies were carried out to clarify the mechanisms behind the mentioned results.

The midpoint potential of +90 mV vs. NHE was obtained in CV measurements of the enzyme adsorbed on an alkanethiol-modified planar gold electrode [147]. This value, probably, belongs to the heme group of adsorbed ThOx, because heme is usually the first electron acceptor/donor in the case of DET reactions of the complex heme-containing redox enzymes [24]. Thus, the titration curves presented in Fig. 6 (Paper 2) can be reconsidered as two blurry redox processes with midpoint potentials of ca. 0 mV and -200 mV. The highest value, most likely, is the redox potential of the heme of ThOx in the solution, whereas the origin of the second redox pair is still unknown.

4.3.3. Sulphite oxidase

Sulphite oxidase (SOx) (EC 1.8.3.1) contains a Mo complex cofactor (MoCo) and a cytochrome b_5-type heme cofactor (Fig. 21). SOx is an intermembrane redox enzyme, which catalyses the two electron oxidation of the natural substrate sulphite

(SO_3^{2-}) to sulphate (SO_4^{2-}) [148]. The enzyme is present in many species including animals, where the rat, chicken and human enzymes have been studied extensively [149-152]. SOx catalyses a physiological highly important degradation of sulphur containing amino acids, such as cysteine and methionine, as well as it is also involved in sulphite and sulphur detoxification [153]. The oxidation of sulphite to sulphate takes place at the MoCo centre with concomitant reduction of Mo(VI) to Mo(IV). The reducing equivalents are passed further to the cyt *b5* heme of the enzyme, and from there to cyt *c*. It is believed that cyt *c* is the physiological electron acceptor for SOx. However, oxygen and transition metal complexes can also function as artificial electron acceptors of the enzyme [154].

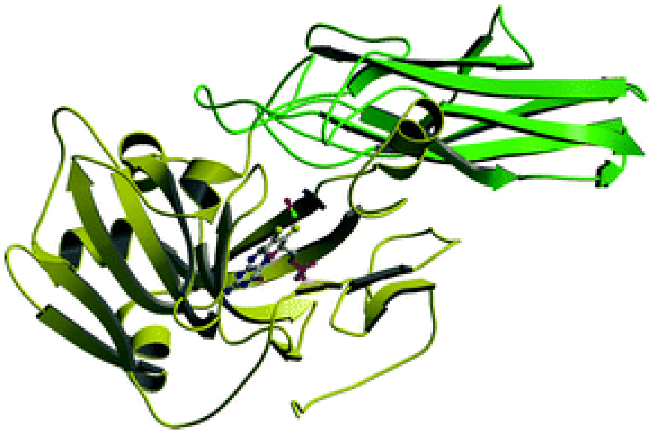


Figure 21. The structure of chicken sulphite oxidase.

The redox potentials of the MoCo centre of SOx were previously determined by potentiometric titration using low-temperature and room-temperature EPR spectroscopy [155]. It was found that the values of the potentials are strongly dependent on pH and the presence of coordinating anions [156]. The midpoint potentials for the Mo(VI/V) couple for the inhibited (low pH, high concentration of anions) and uninhibited (high pH, no coordinating anions) forms of the enzyme were reported as 131 mV and -57 mV vs. NHE, respectively; and for the Mo(V/IV) couple as 86 mV and -233 mV, respectively [156]. The potentials of the heme group of SOx from different sources (chicken liver and beef liver) were also measured using a MET-based UV-VIS spectroelectrochemistry and CV. They were found to be ~90 mV and ~50 mV for the inhibited and uninhibited forms of the enzyme, respectively [61,155-157].

The aim of our work was to obtain a catalytic DET reaction between the enzyme and a gold electrode in the presence of substrate, which has been somewhat unclear [158-160]. DET was proven by the conducted spectroelectrochemical experiments as well as by cyclic voltammetry. Furthermore, the formal potential of the heme centre was found to be ca. 85 mV vs. NHE in an $n = 0.98$ redox transformation. Our spectroelectrochemical measurements have also proven that the heme domain of the enzyme is responsible for DET at the gold electrode. For more information about this study, the reader is referred to Paper 3.

4.4. Spectroelectrochemistry of copper redox centres in proteins and enzymes

4.4.1. Azurin

Spectroelectrochemical studies of azurin were carried out to examine the spectroelectrochemical features of the relatively simple protein with a single copper redox centre as well as for additional testing of our spectroelectrochemical system as well as procedure of titration. This work has given substantial experience and data for interpretation of spectroelectrochemical results of laccases and billirubin oxidases hosting multicopper redox centres, see below and Papers IV, V, VI.

Azurin belongs to a family of small blue-copper proteins (9-14 kDa) functioning as electron carriers in electron-transfer chains of plants and bacteria [161]. It contains a mononuclear copper of the T1 type [162,163]. The T1 copper site exhibits several distinguishing spectroscopic characteristics, which include an intense blue colour (in its oxidised state) with an absorption band in the visible region of *ca.* 600 nm. The protein was crystallised and the 3-D structure of *Pseudomonas aeruginosa* azurin is presented in Fig. 22. The copper ion is coordinated by one cysteine, two histidines, and one axial methionine [65,161,164-166]. The electron transfer function involves a redox state transition of the copper ion between +1 and +2.

The wild type and several different mutants of the protein have been intensively studied using spectroelectrochemistry [167-169], including a mediated thin-layer electrolysis technique [170]. The main purpose of these studies was to determine the redox potentials of azurins from different sources in order to understand the possible effect of the copper-ligand interactions to the redox potential based on the electrochemical and spectral properties of the protein.

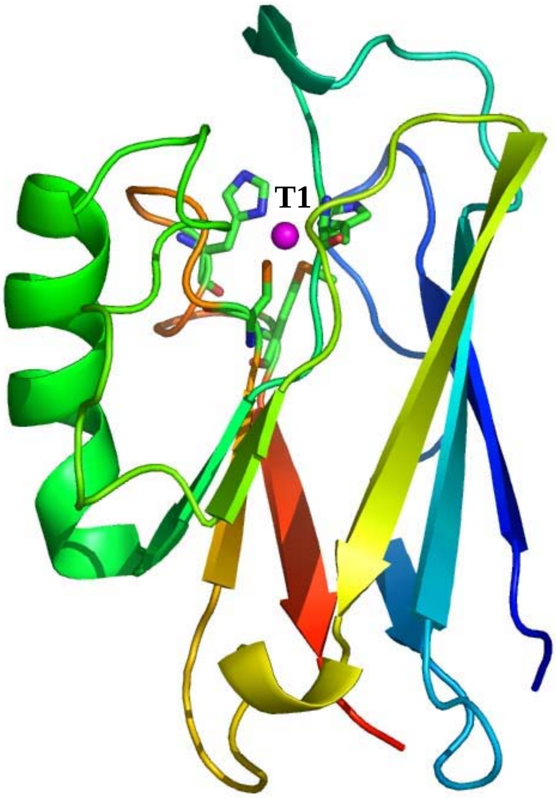


Figure 22. The structure of Pseudomonas aeruginosa azurin.

The redox potentials of the T1 site of azurin from different sources were estimated and found to be in the range from 270 to 320 vs. NHE [162,171,172]. In spite of many publications describing DET between azurin and different types of electrodes, e.g. [163,173-175], to our best knowledge, DET-based spectroelectrochemical studies of the protein was not performed. In our spectroelectrochemical studies *Pseudomonas aeruginosa* azurin was used as the model small T1 site-containing protein.

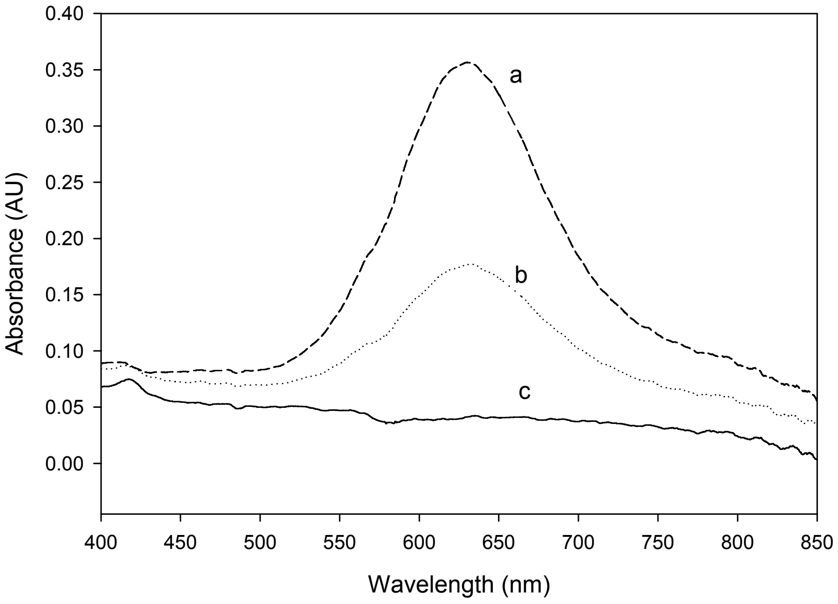


Figure 23. Spectra of azurin from Pseudomonas aeruginosa from DET titration corresponding to oxidised (spectra a), half oxidised (spectra b), and fully reduced (spectra c) enzyme.

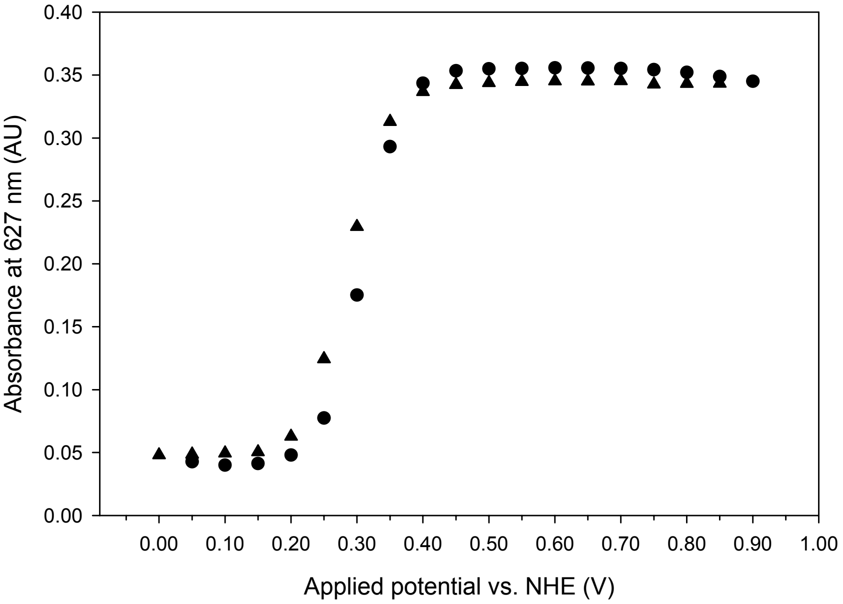


Figure 24. Absorbance vs. applied potential of the DET redox titration experiment of Pseudomonas aeruginosa azurin (bare gold capillary electrode, pH 7.0). (●) *represent the oxidative titration and* (▲) *represent the reductive titration.*

Direct (mediatorless) redox titrations of azurin have been performed in our studies. This was achieved by injecting the azurin solution inside the gold capillary electrode followed by stepwise changing the applied potential for electrolysis for electrolysis to occur and simultaneously the spectral changes were followed. Reversible, fast, and reproducible DET-based electrochemistry of the redox protein inside the bare gold capillary electrode was observed (Figs. 23 and 24). Spectroelectrochemical data for the solution containing azurin without any mediators inside the capillary clearly show that the blue colour vanishes when the applied potential is switched from +900 mV to + 0 mV vs. NHE. The fading of the colour can only be explained by the direct reduction of the blue copper site. Typical absorbance spectra of the oxidised, partly reduced, and fully reduced form of the redox protein in the absence of any mediators are presented in Fig. 23. The spectroelectrochemical titration curve (optical absorbance vs. applied potential) is shown in Fig. 24. From the data obtained in this experiment it is possible to extract the $E^{0'}$ of the T1 site and n, which were found to be 307 mV vs. NHE and $n = 0.92$. These values are in very good agreement with previously published data [162,171,172,176].

