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Regulation of cytochrome P450 activity by physicochemical methods

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Contents

I.	Introduction	881
II.	The use of electrochemical methods in the study of hemoproteins	881
III.	Photoinduced electron transport	883
IV.	Changes in the microenvironment of the protein and its active centre	884
V.	Chemical modification of proteins for triggering new catalytic functions	884
VI.	Effect of temperature on hemoprotein-catalysed reactions	885
VII.	Effect of high pressure on the catalytic activity of hemoproteins	886
VIII.	Conclusion	886

Abstract. Physicochemical factors influencing the catalytic activities of hemoproteins (of cytochrome P450, in particular) that find use in biosensors and bioreactors are considered. The authors' data on the preparation of semisynthetic hemoproteins based on cytochrome P450 2B4 and NADPH-dependent cytochrome P450 reductase are presented. The use of alternative electron sources (electrochemical reduction and photoreduction) for the redox cycle of hemoproteins is discussed. The effects of temperature, pressure, chemical modification of proteins and organic solvents on the efficiency of hemoprotein-catalysed enzymic reactions are analysed. The bibliography includes 86 references.

I. Introduction

Cytochromes P450 are unique enzymes in that they are able to hydroxylate substrates with nonactivated C – H bonds. Cytochromes metabolise up to 200 000 compounds and catalyse about 60 types of chemical reactions, such as hydroxylation, N-, O- or S-demethylation, dealkylation, epoxidation, *etc.*[1] This feature of cytochromes P450 can be employed for quantification of medicinals and/or their metabolites and xenobiotics in different media as well as for stereodirected synthesis of steroids and other classes of biologically active compounds. The increase in the efficiency of catalysis by cytochromes P450 is a problem of considerable practical importance. The principles of bioelectrocatalysis have been discussed in detail.[2, 3]

The present review is devoted to the analysis of effects of various physicochemical factors on different stages of enzymic reactions with special emphasis on the advantages of alternative electron sources (electrochemical and photoreduction) over traditional reducing agents, such as NADH or NADPH. Reduction with the use of pyridine nucleotides is not a continuous process due to rapid exhaustion of NADH or NADPH. NADPH-regenerating enzymes are normally introduced into a biochemical system to maintain the required concentration of reducing agents, which makes the system more complicated. The use of cathodic current instead of NADH or NADPH allows the reduction process to become more long-lasting and controllable by means of physicochemical factors, such as voltage and electric current. In the case of photoreduction, this process is monitored by recording the generated photocurrent and can be controlled by changing the excitation wavelength. The published data obtained in the past decade on cytochromes P450 are surveyed.

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II. The use of electrochemical methods in the study of hemoproteins

1. The use of electrolysis for reduction of hemoproteins

Electron transport in biological systems normally occurs in the presence of electron donors (*e.g.,* NADH and NADPH) and an electron mediator, such as flavin nucleotides. In recent years, electrical systems are finding increasing use in the study of redox conversions of enzymes (of metalloenzymes, in particular).[4] Electrons can be transferred to the electrode either directly, by electrochemical transport from the protein redox centre, or in the presence of mediator molecules.[5] Different types of electrodes, such as graphite (glassy carbon, pyrolytic graphite), gold, platinum, semiconducting (indium oxide, tin oxide) and chemically modified graphite electrodes combined with redox catalysts (*e.g.,* rhodium catalysing oxygen reduction to hydrogen peroxide or water, metal complexes which efficiently mediate electron transport, *etc.*).[6 – 8]

The electron transfer in enzyme systems containing cytochrome P450 occurs according to the scheme:

$$\text{NADPH} \longrightarrow \text{NADPH-dependent cytochrome P450 reductase} \longrightarrow \longrightarrow \text{cytochrome P450.}$$

Here, NADPH is the electron donor and the microsomal flavoprotein (NADPH-dependent cytochrome P450 reductase) or a mitochondrial system consisting of a flavoprotein and a protein containing a Fe – S cluster is the electron carrier from NADPH to the hemoprotein (cytochrome P450).

The use of cathodic current instead of NADPH for electroreduction of flavins as mediators in oxygenase reactions has made it possible to obtain highly efficient electrochemical systems (Table 1).[9] The hydroxylation of lauric acid in the presence of an

Table 1. Comparison of rates of electrochemical and NADPH-dependent substrate metabolism reactions catalysed by recombinant proteins.[9]

Protein (reductase + cytochrome P450)	Substrate	Reaction	Reaction rate /nmol min^{-1} (nmol of P450)$^{-1}$	
			electrochemical	NADPH-dependent
rF450(mBov17α/mRatOR)L1	Progesterone	17α-Hydroxylation	20	28
(reductase + P450 17α)	Pregnenolone	"	5	5
rF450(mHum3A4/ mRatOR)L1	Testosterone	6β-Hydroxylation	2.6	10.0
(reductase + P450 3A4)	Erythromycin	N-Demethylation	1.2	2.2
	Benzphetamine	"	2.0	4.6
rF450(mHum1A2/mRatOR)L1	Caffeine	"	0.75	1.5
(reductase + P450 1A2)	Imipramine	"	1.2	2.0
CYP102-BM3 (P450 BM3)	Lauric acid	ω-Hydroxylation	110	900

electrochemical system consisting of a gold or platinum electrode and a purified fused protein containing P450 4A1 and NADPH-dependent cytochrome P450 reductase has been studied.[6, 9, 10] In addition to the fused protein, the electrochemical system contained a coordination compound of Co(III), *viz.*, [(*S*)-1,3,6,8,10,13,16,19-octaazabicyclo[6.6.6]eicosanocobalt(III)] as a mediator. The standard redox potential, U, of this compound is -350 mV (*vs.* standard hydrogen electrode). Presumably, the reduction of the Co(III) ion on the cathode occurs within the coordination compound according to the scheme: $Co^{3+} + e \rightarrow Co^{2+}$. If the electrolysis is carried out in a system containing only the flavoprotein (reductase) or a hemoprotein (cytochrome P450) no hydroxylation of lauric acid occurs, which suggests that the Co^{2+} ion plays the role of an activator. This reduces flavin nucleotides of the reductase, which, in turn, reduce the heme iron of cytochrome P450. Oxygen activation occurs on the reduced heme iron within the triple complex 'substrate$-Fe^{2+}-O_2$'; in this case, the oxidation of various substrates under physiological conditions occurs at high rates.[1] Electrochemical reduction of the fusion protein generates hydrogen peroxide, which destroys the enzyme system. The addition of catalase increases the efficiency of the system in the hydroxylation reaction. It was shown that the highest rate of lauric acid hydroxylation is observed at -450 mV (*vs.* standard hydrogen electrode).[6, 9, 10]

A semiartificial flavohemoprotein (flavocytochrome P450 2B4) possessing reductase and oxygenase activities was obtained earlier in our laboratory.[11, 12] Using an electrochemical system consisting of flavocytochrome 2B4 or 1A2 and rhodium–graphite microelectrodes, we performed successful aniline *p*-hydroxylation, aminopyrine N-demethylation and 7-ethoxy- and 7-pentoxyresorufin O-dealkylation. The reduction was carried out at $U = -450$ mV (*vs.* AgCl electrode).[13] The catalytic constants of NADPH-dependent and electrochemical reactions catalysed by flavocytochromes 1A2 and 2B4 were comparable.

Several factors have plagued direct electron transfer between electrodes and proteins. These include: (i) electroactive prosthetic groups deep within the protein structure, (ii) adsorptive denaturation of proteins onto electrodes, and (iii) unfavourable orientations at electrodes. Remarkable recent progress provides several strategies for achieving direct electron exchange between electrodes and proteins.

Immobilisation of enzymes on the electrode surface is used to increase the efficiency of the electron transfer from the electrode to the electroactive prosthetic group of the protein [14–16] by means of adsorption, covalent binding and incorporation of enzymes into polymeric films coating the electrodes. For example, positively charged proteins are immobilised by adsorption on electrodes of pyrolytic graphite containing negatively charged carboxy groups, whereas immobilisation of hemoproteins on graphite electrodes involves covalent binding in the presence of a cross-linking bifunctional reagent (glutaraldehyde) and albumin.[17] In the latter case, albumin is strongly adsorbed on the surface of graphite electrodes, while glutaraldehyde cross-links the redox protein with albumin through the amino groups thereby binding the former to the electrode.