The DET-based studies of the T1 site containing redox protein additionally confirmed that our spectroelectrochemical equipment is sufficiently good to conduct DET studies of redox proteins and enzymes. Another important conclusion from this test studies was that DET-based redox titration of the simple copper-containing protein showed a single redox Nernstian dependence (slope) with excellent titration parameters.

4.4.2. Laccase

Laccase (*p*-diphenol:oxygen oxidoreductase; EC 1.10.3.2) is a multi-copper oxidase that catalyses the oxidation of different aromatic compounds and some inorganic substances with the concomitant reduction of dioxygen directly to water without the formation of highly reactive oxygen species [177,178]. Laccases are found in plant [179], fungi [180], bacteria [181], and insects [182]. In plants laccase is known to be involved in lignin biosynthesis [183], whereas in fungi, laccase is involved in lignin degradation [184,185]. Owing to the catalytic and electrochemical properties laccases possess, the enzyme is of interest in different areas of biotechnology, like biobleaching [186], bioremediation and biodegradation [186-188], biosensors [64,177,189-193], and biofuel cells [194-197].

The 3-D structure of laccase has been solved only for a limited number of the enzymes from fungi [198-201] and bacterium [202]. The crystal structure of *Trametes versicolor* laccase is presented in Fig. 25.

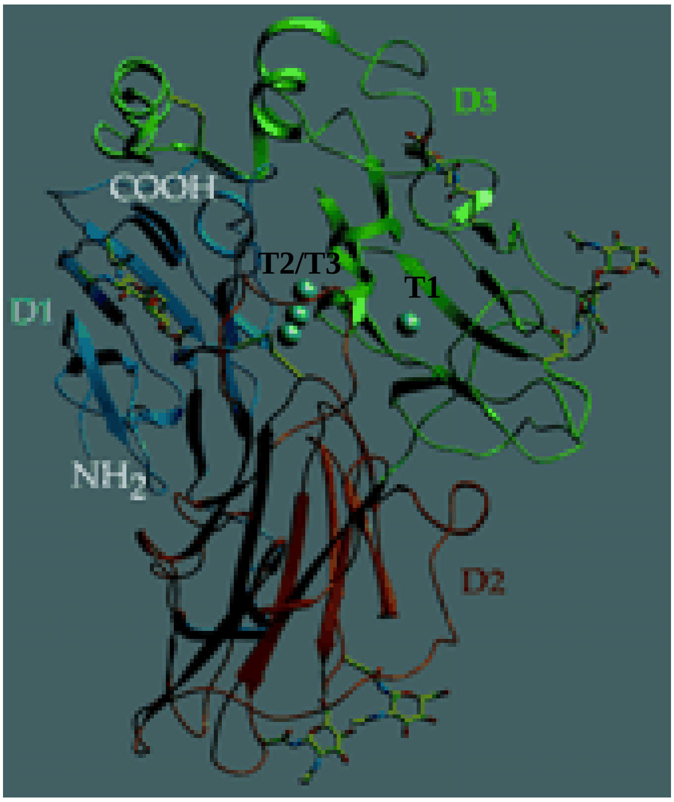


Figure 25. Crystal structure of Trametes versicolor laccase.

Since the work reported herein is focused to a large extent on the metal centres of two blue multicopper oxidases, namely laccases and bilirubin oxidases, the current picture of these centres, which are classified into three types according to their spectroscopic characteristics [178], should be briefly reviewed. The Type 1 (T1) centre, which is the electron donor oxidising site, is characterised by an intense blue absorption maximum at approximately 610 nm arising from Cys S → Cu charge-transfer (CT) transitions as well as an EPR signal displaying a small hyperfine coupling. The Cys ligand at the T1 Cu is flanked on either side in the polypeptide sequence by histidines, which in turn are ligands to the Cu atoms located at the binuclear the Type 3 (T3) centre. The bi-nuclear T3 site is diamagnetic, containing two antiferromagnetically-coupled copper atoms, and therefore it is EPR-silent, but the site does exhibit a characteristic 330 nm absorption shoulder arising from CT transitions of an OH bridging between the two Cu (II) ions and displays a characteristic fluorescence spectrum. The oxidised Type 2 (T2) centre exhibits a Cu (II) EPR signal. The T2 and T3 centres are close to each other and, thus, form the so-called trinuclear Cu cluster, which comprises the site of dioxygen reduction (Fig. 25).

For all blue multicopper oxidases, including laccase and bilirubin oxidase, the copper ligands for the T2/T3 cluster are exclusively histidines, whereas for the T1 site cysteine, two histidines, and different axial ligands, depending on the source of the enzyme [65]. E.g. methionine is an axial ligand in the case of tree laccases, some bilirubin oxidases, ascorbate oxidase, and the T1A site of ceruloplasmin. Leucine is the axial ligand in the T1 site of some fungal laccase and the T1B site of ceruloplasmine, whereas a phenylalanine is the axial ligand in the case of high redox potential fungal laccases and bilirubin oxidases.

A rather high redox potential of the T1 site, ranging from 430 mV for tree laccases up to 790 mV for the enzymes isolated from fungi [65], enables the enzymes to be an efficient electron acceptor for different substrates. The measurements of the redox potential of the T3 copper were performed by Reinhammer and Vänngård, two Swedish researchers from Göteborg, in 1971-1972 [87,88]. The values of the T3 copper site of *Rhus vernicifera* low-redox potential tree laccase and *Polyporus versicolor* high redox potential fungal laccase were found to be 480 and 780 mV vs. NHE, respectively [88]. The redox potential of the T2 site was, though, estimated only approximately for the low-potential laccase and was found to be about 390 mV vs. NHE [87,88]. In this thesis we assessed the redox potential of the T2 site of a high-potential laccase (Paper 4).

A number of MET-based spectroelectrochemical studies of laccases from different sources have been previously performed in order to determine the redox potentials of the T1, T2, and T3 sites of the enzymes, their pH and temperature dependence (e.g., [87,88,203-205,206, Papers 4 and 5). Moreover, the redox potentials of the T1 site of multiforms of laccase were also measured using MET-based spectroelectrochemistry ({Schneider, 1999 #213,207,208], Paper 5). It should be noticed that laccase is the first enzyme for which direct electrochemistry was reported as early as in 1978 [35]. After that time numerous papers about DET-based electrochemistry of laccases from different sources were published (see our recent review [65]) due to possible applications of the enzyme in biosensors and biofuel cells. In spite of this fact no DET-based spectroelectrochemistry of laccases has been reported till 2005 (Paper 4), as well as the redox potential of the T2 site for fungal high redox potential laccases was unknown (Paper 4).

For biotechnological applications high-potential (the T1 site) fungal laccases, such as those from *Trametes versicolor, Trametes hirsuta, Cerrena maxima*, are important [206]. To understand heterogeneous and intramolecular ET processes in laccases numerous spectroelectrochemical studies of *Trametes hirsuta* laccase have been performed during the time of my PhD studies. The redox titration of the T1 site of the enzyme using $K_3[Mo(CN)_8]/K_4[Mo(CN)_8]$ as redox couple resulted in the expected value of the redox potential of 780 mV and with an *n* equal to 1.0 (Fig. 26).

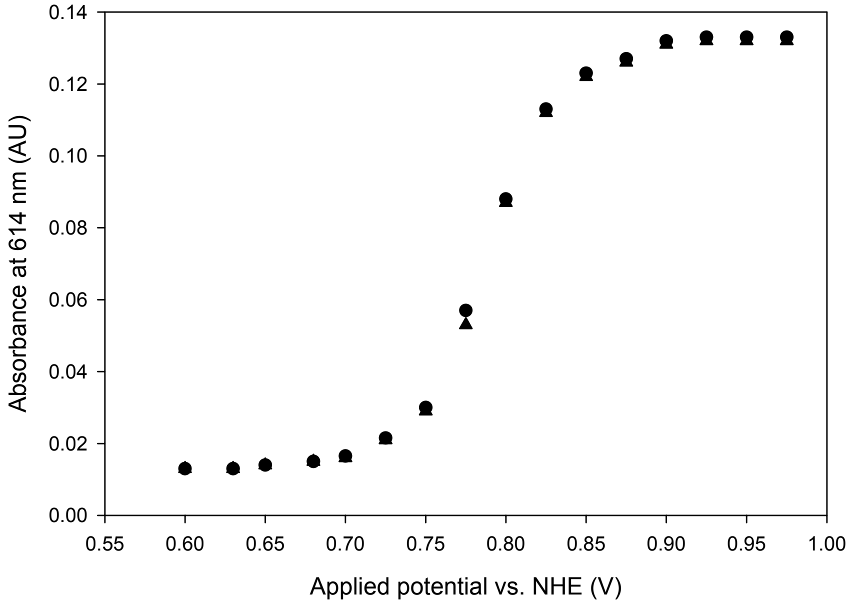


Figure 26. Absorbance vs. applied potential, representing the Mediated redox titration of Trametes hirsuta laccase. (●) represent the oxidative titration and (▲) represent the reductive titration. (0.02 mM laccase and 0.02 mM $K_4[Mo(CN)_8]$, pH 5.0).

However, the titration of the same T1 site by exploiting DET resulted in an absorbance-potential relationship with well-pronounced hysteresis (Paper 4, Fig. 4B). This hysteresis could be interpreted as a poor electron transfer and a non Nernstian equilibrium situation. However, the absorbance change going from the reduced to the oxidised enzyme gave a 0.9 e^- process with a redox potential of around 800 mV, parameters that are very close to those obtained for MET-base T1 redox titrations. From this argument we concluded that an additional redox centre with a redox potential of 400 mV vs. NHE is involved in the DET between the T1 site and the gold surface. The origin of this redox centre was ascribed to the T2 copper based on CV measurements and EPR-spectroelectrochemistry (Paper 4, [54,55]). Several other fungal laccases, such as those from *Trametes ochracea* and *Cerrena maxima*, were also spectroelectrochemically studied for investigations of their DET characteristics on gold. The titrations always gave hysteresis-like dependencies of the absorbance vs. applied potential.

To summarise the results the mechanism of DET of laccases on gold was proposed as presented in Fig. 27. From this figure it can be seen that the laccase-Au electronic communication is totally different from that for laccase-carbon. When laccase is adsorbed on a carbon electrode the natural electron transfer pathway (T1 site → T2/T3) is realised resulting in the bioelectrocatalytic activity of the enzyme (Fig. 27), e.g. a well-pronounced high potential bioelectroreduction of oxygen [53]. However, when laccase is immobilised on gold, the T2/T3 cluster is the first electron acceptor (Fig. 27). Thus, bioelectrocatalytic reduction of oxygen starting at the potential of the T1 site cannot be achieved [54,55]. Moreover, the reduction of oxygen to hydrogen peroxide is realised in the case of laccase directly adsorbed on bare gold electrodes [54]. Furthermore, during this work the hypothesis that the redox potential of the T2 copper site in both low- and high-potential laccases and possibly other multicopper oxidases might be very similar, i.e. approximately 400 mV, was proposed. To test this hypothesis, additional spectroelectrochemical studies of another blue multicopper oxidases, bilirubin oxidases, were carried out.

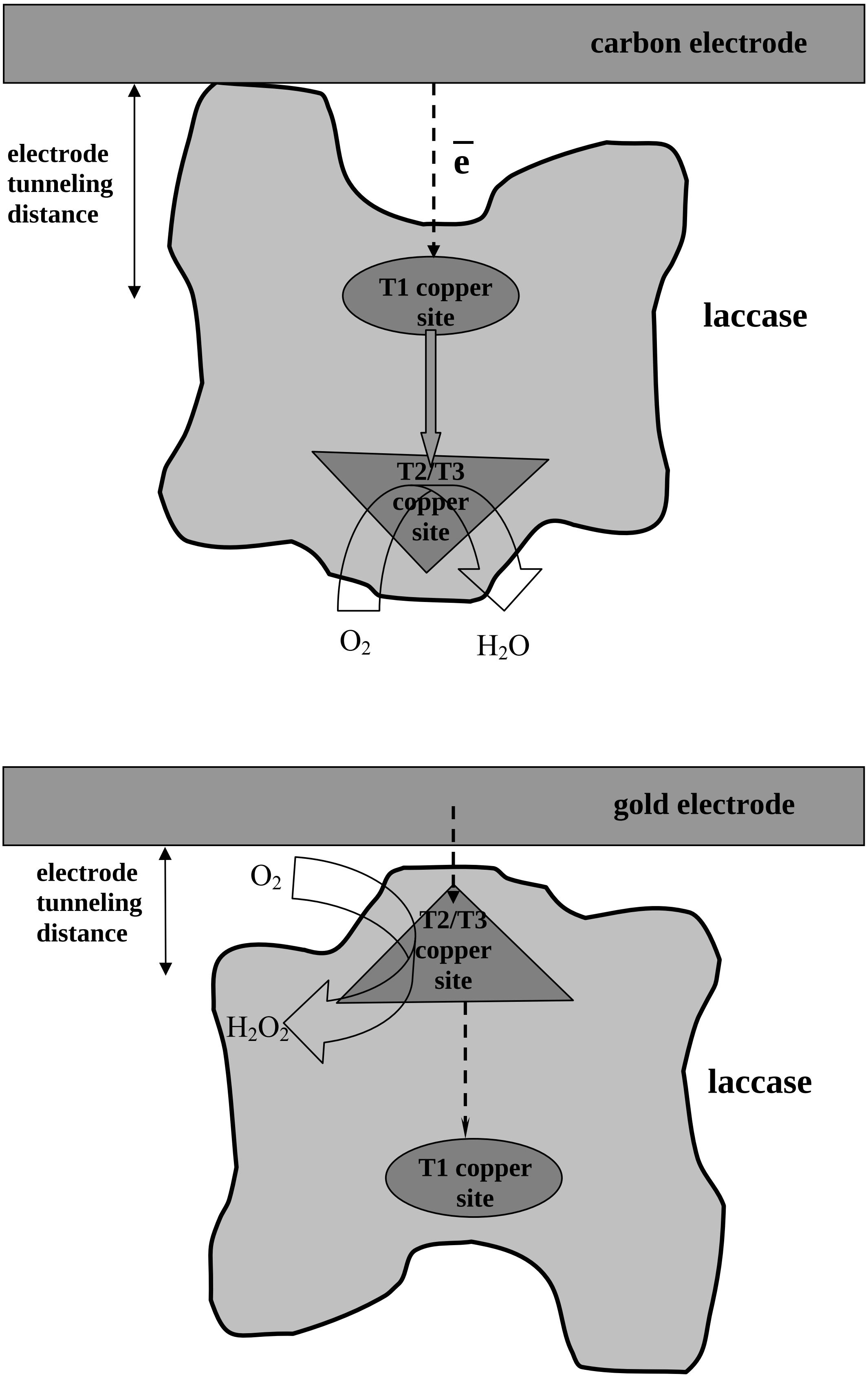


Figure 24. Proposed schemes for electroreduction of oxygen at a carbon electrode with adsorbed laccase (top) and redox transformation of the enzyme on gold electrode (bottom).

4.4.3. Bilirubin oxidase

Bilirubin oxidase (BOD) (bilirubin:oxygen oxidoreductase, EC 1.10.3.2) is a blue multi-copper oxidase catalysing the oxidation of bilirubin to biliverdin as well as others tetrapyrroles, diphenols, and aryl diamines with the concomitant reduction of molecular oxygen to water [178]. The 3-D structure of any BOD has not been solved yet. However, based on the known biochemical and spectral information it is believed that BOD contains four copper ions per molecule, classified into three types: one type 1 (T1), one type 2 (T2) and two type 3 (T3), and that is in analogy with ascorbate oxidase, laccase, and ceruloplasmin [178,209]. As all other blue multicopper oxidases this enzyme exhibits a strong absorption peak near 600 nm originating from the T1 site and a shoulder at 330 nm originating from the T3 site [210-214].

Two BODs from the fungi *Myrothecium verrucaria* and *Trachyderma tsunodae* were used in the present study for the following reasons. First of all, only these enzymes are well characterised. For instance, detailed biochemical, spectral, and kinetic characterisations of the two BODs were previously performed [204,210-213,215-218]. The primary structures of *M. verrucaria* and *T. tsunodae* BODs have been published (see GenBank website). Furthermore, the accessibility of two highly purified and concentrated BOD preparations obtained via collaboration with the University of Texas, Austin, USA (Prof. Adam Heller and Dr. Nicolas Mano) was also a determining factor. Finally, large discrepancies between the values of the redox potentials of the T1 site for identical BODs were previously reported, e.g., ~180 mV difference between the reported values of the T1 sites of *M. verrucaria* BOD and ~95 mV of *T. tsunodae* BOD (see Table 2 in Paper 6). It is important to note that the ligands of the copper ion of the T1 site of *T. tsunodae* BOD are those found in high redox potential laccases (e.g. *T. versicolor* and *T. hirsuta* [65,201,206], Paper 4), e.g., Cys2HisPhe [214,218]. Thus, the previously reported high values of the redox potential of the T1 site of this enzyme (615-710 mV vs. NHE) are expected [113,214]. However, the ligands of the T1 copper for *M. verrucaria* are Cys2HisMet, similar to those found in low-potential laccases, ascorbate oxidase, and ceruloplasmin [209,216,217,219]. The estimation of the value of the redox potential of the T1 site of *M. verrucaria* BOD (480 mV [204]) is consistent with the values measured for other low-potential oxidases. However, two other studies reported more positive redox potentials (570 mV and 660 mV vs. NHE) for this enzyme [113,209]. Thus, an additional aim of the spectroelectrochemical studies of BODs was to carefully determine the redox potentials of the T1 sites of both BODs and to reconcile the differences in the reported values.

The first task of these spectroelectrochemical studies was, however, the investigation of DET between BOD and gold surface. It should be noticed that BOD is used nowadays for the development of MET- and DET-based cathodes for biofuel cells [220-225] targeting their implantation [226,227]. The enzyme is not inhibited by physiological concentrations such as chloride ions as well as it has one of its maxima of bioelectrocatalytic activity at neutral pH [206]. Establishment of efficient DET of BOD would offer the opportunity to design biofuel cells with a maximal open circuit voltage since the electrode processes will occur with minimal overvoltage [228].

In order to get precise information about the values of the redox potentials of the T1 sites of *T. tsunodae* and *M. verrucaria* BODs sufficient electron transfer communication should be established between the electrode and enzymes. Thus, small redox mediators, $K_4[Fe(CN)_6]$ and $K_4[Mo(CN)_8]$, which are optically not absorbing above 500 nm and cover a broad potential range (300 mV - 900 mV) were used for titration. Each redox titration was carried out in two directions, i.e. from the fully oxidised to the fully reduced states of the enzymes and vice versa (Figs. 1 and 2, Paper 6). The redox potentials of the T1 site of *T. tsunodae* and *M. verrucaria* BODs were found to be 660 mV and 670 mV, respectively. A very interesting pattern of MET was found in the case of both enzymes, but especially in MET titration of *T. tsunodae* BOD. It should be emphasised that *n*-values of the redox titrations (0.66 and 0.80 for *T. tsunodae* and *M. verrucaria* BODs, respectively) were far away from the expected one electron. In addition, a sigmoidal Nernstian plot of the titration curves was obtained (Figs. 1 and 2, Paper 6). Taking into account the fact that the oxidative and the reductive titrations almost coincided it was suggested that a redox transformation of the two independent, closely spaced one-electron redox pairs were monitored in our experiments. One of these pairs would be the T1 site of the enzyme and the origin of the other one was clarified in further DET spectroelectrochemical studies (Paper 6).