Yet another approach consists of the use of promoter molecules which favour the electron transport through proper orientation of the proteins on the electrode, which thus blocks their adsorptive denaturation. The role of promoters can be played by alkanethiols containing terminal functional groups (*e.g.,* mercaptoundecanoic acid).[18] The chemosorption of mercaptoundecanoic acid on the surface of gold electrodes is accompanied by the formation of a stable monolayer, which facilitates subsequent binding (both covalent and ionic) of proteins to the electrode surface.

$$Au(electrode)-S(CH_2)_{10}COO^- \ (NH_3)^+\text{-hemoprotein},$$

$$Au(electrode)-S(CH_2)_{10}COO^- \ (NH_3)^+(CH_2)_n(NH_3)^+ \ ^-OOC\text{-hemoprotein}.$$

Double-stranded DNA, which is strongly adsorbed on electrodes of pyrolytic graphite, can also be used as a promoter molecule.[19] Such DNA-containing electrodes can readily extract hemoproteins from the solution.

The rate of the electron transfer from the electrode to the redox protein, can be increased if the electrodes containing covalently bound or adsorbed electron transport mediators, such as riboflavin or methylviologen, are chemically modified;[20, 21] the stability of hemoproteins in electrochemical systems is increased if they are incorporated into phospholipid films which coat the electrodes.[22–24] Surfactants containing charged or polar head groups and a hydrophobic tail (didodecyldimethylammonium bromide, polystyrenesulfonate, dihexadecyl phosphate, dimyristoylphosphatidylcholine) are used as films mimicking natural biomembranes.

It is of note that electrochemical processes involving redox proteins are reversible only in liquid-crystalline films.[22] If cytochrome $P450_{cam}$ is incorporated into liquid-crystalline films formed by dimyristoyl-L-α-phosphatidylcholine or didodecyldimethylammonium bromide, direct reversible electron transfer occurs from the electrode to the heme iron of the enzyme as is judged by direct current cyclic voltammograms.[22–24] Thus myoglobin reduction in liquid-crystalline didodecyldimethyldiammonium bromide films coating the pyrolytic graphite electrodes is reversible in contrast with its reduction on electrodes without biomembrane-like surfactant films.[24] On addition of a substrate (*e.g.,* trichloroacetic acid), this is reduced, as follows from the increase in the cathodic current. The incorporation of cytochrome $P450_{cam}$ into DNA films adsorbed onto gold electrodes has made it possible to perform direct reduction of the heme iron and catalytic epoxidation of styrene.[25]

Electrochemical reduction of metmyoglobin ($X-MbFe^{III}$), which is a perfect functional model of cytochrome P450 (the catalytic activity of myoglobin is close to that of cytochrome P450), was studied by Onuoha *et al.*[24] The electroreduction was carried out on electrodes of pyrolytic graphite at the potential, $U = -400$ mV (*vs.* SCE) in aqueous buffers or microemulsions of

hydrocarbons, water and cationic detergents. Under these conditions metmyoglobin is reducted on the electrode [Scheme 1, reaction (1)]. The reduced metmyoglobin rapidly reacts with oxygen to yield an $X-MbFe^{II}-O_2$ complex, which is reduced with the liberation of hydrogen peroxide on the electrode. The hydrogen peroxide evolved effects stepwise oxidation of metmyoglobin ($X-MbFe^{III}$) to ferrylmyoglobin ($HX-MbFe^{IV}=O_2$), an active oxidised form of myoglobin [Scheme 1, reactions (4) and (5)].