The mediatorless redox titrations presented in Figs. 3 and 4 (Paper 6) revealed undoubted proof of DET on Aufor both BODs. Moreover, a clear hysteresis, corresponding to a diode-like behaviour of the enzymes, was also observed. Furthermore, DET-based spectroelectrochemistry of *M. verrucaria* BOD resulted in several slopes on the titration curves (Fig. 3A, Paper 6). A low redox potential process with a midpoint potential of about 390 mV vs. NHE, similar to the one that was obtained during the reductive titration of laccase (Fig. 4, Paper 4), was also found in the case of *T. tsunodae* BOD indicating that there might be another one than the T1 redox component participating in the redox transformation monitored at 610 nm. These findings along with the additional CV measurements [57] confirmed the previously proposed hypothesis about the presence of a low redox potential copper site in all blue multicopper oxidases including high redox potential enzymes.

4.4.5. Succinate:quinone oxidoreductase (Complex II)

Succinate:quinone oxidoreductase, SQR, is also referred to as respiratory chain Complex II, EC 1.3.5.1. SQR is present in the respiratory chain of mitochondria and in many microorganisms [20]. SQR and quinol:fumarate reductase (QFR) comprise a family of enzymes and have a preserved general structure in both pro- and eukaryotes. SQR catalyses a two electron succinate oxidation, transferring the electrons to the quinone binding site(s) for concomitant two electrons reduction of quinone to quinol. QFRs catalyses the reverse reaction. Generally both SQRs and QFRs can catalyse both the forward and the reverse reaction depending on conditions, i.e. the redox potential of the quinones used in the assay. The high resolution structure of *E. coli* SQR, that contains heme*,* was solved by Yankovskaya and coauthors in 2003 [229]. In addition, the structure of two QFRs, the QFR from *E. coli* (that lacks heme, [230]) and the QFR from *Wolinella succinogenes* (that contains two hemes, [231]) are known. The latter enzyme closely resembles *Bacillus subtilis* SQR that has been studied in this work (Fig. 28).

The extrinsic part of the SQR enzyme complex, i.e., succinate dehydrogenase (SDH), is composed of a flavoprotein (FP) containing a covalently bound FAD and an iron-sulphur protein (IP) harbouring three iron-sulphur clusters. At the FAD catalytic site succinate oxidation or fumarate reduction occurs. The extrinsic part of the enzyme, i.e. SDH, shows a high degree of primary sequence conservation, even though the redox potentials of the iron-sulphur clusters are differently tuned in SQRs and QFRs [20]. The membrane anchor domain shows a much greater primary sequence variability and may consist of one larger or two smaller polypeptides and the domain can contain one or two hemes of cytochrome *b* type with bis-histidine axial ligation, or may lack heme [232]. Nevertheless, the structure of the membrane anchor domain is well conserved. When two hemes are present they are ligated by histidine residues located on individual transmembrane helices, forming a four helix bundle with the hemes located on top of each other perpendicular to the membrane plane [20]. This cytochrome *b* structure, fundamentally different from that of cytochrome *b* of the bc_1 complex, was confirmed by the high resolution structure of *Wolinella succinogenes SQR* [231], that has a membrane anchor domain composed of one polypeptide that contain two hemes. A comparison with the high resolution structure of *E. coli* QFR, that has an anchor domain composed of two polypeptides but lacking heme, shows that the membrane spanning domains are indeed structurally conserved in these two extreme cases [230,231]. The classical mitochondrial SQR that catalyses succinate oxidation and ubiquinone (E_m = +120 mV) reduction contains one heme group in the membrane spanning domain, whereas SQRs that operate with menaquinone (E_m = -75 mV) always contain two hemes. QFRs, on the other hand, are always using a low potential quinone as electron donor, and may come in any variety. It has been postulated that two hemes are necessary in the SQR enzymes that catalyse menaquinone reduction by succinate and thus rely on energy from the transmembrane electrochemical potential for function [20].

The arrangement of two hemes with different redox potentials ligated on top of each other predicts that redox events may trigger protein conformational changes related to enzyme function. At physiological pH, only the proximal, high-potential heme group of diheme SQRs is reduced by succinate, whereas the distal, low potential heme group remains in its oxidised state [233]. Thus, it is particularly interesting to undertake further investigations on the electrochemical behaviour of the diheme SQRs. Spectroelectrochemical (redox mediator potentiometry) measurements of the two hemes in *Bacillus subtilis* complex II was found to be + 64 mV for heme $_H$ and -96 mV for heme $_L$ [234,235]. In addition, redox mediator EPR titrations were utilised for the determination of the redox potentials of Fe-S centres [236].

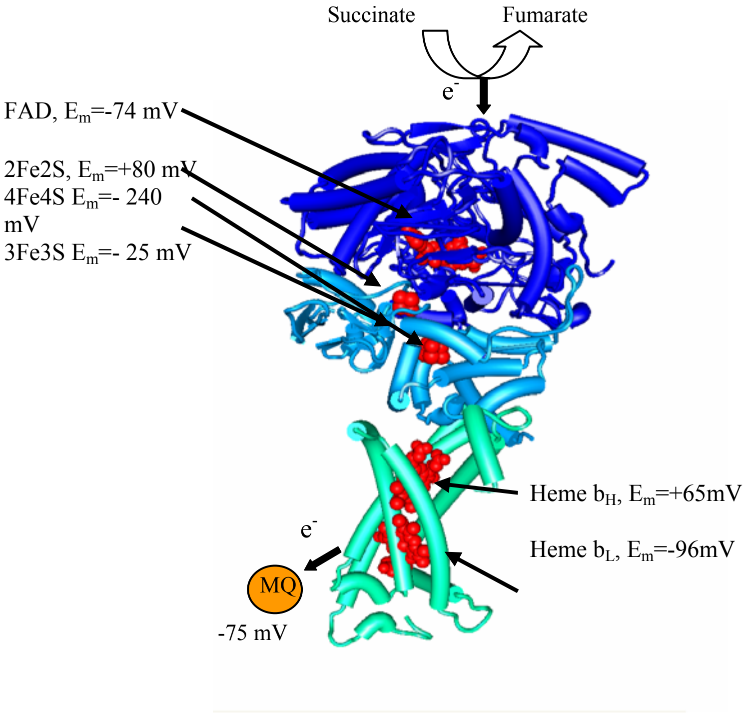


Figure 28. Schematic representation of the structure of the W. succinogenes QFR (figure kindly provided by Tobias Gustavsson).

The aim of this work was initially to study electron transfer reactions between an electrode and intact SQR by UV-Vis spectroelectrochemistry. The reason for the choice of spectroelectrochemistry was that this technique enabled to control the driving force (by the applied potential of the electrode) and simultaneously to follow the redox states of the redox enzyme by the spectral changes of the distal and proximal hemes. The redox potentials of both hemes, heme$_H$ and heme$_{L,}$ were determined using MET-based spectroelectrochemical titration and found to be +65 mV and –96 mV, respectively. These values are in very good agreement with the midpoint potentials previously reported for these hemes using the conventional mediated redox titrations [233].

Then the experiments were repeated in the absence of mediators. Direct heterogeneous electron transfer between the enzyme and a thiol coated gold electrode was seen. Interestingly, reduction of both hemes occurred roughly at the midpoint potential of heme $_L$. Reoxidation, on the other hand, showed a peculiar hysteresis, with a very slow reoxidation of heme $_L$, and delayed reoxidation of heme $_H$ until after +200 mV (Manuscript 1). This diode-like behaviour with a fast electron transfer reducing the enzyme, but slow, when Complex II was reoxidised indicates that there is one electron acceptor in the DET-enzyme reaction. It seemed like the enzyme in the absence of mediators communicated with the electrode exclusively via heme $_L$. In the light of the flexible structural arrangement of the cytochrome, it could further be postulated that the drastically different behaviours in the red to ox titration direction compared to the ox to red titration direction could be caused by a conformational change. In the presence of mediators, the rates of the two reactions were always very similar.

It has long been known that the inhibitor HQNO binds to the quinone-binding site Q_d, close to heme $_L$, and causes a shift of its E_m with about –60 mV. When we monitored the rate of heme reduction in SQR alone +/- HQNO, we saw no reduction at the set potential of -200 mV, but the block was relieved, when the potential was set to –400 mV (Manuscript 1). If redox mediators were added, reduction was again possible at -200 mV, even if HQNO was present. This indicated that the quinone-like mediators were able to bypass the HQNO block at the Q_d site, presumably via Q_p, indicating that this second proximal quinone binding site is indeed present in *Bacillus subtilis SQR*. The quinone-mediated redox titration was then repeated in the presence of HQNO. As expected, the redox midpoint potentials were about 50 mV lower than in the absence of HQNO, but both hemes were clearly resolved. Surprisingly, the subsequent red to ox titrations revealed a completely different behaviour than the normal mediated reaction, and instead resembled the unmediated red to ox titration, with the same delayed reoxidation of heme$_H$ (Paper VII). Thus, the mediated red to ox reaction in the presence of HQNO seems to be equivalent to the completely unmediated reaction. This corroborates that mediation really occurs via the quinone-binding site, and not from the general enzyme surface. Furthermore, it reveals the nature of the conformational change more specifically, since Qp was open and accessible during the oxidative-reductive titration, but became unaccessible during the reductive-oxidative titration.

We conclude that intact purified *B. subtilis* SQR can communicate directly with a gold capillary electrode, and that it does so only via the most distal heme, namely heme$_L$. Two quinone-binding sites are present, one distal site that previously has been shown to bind the specific inhibitor HQNO, and another proximal site located close to heme$_H$ at the interface between the IP subunit and the membrane anchor. The latter site is open when the enzyme is in its oxidised state, but closed when both hemes are reduced. This might be the first proof of a redox induced conformational change in an SQR/QFR enzyme.

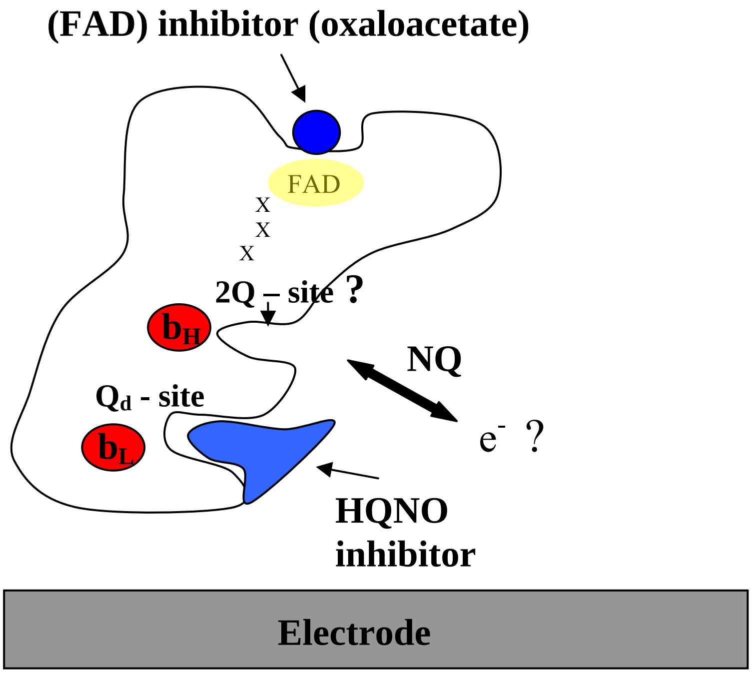


Figure 28. A principal scheme of the electron transfer experiment of Complex II involving the suggested second Q-site.