Scheme 1

$$X-MbFe^{III} + e \longrightarrow X-MbFe^{II}\ \text{(electrode)}, \quad (1)$$

$$X-MbFe^{II} + O_2 \longrightarrow X-MbFe^{II}-O_2, \quad (2)$$

$$X-MbFe^{II}-O_2 + 2e + 2H^+ \longrightarrow$$

$$\longrightarrow X-MbFe^{III} + H_2O_2\ \text{(electrode)}, \quad (3)$$

$$X-MbFe^{III} + H_2O_2 \longrightarrow {}^{\bullet}X-MbFe^{IV}=O + H_2O, \quad (4)$$

$$2\,{}^{\bullet}X-MbFe^{IV}=O + H_2O_2 \longrightarrow 2\,HX-MbFe^{IV}=O + O_2, \quad (5)$$

where X is an amino acid residue, *e.g.*, tyrosine-130. Styrene added to the reaction system is oxidised by hydrogen peroxide to give styrene oxide and benzaldehyde.

The efficiency of electrolysis used for the reduction of hemoproteins is comparable with the traditional reduction with pyridine nucleotides. The results obtained in the study of electrochemical and NADPH-dependent reactions of several isoforms of cytochrome P450 are presented in Table 1.

At present, attempts are being undertaken aimed at designing cytochrome P450-containing bioreactors and biosensors based on electrochemical systems.[9, 22, 26]

2. The use of inorganic semiconductors in redox systems

The effectiveness of inorganic semiconductors (TiO_2, ZnO, CdS) in combination with enzymes (hydrogenases, dehydrogenases) in photoproduction of hydrogen, photoreduction of $NADP^+$ and photosynthesis of organic acids and amino acids has been demonstrated in a number of studies.[27–31] The system 'inorganic semiconductor–enzyme' can transform light energy by semiconductor-photosensitised oxidation of organic electron donors and by enzyme-catalysed reduction of substrates (H^+, $NADP^+$, organic acids and amino acids) with electrons photogenerated in the semiconductor. Coupling of an inorganic semiconductor with an enzyme is usually attained with the help of electron carriers (mediators), *e.g.*, methylviologen, a rhodium bipyridyl complex and other compounds subject to reversible oxidation–reduction. Direct mediator-free transfer of electrons photogenerated in the conduction band of the semiconductor to the enzyme reaction centre has been described by Nikandrov *et al.*[28] A prerequisite for the conjugate effect of a semiconductor and an enzyme in the absence of an electron carrier is the sorption of the enzyme on the semiconductor surface.

3. The construction of an enzymic 'galvanic couple' for redox reactions

The operating principle of a galvanic element is based on the difference in the electrode potentials of the oxidant and the reducing agent. Active metals (*e.g.*, zinc) are most commonly used as reducing agents, while hemoproteins are used as oxidants. Normally, hemoproteins have a more positive standard electrode potential than zinc [$E_0(Zn^0/Zn^{2+}) = -763$ mV]; therefore zinc serves as a reducing agent in a zinc–hemoprotein couple. We used metallic zinc as an electron donor and a hemin–human serum albumin complex as an artificial hemoprotein and succeeded in effecting N-demethylation of aromatic amines.[32] The rates of these reactions were comparable with those of NADH-dependent reactions carried out in the presence of flavin nucleotides or riboflavin as electron transfer mediators.

Metallic cadmium [$E_0(Cd^0/Cd^{2+}) = -403$ mV, $E_0(NAD^+/NADH) = -340$ mV] was used as a reducing agent in a system containing NAD^+-dehydrogenase.[33] In this case, too, the 'galvanic couple' principle is realised.

III. Photoinduced electron transport

Light energy is utilised not only in traditional photosynthetic reactions or in photoinduction of semiconductors, it also triggers electron transport in redox systems. Direct photoreduction of hemoproteins in the absence of electron donors has been described by Gu *et al.*[34] Metmyoglobin, cytochromes *c* and b_5 and heme complexes with octapeptides and cytochrome P450 were photoreduced upon laser irradiation at wavelengths of 430–254 nm. UV irradiation resulted in photooxidation. This study has shown that the heme ligands play a crucial role in photoreduction. The photoreduction products were characterised by UV and visible absorption spectra and Raman spectra and compared with the products formed upon chemical reduction.