5. Conclusions

A spectroelectrochemical cell was constructed and tested in a number of applications such as detection of direct electron transfer (DET) between redox enzymes and gold electrodes (Papers 1-4, 6 and Manuscript 1), determination of the formal potentials of redox centres in proteins and enzymes (Papers 1-3, 5, 6 and Manuscript 1), as well as mechanistic studies of heterogeneous and intermolecular electron transfer of copper oxidases and Complex II. The cell appeared to be robust and was reasonably convenient to work with. The low volume and long optical path of the cell were very important features, considering the usually limited available amount of enzymes and the high concentrations needed for obtaining sufficient optical absorbance.

The existence of DET between enzymes and electrodes has been demonstrated for enzymes such as theophylline oxidase, cytochrome P450 cam and sulphite oxidase. The results are important for applications of these enzymes in biosensors as well as for future fundamental investigations of heterogeneous ET properties of these and other redox enzymes at the surfaces of different electrodes.

Spectroelectrochemical investigations of the ET between blue multi-copper oxidases and gold electrodes (Papers 4 and 6) yielded some of the most fundamental results of this thesis. It was demonstrated that the mechanism of heterogeneous ET of laccases on gold is totally different from that usually observed on carbon electrodes. At carbon electrodes laccases are electronically connected to the conducting electrode through the T1 copper centre. At gold surfaces, by contrast, the electronic connection of the laccase redox centre to the surface is established through the T2 copper centre. Such an ET connection between the gold surface and the T2 copper centre, severely disturbs the oxygen reduction reaction catalysed by laccase. Similar observations have been made for two bilirubin oxidases. These findings are extremely important in the development of biofuel cells, in which blue copper oxidase modified electrodes are promising candidates as cathodes.

Spectroelectrochemical studies of Complex II (SQR) (Manuscript 1) gave highly interesting results. DET for intact Complex II has been demonstrated, where heme$_L$ was found to be the first electron acceptor in the ET reaction between the electrode and the enzyme. The result of the DET reaction experiments together with the results from studies of specific mediated ET and the use of specific inhibitors, suggest the existence of a second unknown quinone site, as well as the important finding that this quinone site seems to close or open depending on the redox states of heme$_H$ and heme$_{L.}$ Such a finding could illuminate a mechanistic regulatory function behind succinate oxidation and allow us to further our understanding of its connection to the citric acid cycle.

Hopefully, a small contribution to the development of spectroelectrochemistry of redox enzymes has been accomplished by this work. The use of electrode-driven DET reactions in multi redox enzyme spectroelectrochemical experiments reveal the main electron transfer entrance/exit of multi redox site enzymes, experimentally proved by the divergence in the number of electrons involved in the redox transitions of the different sites. In addition to the DET studies, in multi redox site enzymes, where specific mediators and specific inhibitors exist (i.e, Complex II), the access and blocking of the different sites enable deep enzyme mechanistic studies of intact soluble redox enzymes. Hysteresis in the obtained redox titrations and the relative electron transfer rate between the different sites and the electrode are the crucial points in all the mechanistic experiments.

6. Acknowledgements

My greatest appreciation to you Taut for introducing me to such an interesting subject as spectroelectrochemistry! It all started with a small diploma work a long time ago and over time we have been developing our cell and a fine technique for the studies of redox proteins and enzymes. Thank you also for always allowing me to study the subject that interested me the most! And for allowing me to go for all interesting and fun conferences abroad! Thank you also for paying for some extra time to me, especially nowadays when I know that it takes effort to raise money.

Lo, thank you for all your generosity during the years, thank you for all the fun conferences we have been to together! I have greatly appreciated the dinners at your place, with always tasty prepared dishes! Thank you also for friendship! You are the main reason for me that I from my side was chose to do my PhD studies here at the department.

Sergey, my dearest friend, none has put so much time with me as you have and you have been helping me a lot during my PhD years, both professionally and as my closest friend. You have been one of few that cared and understood my hard problem that I got some years ago. I think we have had many fun, interesting and loooong! 48 hours experiments together during the years! and I really hope that you can put all the pieces of research of blue multi copper oxidases together and get a reward as a great step forward in this field! I also hope to run more fun experiments together with you soon and to make some beer sandwiches, drinking Heineken!

Special thanks to you Barbro for all your kindness during the years! and for your help with my never ending questions to you about formalities! Great thanks to you Britt for all your help during the years with consumables, solving problems of different kind and helping all us PhD students preparing for lab courses that our department keeps!

Thank you Jenny for always being positive to me!

Eva, you are true fine person, who always wishes the best for people around you and you have always been giving me a good mood, encouragement and help whenever I needed it. Greatest thanks to you for that!! If you wish or need my help I will always gladly help you!

My greatest appreciation to you Tobias for the collaboration, you and I have been fighting hard for the Complex II studies during all the years! Thank you for all the interesting discussions! You made my PhD time interesting and positive! I hope I can do something for you in the future.

Great appreciation to you Cecilia! For all long discussions and for enabling me to work with such interesting enzyme!

Curt, great thanks to you! I have had many interesting and good conversations with you during the years and you have contributed great to my stay at the department! In addition I would like to thank you for professional help regarding some of my manuscripts and parts of this thesis! Thank you Axel for collaboration and your kind party invitations that I unfortunately never had the possibility to attend! I wish you the best luck!

The greatest appreciation to you Niklas, you are the one accepting me in a project work at the department, without you I wouldn't stand here today, thank you! and the same holds for you Per (you were the first to introduce me to analytical chemistry) and thank you for many, many nice times together and a lot of fun innebandy games during our PhD student years, winning many tough games! It doesn't exist any penalty term "kind but resolute screening" in innebandy, luckily for our team…

Great thanks to Annika and Tomas Buch-Rasmussen! at Novo Nordisk, without you, there would not have been any finance and position for me.

Many thanks to all my collaborators during the years Nikitas, Elena, Stepan, Curt, Renata…and all coauthors in the papers I am involved in.

Great thanks to all colleagues during the years, Tobias (for delicious dinners!), Claes (solving computer problems and initiating football games!) Staffan, Leonard, Arieh, Erland, Sune, Florentina, Yirgalem, Carina, Jan,

To Vasile and Federico, I wish you the best luck with discovering new interesting mechanisms of enzymes using spectroelectrochemistry! Perform a lot of interesting studies and tell me the result.

To Christer, Rikard, Lars-Henric, Arto, Niklas, Saioa, Pille, Barri, Pär, Lars, Karolina (when your modeling finally is developed, I am sure it will be a highly useful tool for many people!) and the "new" people Anders, Peter, Peter, Laura and Kinga… I wish you all good luck with your research and writing. Konstantin, good luck with the virtual labs! Thank you Edita for small chats during the years!

I take the opportunity to show my appreciation for some other people that mean a lot to me which is somewhat besides the PhD…

Great thanks to you my cousin Johan! we have had some really nice moments together when you visited Lund during your research and thanks for joining our innebandy team scoring many goals!

Thank you Daniel for just being a nice guy and I am happy that you got a fine PhD position!

And so to one of the finest person I have met, my uncle Atte, I thank you for always wishing to help all people around you. You have always been there wishing and doing the best for our family during all the years. I will always remember that.

Great thanks to you Anna-Karin Thulin and Dr. Mikael Nornholm for understanding my hard problem that has been causing so much trouble during my whole PhD time and I am most grateful to your important support!

Finally to my dearest family

I am lucky to have you Mamma, thank you for all your big support, help and great, great generosity during the years and not at least the last month!

Maria ett fantastiskt stort tack till dig! för att du hjälpt mig att kunna jobba så fritt under dessa år som ibland krävts när lååånga experiment varit enda vägen till resultat!

Till dig min älskade pojk som jag älskar mest i hela världen. Nu strax ska vi hitta på mycket skoj tillsammans!! Det är mycket för din skull jag gjort detta.

Älskade Anny, vilken fin tjej jag har! Det allra största tack till dig för att du alltid står vid min sida och för att man inte kan tänka sig en finare person som flickvän. Du har alltid stött mig i alla lägen och strax ska du få träffa mig mycket och vi ska sticka iväg på härliga konst, mat och vinresor tillsammans!

7. References

[1] G.M. Soriano, M.V. Ponamarev, C.J. Carell, D. Xia, J.L. Smith, W.A. Cramer (1999) J. Bioenerg. Biomembr., 31, 201-213.

[2] A.J. Bard, L.R. Faulkner (1980) Electrochemical Methods, John Wiley & Sons, Inc., New York.

[3] D.G. Nicholls, S.T. Ferguson (2002) Bioenergetics 3, Academic Press, New York.

[4] F.Q. Schafer, G.R. Buettner (2001) Free Rad. Biol. Med., 30, 1191-1212.

[5] J. Koryta, J. Dvorák, L. Kavan (1993) Principles of Electrochemistry, John Wiley & Sons, Inc., Chichester.

[6] W.M. Clark (1960) Oxidation-Reduction Potentials of Organic Systems, Williams and Wilkins, Baltimore.

[7] R. Szentrimay, P. Yeh, T. Kuwana (1977) Electrochemical Studies of Biological Systems, ACS Symp. Ser. Vol. 38, Washington.

[8] Y. Zu, R.J. Shannon, J. Hirst (2003) J. Am. Chem. Soc., 125, 6020-6021.

[9] L.A. Sazanov, P. Hinchliffe (2006) Science, 311, 1430-1436.

[10] S.L. Mayo, W.R. Ellis, Jr., R.J. Crutchley, H.B. Gray (1986) Science, 233, 948-52.

[11] C.C. Moser, J.M. Keske, K. Warncke, R.S. Farid, P.L. Dutton (1992) Nature, 355, 796-802.

[12] A.A. Stuchebrukhov (2003) Theoret. Chem. Acc., 110, 291-306.

[13] J.R. Winkler, H.B. Gray (1997) J. Biol. Inorg. Chem., 2, 399-404.

[14] D.N. Beratan, S.S. Skourtis (1998) Curr. Opin. Chem. Biol., 2, 235-243.

[15] C.C. Page, C.C. Moser, X. Chen, P.L. Dutton (1999) Nature (London), 402, 47-52.

[16] R.A. Marcus, N. Sutin (1985) Biochim. Biophys. Acta, 811, 265-322.

[17] R.A. Marcus (1994), Theory of Electron Transfer Reactions, California Inst. of Tech., Pasadena, 15 pp.

[18] U. Brandt (1998) Biochim. Biophys. Acta, 1364, 89-296.

[19] M. Saraste (1999) Science, 283, 1488-1493.

[20] C. Hagerhall (1997) Biochim. Biophys. Acta, 1320, 107-141.

[21] H.O. Finklea (1996) Electroanal. Chem., 19, 109-335.

[22] R.G. Nuzzo, D.L. Allara (1983) J. Am. Chem. Soc., 105, 4481.

[23] C.D. Bain, E.B. Troughton, Y.T. Tao, J. Evall, G.M. Whitesides, R.G. Nuzzo (1989) J. Amer. Chem. Soc., 111, 321-35.

[24] L. Gorton, A. Lindgren, T. Larsson, F.D. Munteanu, T. Ruzgas, I. Gazaryan (1999) Anal. Chim. Acta, 400, 91-108.

[25] F.A. Armstrong, G.S. Wilson (2000) Electrochim. Acta, 45, 2623-2645.

[26] I. Willner, E. Katz (2000) Ang. Chem.-Int. Ed. 39, 1180-1218.

[27] I. Willner (2002) Science, 298, 2407-2408.

[28] A. Heller (1992) Electrical Connection of Enzyme Redox Centres to Electrodes, Dep. Chem. Eng.,Univ. Texas, Austin, TX, 53 pp.

[29] T. Vo-Dinh, B. Cullum (2000) Fresenius J. Anal. Chem., 366, 540-551.

[30] G.S. Wilson, Y.B. Hu (2000) Chem. Rev., 100, 2693-2704.

[31] A. Moody, S. Setford, S. Saini (2001) Analyst, 126, 1733-1739.

[32] M.J. Eddowes, H.A.O. Hill (1977) J. Chem. Soc. Chem. Commun., 21, 771-772.

[33] P. Yeh, T. Kuwana (1977) Chem. Lett., 1145-1148.

[34] Y. Gui, T. Kuwana (1987) J. Electroanal. Chem., 226, 199-209.

[35] I.V. Berezin, V.A. Bogdanovskaya, S.D. Varfolomeev, M.R. Tarasevich, A.I. Yaropolov (1978) Dokl. Akad. Nauk, 240, 615-618.

[36] M.R. Tarasevich, A.I. Yaropolov, V.A. Bogdanovskaya, S.D. Varfolomeev (1979) Bioelectrochem. Bioenerg., 6, 393-403.

[37] A.I. Yaropolov, V. Malovik, S.D. Varfolomeev, I.V. Berezin (1979) Dokl. Akad. Nauk. SSSR, 249, 1399-1401.

[38] M.C. Rhoten, J.D. Burgess, F.M. Hawkridge (2000) Electrochimica Acta, 45, 2855-2860.

[39] A. Sucheta, B.A.C. Ackrell, B. Cochran, F.A. Armstrong (1992) Nature, 356, 361-362.

[40] J. Hirst, A. Sucheta, B.A.C. Ackrell, F.A. Armstrong (1996) J. Am. Chem. Soc., 118, 5031-5038.

[41] L.J.C. Jeuken, A.K. Jones, S.K. Chapman, G. Cecchini, F.A. Armstrong (2002) J. Am. Chem. Soc., 124, 5702-5713.

[42] F.A. Armstrong, H.A.O. Hill, N.J. Walton (1988) Acc. Chem. Res., 21, 407.

[43] F.A. Armstrong, A.M. Lannon (1987) J. Am. Chem. Soc., 109, 7211.

[44] R.M. Paddock, E.F. Bowden (1989) J. Electroanal. Chem., 260, 487.

[45] D.L. Scott, E.F. Bowden (1994) Anal. Chem., 66, 1217.

[46] M.S. Mondal, H.A. Fuller, F.A. Armstrong (1996) J. Am. Chem. Soc., 118, 263.

[47] M.S. Mondal, D.B. Goodin, F.A. Armstrong (1998) J. Am. Chem. Soc., 120, 6270.

[48] D.L. Scott, R.M. Paddock, E.F. Bowden (1992) J. Electroanal. Chem., 341, 307.

[49] T. Ikeda, F. Matsushita, M. Senda (1991) Biosens. Bioelectron., 6, 299.

[50] G.F. Khan, H. Shinohara, Y. Ikariyama, M. Aizawa (1991) J. Electroanal. Chem., 315, 263.

[51] T. Ruzgas, A. Lindgren, L. Gorton, H.-J. Hecht, J. Reichelt, U. Bilitewski (2002) Electroanal. Methods Biol. Mater., 233-254.

[52] T. Ruzgas, L. Gorton, J. Emneus, G. Marko-Varga (1995) J. Electroanal. Chem., 391, 41-49.

[53] S. Shleev, A. Jarosz-Wilkolazka, A. Khalunina, O. Morozova, A. Yaropolov, T. Ruzgas, L. Gorton (2005) Bioelectrochemistry, 67, 115-124.

[54] M. Pita, S. Shleev, T. Ruzgas, V.M. Fernandez, A.I. Yaropolov, L. Gorton (2006) Electrochem. Commun, 8, 747-753.

[55] S. Shleev, M. Pita, A. Yaropolov, T. Ruzgas, L. Gorton (2006) Electroanalysis, 18, 1901 – 1908.

[56] A. Christenson, N. Dimcheva, E.E. Ferapontova, L. Gorton, T. Ruzgas, L. Stoica, S. Shleev, A.I. Yaropolov, D. Haltrich, R.N.F. Thorneley, S.D. Aust (2004) Electroanalysis, 16, 1074-1092.

[57] S. Shleev, A. El Kasmi, T. Ruzgas, L. Gorton (2004) Electrochem. Commun., 6, 934-939.

[58] S. Tsujimura, T. Nakagawa, K. Kenji, T. Ikeda (2004) Chem. Sens., 20, 770-771.

[59] A. Lindgren, T. Larsson, T. Ruzgas, L. Gorton (2000) J. Electroanal. Chem., 494, 105-113.

[60] A. Lindgren, L. Gorton, T. Ruzgas, U. Baminger, D. Haltrich, M. Schulein (2001) J. Electroanal. Chem., 496, 76-81.

[61] E.E. Ferapontova, T. Ruzgas, L. Gorton (2003) Anal. Chem., 75, 4841-4850.

[62] L.H. Guo, H.A.O. Hill (1991) Adv. Inorg. Chem., 36, 341-75.

[63] H.A.O. Hill, C.B. Moore, D.M.A. NabiRahni (1997) Electrochemistry of Redox Proteins. Bioelectrochemistry: Principles and Practice, 5, Bioelectrochemistry of Biomacromolecules, 183-204.

[64] A.L. Ghindilis, P. Atanasov, E. Wilkins (1997) Electroanalysis, 9, 661-674.

[65] S. Shleev, J. Tkac, A. Christenson, T. Ruzgas, A. Yaropolov, J. Whittaker, L. Gorton (2005) Biosens. Bioelectron., 20, 2517-2554.

[66] L.H. Dubois, R.G. Nuzzo (1992) Ann. Rev. Phys. Chem., 43, 437-463.

[67] A. Cass, G. Davis, G.D. Francis, H.A.O. Hill, W.J. Aston, I.J. Higgins, E.V. Plotkin, L.D.L. Scott, A.P.F. Turner (1984) Anal. Chem., 56, 667-671.

[68] P.N. Bartlett, T. P., R.G. Whitaker (1991) Progr. React. Kinet., 16, 55-155.

[69] A. Cass, D. Davis, H.A.O. Hill (1985) J. Electroanal. Chem., 190, 117-127.

[70] A. Rondeau, N. Larsson, M. Boujtita, L. Gorton, N. El Murr (1999) Analusis, 27, 649-656.

[71] P.L. Dutton (1978) Methods Enzymol., 54, 411-435.

[72] M.L. Fultz, R.A. Durst (1982) Anal. Chim. Acta, 140, 1-18.

[73] M.V. Pishko, A.C. Michael, A. Heller (1991) Anal. Chem., 63, 2268-2272.

[74] N. Mano, V. Soukharev, A. Heller (2006) J. Phys. Chem. B, 110, 11180-7.

[75] S. Timur, Y. Yigzaw, L. Gorton (2006) Sens. Actuat. B, B113, 684-691.

[76] I. Vostiar, E.E. Ferapontova, L. Gorton (2004) Electrochem. Commun., 6, 621-626.

[77] J.J. Kulys, N.K. Cénas (1983) Biochim. Biophys. Acta, 744, 57.

[78] L. Yan, W.T.S. Huck, G.M. Whitesides (2004) J. Macromol. Sci., Pol. Rev., C44, 175-206.

[79] L. Stoica, T. Ruzgas, R. Ludwig, D. Haltrich, L. Gorton (2006) Langmuir, ACS ASAP, DOI: 10.1021/ia061190f.

[80] F.A. Armstrong (2002) Russ. J. Electrochem., 38, 49-62.

[81] S. Dong, J. Niu, T.M. Cotton (1995) Methods. Enzymol., 246, 701-732.

[82] R.J. Gale (1988) Spectroelectrochemistry, Theory and Practice, Plenum Press, New York.

[83] A. Carrington, A.D. McLachlan. (1979) Introduction to Magnetic Resonance: with Applications to Chemistry and Chemical Physics, Chapman and Hall, London.

[84] J.W. Whittaker (2003) Chem. Rev., 103, 2347-2363.

[85] K.E. Paulsen, A.M. Orville, F.E. Frerman, J.D. Lipscomb, M.T. Stankovich (1992) Biochemistry, 31, 11755-11761.

[86] P.E. Jablonski, W.P. Lu, S.W. Ragsdale, J.G. Ferry (1993) J. Biol. Chem., 268, 325-329.

[87] B. Reinhammar, T.I. Vänngård (1971) Eur. J. Biochem., 18, 463-8.

[88] B.R.M. Reinhammar (1972) Biochim. Biophys. Acta, 275, 245-59.

[89] D.E.G. Austen, P.H. Given, D.J.E. Ingram, M.E. Peover (1958) Nature, 182, 1784-1786.

[90] P.W. Atkins (1994) Physical Chemistry, 5th Edition, Oxford University Press, Oxford.

[91] F.B. Kaufman, A.H. Schroeder, E.M. Engler, S.R. Kramer, J.Q. Chambers (1980) J. Am. Chem. Soc., 102, 483.

[92] Y. Zhu, S. Dong (1992) J. Catal. (Cuihau, Xuebao), 13, 209.

[93] K.A. Rubinson, J. Mark (1982) Anal. Chem., 54, 1204.

[94] G.P. Kreishman, C.-H. Su, C.W. Anderson, H.B. Halsall, W.R. Heineman (1980) Adv. Chem. Ser., 188,

[95] V.T. Taniguchi, N. Sailaisuta-Scott, F.C. Anson, H.B. Gray (1980) Pure Appl. Chem., 52, 2275.

[96] W.R. Heineman, C.W. Anderson, H.B. Halsall, M.M. Hurst, J.M. Johnson, G.P. Kreishman, B.J. Norris, M.J. Simone, C.H. Su (1982) Advances Chem. Series, 201, 1-21.

[97] S. Kwee (1986) Bioelechtrochem. Bioenerg., 16, 99.

[98] H.L. Landrum, R.T. Salmon, F.M. Hawkridge (1977) J. Am. Chem. Soc., 99, 3154.

[99] N. Sailaisuta, F.C. Anson, H.B. Gray (1979) J. Am. Chem. Soc., 101, 455.

[100] W. Heineman, M.L. Meckstroth, B.J. Norris, C.-H. Su (1979) Bioelechtrochem. Bioenerg., 6, 577.

[101] S. Song, S. Dong (1988) Bioelectrochem. Bioenerg., 19, 337-346.

[102] L.R. Sharpe, W.R. Heineman, R.C. Elder (1990) Chem. Rev., 90, 705-722.

[103] R.W. Murray, W.R. Heineman, G.W. O'Dom (1967) Anal. Chem., 39, 1666-1668.

[104] M.L. Meyer, T.P. DeAngelis, W.R. Heineman (1977) Anal. Chem., 49, 602-606.

[105] C.W. Anderson, M.R. Cushman (1982) Anal. Chem., 54, 2122-2123.

[106] S.S. Sawant, M. Sundaresan, K.A. Khasgiwale, A.W. Sahani (1990) J. Electrochem. Soc. India, 39, 1-7.

[107] F. Hartl, H. Luyten, H.A. Nieuwenhuis, G.C. Schoemaker (1994) Appl. Spectros., 48, 1528-8.

[108] M.M. da Silva Paula, C.V. Franco (1994) Qúim. Nova, 17, 451-453.

[109] D.D. Schlereth, W. Maentele (1992) Biochemistry, 31, 7494-7502.

[110] J. Zak, M.D. Porter, T. Kuwana (1983) Anal. Chem., 55, 2219-2222.

[111] M.D. Porter, T. Kuwana (1984) Anal. Chem., 56, 529-35.

[112] J.Y. Gui, G.W. Hance, T. Kuwana (1991) J. Electroanal. Chem., 309, 73-89.

[113] S. Tsujimura, A. Kuriyama, N. Fujieda, K. Kano, T. Ikeda (2005) Anal. Biochem., 337, 325-331.

[114] T. Larsson, A. Lindgren, T. Ruzgas (2001) Bioelectrochemistry, 53, 243-249.

[115] E.A. Kabat, T.T. Wu (1974) Proc. Nat. Acad. Sci. USA, 71, 899-900.

[116] A.A. Timchenko, A.I. Denesiuk, B.A. Fedorov (1981) Biofizika, 26, 32-36.

[117] M.J. Romao, M. Archer (2001) Cytochrome *c*. Handbook of Metalloproteins, 1, 44-54.

[118] G. Battistuzzi, M. Borsari., J.A. Cowan, C. Eicken, L. Loschi, M. Sola (1999) Biochemistry, 38, 5553-62.

[119] G.W. Pettigrew, G.R. Moore (1987) Cytochromes c, Biological Aspects, Springer-Verlag., Berlin.

[120] A. Wolf, D.A. Moss (1999) Spectrosc. Biol. Mol.: New Directions, European Conference on the Spectroscopy of Biological Molecules, 8th, Enschede, Netherlands, Aug. 29-Sept. 2, 1999, 75-76.

[121] A. Bonifacio, D. Millo, C. Gooijer, R. Boegschoten, G. van der Zwan (2004) Anal. Chem., 76, 1529-1531.

[122] S. Bernad, W. Maentele (2006) Anal. Biochem., 351, 214-218.

[123] H. Park, J.-S. Park, Y.-B. Shim (2001) J. Electroanal. Chem., 514, 67-74.

[124] X. Jiang, Y. Wang, X. Qu, S. Dong (2006) Biosens. Bioelectron., 22, 49-55.

[125] T. Erabi, Y. Yamashita, K. Nishimura, M. Wada (1987) Bull. Chem. Soc. Japan, 60, 2251-2252.

[126] J.L. Anderson (1979) Anal. Chem., 51, 2312-2315.

[127] H.D. Dewald, J.W. Watkins, II, R.C. Elder, W.R. Heineman (1986) Anal. Chem., 58, 2968-2975.

[128] W.R. Heineman, C.W. Anderson, H.B. Halsall, M.M. Hurst, J.M. Johnson, G.P. Kreishman, B.J. Norris, M.J. Simone, C.-H. Su (1982) Adv. Chem. Ser., 201, 1-21.

[129] D.R. Nelson, L. Koymans, T. Kamataki, J.J. Stegeman, R. Feyereisen, D.J. Waxman, M.R. Waterman, O. Gotoh, M.J. Coon (1996) Pharmacogenetics, 6, 1-42.

[130] W.H. Bradshaw, H.E. Conrad, E.J. Corey, I.C. Gunsalus, D. Lednicer (1959) J. Am. Chem. Soc., 81, 5507.

[131] T.L. Poulos, B.C. Finzel, I.C. Gunsalus, G.C. Wagner, J. Kraut (1985) J. Biol. Chem., 260, 16122-30.

[132] W.-D. Woggon (2001) Chimia, 55, 366-369.

[133] R. Murugan, S. Mazumdar (2005) ChemBiochem., 6, 1204-1211.

[134] V. Reipa, M.P. Mayhew, M.J. Holden, V.L. Vilker (2002) Chem. Commun., 318-319.

[135] I.C. Gunsalus, J.R. Meeks, J.D. Lipscomb (1973) Ann. NY Acad. Sci., 212, 107-121.

[136] M.T. Fisher, S.G. Sligar (1985) J. Am. Chem. Soc., 107, 5018-5019.

[137] S.A. Martinis, S.R. Blanke, L.P. Hager, S.G. Sligar, G.H.B. Hoa, J.J. Rux, J.H. Dawson (1996) Biochemistry, 35, 14530-14536.

[138] S.G. Sligar (1976) Biochemistry, 15, 5399-5406.

[139] M.J. Honeychurch, H.A.O. Hill, L.-L. Wong (1999) FEBS Lett., 451, 351-353.

[140] E.E. Ferapontova, S. Shipovskov, L. Gorton (2006) Biosens. Bioelectron., in press.

[141] T. Ikeda, D. Kobayashi, F. Matsushita, T. Sagara, K. Niki (1993) J. Electroanal. Chem., 361, 221-228.

[142] J. Wang, E. Dempsey, M. Ozsoz, M.R. Smyth (1991) Analyst, 116, 997-999.

[143] C.J. McNeil, J.M. Cooper, J.A. Spoors (1992) Biosens. Bioelectron., 7, 375-80.

[144] D.J.F. Rowe, I.D. Watson, J. Williams, D.J. Berry (1988) Ann. Clinic. Biochem., 25, 4-26.

[145] M. Kawai, M. Kato (2000) Methods Findings Experiment. Clinic. Pharmacol., 22, 309-320.

[146] A.F. Decastro, S.K. Gupta, A.K. Agarwal (1989) PCT Int. Appl., GDS Technology, Inc., USA, 43 pp.

[147] E.E. Ferapontova, L. Gorton (2005) Bioelectrochemistry, 66, 55-63.

[148] A. Messerschmidt, R. Huber, T. Poulos, K. Wieghardt (2001) A. Messerschmidt, R. Huber, T. Poulos, K. Wieghardt (2001) Handbook of Metalloproteins, John Wiley & Sons, Inc., New York.

[149] J.L. Johnson, K.V. Rajagopalan (1980) The oxidation of sulfite in animal systems. Ciba Foundation Symposium, 72, 119-133.

[150] J.H. Enemark, M.M. Cosper (2002) Molybdenum enzymes and sulfur metabolism. Metal Ions in Biological Systems, Vol. 39, 621-654.

[151] B.A. Ganai, A. Masood, M.A. Zargar, S.M. Bashir (2005) Asian J. Microbiol. Biotechnol. Environ. Sci., 7, 891-894.

[152] R. Hille (2003) Structure, 11, 1189-1190.

[153] H.J. Cohen, R.T. Drew, J.L. Johnson, K.V. Rajagopalan (1973) Proc. Natl. Acad. Sci. USA, 70, 3655-3659.

[154] H.J. Cohen, I. Fridovich (1971) J. Biol. Chem., 246, 359-366.

[155] S.P. Cramer, H.B. Gray, N.S. Scott, M. Barber, K.V. Rajagopalan (1980) Molybdenum Chem. Biol. Significance, Proc. Int. Symp., 157-168.

[156] J.T. Spence, C.A. Kipke, J.H. Enemark, R.A. Sunde (1991) Inorg. Chem., 30, 3011-3015.

[157] S.J. Elliott, C. Leger, H.R. Pershad, J. Hirst, K. Heffron, N. Ginet, F. Blasco, R.A. Rothery, J.H. Weiner, F.A. Armstrong (2002) Biochim. Biophys. Acta, 1555, 54-59.

[158] L.A. Coury, Jr., R.W. Murray, J.L. Johnson, K.V. Rajagopalan (1991) J. Phys. Chem. B, 95, 6034-6040.

[159] L. Coury, B.N. Oliver, J.O. Egekeze, C.S. Sosnoff, J.C. Brumfield, R.P. Buck, R.W. Murray (1990) Anal. Chem., 62, 452-458.

[160] L.A. Coury, Jr., L. Yang, R.W. Murray (1993) Anal. Chem., 65, 242-246.

[161] E.T. Adman (1985) Top. Mol. Struct. Biol., 6, 1-42.

[162] E.I. Solomon, M.J. Baldwin, M.D. Lowery (1992) Chem. Rev., 92, 521–542.

[163] L.J.C. Jeuken, P. van Vliet, M.P. Verbeet, R. Camba, J.P. McEvoy, F.A. Armstrong, G.W. Canters (2000) J. Am. Chem. Soc., 122, 12186–12194.

[164] E.N. Baker (1988) J. Mol. Biol., 203, 1071–1095.

[165] H. Nar, A. Messerschmidt, R. Huber, M. van de Kamp, G.W. Canters (1991) J. Mol. Biol., 218, 427–447.

[166] E.T. Adman, R.E. Stenkamp, L.C. Sieker, L.H. Jensen (1978) J. Mol. Biol., 123, 35-47.

[167] E.W. Ainscough, A.G. Bingham, A.M. Brodie, W.R. Ellis, H.B. Gray, T.M. Loehr, J.E. Plowman, G.E. Norris, E.N. Baker (1987) Biochemistry, 26, 71-82.

[168] C.S. St. Clair, W.R. Ellis, Jr., H.B. Gray (1992) Inorg. Chim. Acta, 191, 149-155.

[169] T. Pascher, B.G. Karlsson, M. Nordling, B.G. Malmström, T. Vänngård (1993) Eur. J. Biochem., 212, 289-296.

[170] W.R. Heineman, B.J. Norris, J.F. Goelz (1975) Anal. Chem., 47, 79-84.

[171] J. Zhang, Q. Chi, A.M. Kuznetsov, A.G. Hansen, H. Wackerbarth, H.E.M. Christensen, J.E.T. Andersen, J. Ulstrup (2002) J. Phys. Chem. B, 106, 1131-1152.

[172] D. Pinho, S. Besson, C.D. Brondino, E. Pereira, B. de Castro, I. Moura (2004) J. Inorg. Biochem., 98, 276-286.

[173] F.A. Armstrong, H.A.O. Hill, B.N. Oliver, N.J. Walton (1984) J. Am. Chem. Soc., 106, 921–923.

[174] T. Sakurai, F. Nose, T. Fujiki, S. Suzuki (1996) Bull. Chem. Soc. Japan, 69, 2855–2862.

[175] L.J.C. Jeuken, J.P. McEvoy, F.A. Armstrong (2002) J. Phys. Chem. B, 106, 2304–2313.

[176] L. Andolfi, D. Bruce, S. Cannistraro, G.W. Canters, J.J. Davis, H.A.O. Hill, J. Crozier, M.P. Verbeet, C.L. Wrathmell, Y. Astier (2004) J. Electroanal. Chem., 565, 21-28.

[177] A. Yaropolov, O.V. Skorobogat'ko, S.S. Vartanov, S.D. Varfolomeyev (1994) Appl. Biochem. Biotechnol., 49, 257-280.

[178] E.I. Solomon, U.M. Sundaram, T.E. Machonkin (1996) Chem. Rev., 96, 2563-2605.

[179] H. Yoshida (1883) J. Chem. Soc. Trans., 43, 472-486.

[180] J. Boidin (1951) Rev. Mycologie, 16, 173-197.

[181] A. Givaudan, A. Effosse, D. Faure, P. Potier, M.L. Bouillant, R. Bally (1993) Microbiol. Lett., 108, 205-210.

[182] F.M. Barrett, S.O. Andersen (1981) Insect Biochem., 11, 17-23.

[183] D.M. O'Malley, R. Whetten, W. Bao, C.L. Chen, R.R. Sederoff (1993) Plant J., 4, 751-757.

[184] M. Smith, C.F. Thurston, D.A. Wood (1997) Multi-Copper Oxidases, 201-224.

[185] H.-D. Youn, Y.C. Hah, S.-O. Kang (1995) FEMS Microbiol. Lett., 132, 183-188.

[186] A.M. Mayer, R.C. Staples (2002) Phytochemistry, 60, 551-565.

[187] H.P. Call, I. Mucke (1997) J. Biotechnol., 53, 163-202.

[188] M. Balakshin, C.-L. Chen, J.S. Gratzl, A.G. Kirkman, H. Jakob (2001) J. Mol. Catal. B, 16, 205-215.

[189] R.C. Minussi, G.M. Pastore, N. Duran (2002) Trends Food Sci. Technol., 13, 205-216.

[190] N. Duran, M.A. Rosa, A. D'Annibale, L. Gianfreda (2002) Enz. Microb. Technol., 31, 907-931.

[191] B. Haghighi, L. Gorton, T. Ruzgas, L.J. Jonsson (2003) Anal. Chim. Acta, 487, 3-14.

[192] A. Jarosz-Wilkolazka, T. Ruzgas, L. Gorton (2004) Enz. Microb. Technol., 35, 238-241.

[193] S. Shleev, P. Persson, G. Shumakovich, Y. Mazhugo, A. Yaropolov, T. Ruzgas, L. Gorton (2006) Enz. Microb. Technol., 39, 835-840.

[194] G.T.R. Palmore, H.-H. Kim (1999) J. Electroanal. Chem., 464, 110-117.

[195] M. Tarasevich, V.A. Bogdanovskaya, N.M. Zagudaeva, A.V. Kapustin (2002) Russ. J. Electrochem., 38, 335.

[196] V. Soukharev, N. Mano, A. Heller (2004) J. Am. Chem. Soc., 126, 8368-8369.

[197] F. Barriere, P. Kavanagh, D. Leech (2006) Electrochim. Acta, 51, 5187-5192.

[198] V. Ducros, A.M. Brzozowski, K.S. Wilson, S.H. Brown, P. Östergaard, P. Schneider, D.S. Yaver, A.H. Pedersen, G.J. Davies (1998) Nat. Struct. Biol., 5, 310-316.

[199] M. Antorini, I. Herpoel-Gimbert, T. Choinowski, J.-C. Sigoillot, M. Asther, K. Winterhalter, K. Piontek (2001) Biochim. Biophys. Acta, 1594, 109-114.

[200] N. Hakulinen, L.-L. Kiiskinen, K. Kruus, M. Saloheimo, A. Paananen, A. Koivula, J. Rouvinen (2002) Nat. Struct. Biol., 9, 601-605.

[201] K. Piontek, M. Antorini, T. Choinowski (2002) J. Biol. Chem., 277, 37663-37669.

[202] F.J. Enguita, L.O. Martins, A.O. Henriques, M.A. Carrondo (2003) J. Biol. Chem., 278, 19416-19425.

[203] V.T. Taniguchi, B.G. Malmstroem, F.C. Anson, H.B. Gray (1982) Proc. Nat. Acad. Sci. USA, 79, 3387-3389.

[204] F. Xu, W. Shin, S.H. Brown, J.A. Wahleithner, U.M. Sundaram, E.I. Solomon (1996) Biochim. Biophys. Acta, 1292, 303-311.

[205] P. Schneider, M.B. Caspersen, K. Mondorf, T. Halkier, L.K. Skov, P.R. Östergaard, K.M. Brown, S.H. Brown, F. Xu (1999) Enz. Microb. Technol., 25, 502-508.

[206] S.V. Shleev, O.V. Morozova, O.V. Nikitina, E.S. Gorshina, T.V. Rusinova, V.A. Serezhenkov, D.S. Burbaev, I.G. Gazaryan, A.I. Yaropolov (2004) Biochimie, 86, 693-703.

[207] A. Klonowska, C. Gaudin, A. Fournel, M. Asso, J. Le Petit, M. Giorgi, T. Tron (2002) Eur. J. Biochem., 269, 6119-6125.

[208] A. Klonowska, C. Gaudin, M. Asso, A. Fournel, M. Reglier, T. Tron (2005) Enz. Microb. Technol., 36, 34-41.

[209] A. Shimizu, J.H. Kwon, T. Sasaki, T. Satoh, N. Sakurai, T. Sakurai, S. Yamaguchi, T. Samejima (1999) Biochemistry, 38, 3034-3042.

[210] N. Tanaka, S. Murao (1982) Agric. Biol. Chem., 46, 2499-2503.

[211] Y. Gotoh, Y. Kondo, H. Kaji, A. Takeda, T. Samejima (1989) J. Biochem. (Tokyo), 106, 621-626.

[212] J. Guo, X.X. Liang, P.S. Mo, G.X. Li (1991) Appl. Biochem. Biotechnol., 31, 135-143.

[213] S. Koikeda, K. Ando, H. Kaji, T. Inoue, S. Murao, K. Takeuchi, T. Samejima (1993) J. Biol. Chem., 268, 18801-18809.

[214] J. Hirose, K. Inoue, H. Sakuragi, M. Kikkawa, M. Minakami, T. Morikawa, H. Iwamoto, K. Hiromi (1998) Inorg. Chim. Acta, 273, 204-212.

[215] S. Murao, N. Tanaka (1981) Agric. Biol. Chem., 45, 2383-2384.

[216] K. Kataoka, K. Tanaka, Y. Sakai, T. Sakurai (2005) Protein Express. Purificat., 41, 77-83.

[217] G. Zoppellaro, N. Sakurai, K. Kataoka, T. Sakurai (2004) Biosci. Biotechnol. Biochem., 68, 1998-2000.

[218] K. Hiromi, Y. Yamaguchi, Y. Sugiura, H. Iwamoto, J. Hirose (1992) Biosci. Biotechnol. Biochem., 56, 1349-1350.

[219] A. Shimizu, T. Sasaki, J.H. Kwon, A. Odaka, T. Satoh, N. Sakurai, T. Sakurai, S. Yamaguchi, T. Samejima (1999) J. Biochem. (Tokyo), 125, 662-668.

[220] N. Mano, H.-H. Kim, A. Heller (2002) J. Phys. Chem. B, 106, 8842-8848.

[221] N. Mano, H.-H. Kim, Y. Zhang, A. Heller (2002) J. Am. Chem. Soc., 124, 6480-6486.

[222] N. Mano, F. Mao, A. Heller (2002) J. Am. Chem. Soc., 124, 12962-12963.

[223] S. Tsujimura, K. Kano, T. Ikeda (2002) Electrochemistry (Tokyo), 70, 940-942.

[224] N. Mano, F. Mao, A. Heller (2003) J. Am. Chem. Soc., 125, 6588-6594.

[225] R. Duma, S.D. Minteer (2006) PMSE Preprints, 94, 592-593.

[226] A. Heller (2004) Phys. Chem. Chem. Phys., 6, 209-216.

[227] S.C. Barton, J. Gallaway, P. Atanassov (2004) Chem. Rev., 104, 4867-4886.

[228] P. Atanassov, D. Ivniski (2006) Abstracts of Papers, 232nd ACS National Meeting, San Francisco, CA, United States, Sept. 10-14, 2006, COLL-618.

[229] V. Yankovskaya, R. Horsefield, S. Toernroth, C. Luna-Chavez, H. Miyoshi, C. Leger, B. Byrne, G. Cecchini, S. Iwata (2003) Science, 299, 700-704.

[230] T.M. Iverson, C. Luna-Chavez, G. Cecchini, D.C. Rees (1999) Science, 284, 1961-1966.

[231] C.R.D. Lancaster, A. Kroger, M. Auer, H. Michel (1999) Nature, 402, 377-386.

[232] C. Hägerhäll, L. Hederstedt (1996) FEBS Lett., 389, 25-31.

[233] C. Hägerhäll, R. Aasa, C. von Wachenfeldt, L. Hederstedt (1992) Biochemistry, 31, 7411-7421.

[234] I.A. Smirnova, C. Hägerhäll, A.A. Konstantinov, L. Hederstedt (1995) FEBS Lett., 359, 23-26.

[235] C. Hägerhäll, R. Aasa, C. von Wachenfeldt, L. Hederstedt (1992) Biochemistry, 31, 7411-7421.

[236] J.J. Maguire, K. Magnusson, L. Hederstedt (1986) Biochemistry, 25, 5202-5208.