Heme photoreduction in hemoproteins can be utilised for 'triggering' redox reactions, such as oxygen activation by cytochrome P450 or binding of ligands (*e.g.*, O_2) with myoglobin or hemoglobin. Studies of model complexes (hemin–octapeptides) corroborated the viewpoint that aromatic amino acids are not the primary electron donors, since the model peptides were devoid of aromatic amino acid residues. In the presence of organic solvents (glycerol and other alcohols), the rate of photoreduction increased.[34] Irradiation with a nonfocused laser ($\lambda = 457.9$ nm, power 40 mV) of a 'riboflavin–hemin–albumin' complex resulted in riboflavin photoreduction as could be evidenced from changes in the absorption spectrum of the complex. Amine demethylation and aniline hydroxylation products were formed in the presence of *N*-substituted amines or aniline; the rate constants (k_{cat}) for dimethylaniline N-demethylation and aniline hydroxylation are equal to 0.5 min^{-1} and 0.13 min^{-1}, respectively, *i.e.,* they are comparable with those of NADPH-dependent reactions.[32]

The photooxidation of the reduced flavocytochrome b_2 † from *Saccharomyces cerevisiae* (lactate dehydrogenase) upon irradiation with a laser ($\lambda = 400$ nm) has been described by Hazzard *et al.*[35] At this wavelength, the oxidised form of 5-deazariboflavin passes into an excited triplet state; it is this state that is involved in the oxidation of the reduced heme. The rate of intermolecular electron transport was 2200 s^{-1}.

A series of investigations [36–40] have been devoted to the study of electron transport in hemoproteins (in cytochromes *c* and b_5, in particular) modified with ruthenium or cobalt complexes. A characteristic feature of ruthenium complexes is their ability to undergo reversible photoreduction and to transfer electrons to other acceptors. Thus a ruthenium complex covalently bound to cysteine-65 of cytochrome b_5 effected rapid reduction of cytochrome *c* according to the scheme.[36]

$$Ru^{2+}-\text{cyt}\,b_5(Fe^{3+}) \xrightarrow{\bar{e}} Ru^{3+}-\text{cyt}\,b_5(Fe^{2+}),$$

$$Ru^{3+}-\text{cyt}\,b_5(Fe^{2+}) + \text{cyt}\,c(Fe^{3+}) \xrightarrow{\bar{e}}$$

$$\longrightarrow Ru^{3+}-\text{cyt}\,b_5(Fe^{3+}) + \text{cyt}\,c(Fe^{2+}).$$

The electron transfer in these systems was studied in direct photoreduction of ruthenium complexes and using a flash-photolysis technique. The photoreduction of the flavoprotein cytochrome P450 reductase in the presence of electron acceptors (ferricyanide, Tetrazolium Nitro Blue) could be carried out in the absence of NADPH.[40]

† Reduced flavocytochrome was obtained by titration of the oxidised enzyme with a 1.5-fold excess of L-lactate.

Thus, photoinduced electron transport can serve as an alternative source of electrons in redox reactions provided the proteins contain photosensitive prosthetic groups or their analogues.

IV. Changes in the microenvironment of the protein and its active centre

1. Modelling of the membrane environment

The environment, especially the cell membrane, strongly influences the enzyme activity in living organisms. The construction of an artificial membrane-like environment may significantly enhance the enzyme activity. Among various synthetic membranes studied, synthetic membrane-like phospholipid bilayers were shown to possess an activating effect.[41] Cytochrome *c* incorporated into such membranes increases the rate of H_2O_2-induced *N,N*-dimethylaniline N-demethylation 10.6-fold. A 20-fold decrease in the Michaelis constant for H_2O_2 is observed, which suggests increased affinity of the enzyme towards hydrogen peroxide. Changes in the spectral characteristics of cytochrome *c* incorporated into the phospholipid bilayer suggest significant structural and functional changes. The oxidised form of the protein is characterised by a short-wave shift of the optical density maximum (from 408 to 406 nm) upon incorporation, the absorption maximum of the reduced form is shifted from 416 to 426 nm and one maximum at 550 nm is observed instead of two maxima at 520 and 550 nm.

The incorporation of myoglobin into membrane-like films results in a decrease in the activation energy of electrochemical reduction of dibromoethylene and trichloroacetic acid, the rate of electron transport increases 1000-fold.[16] Acetylcholinesterase, cholinesterase and horseradish peroxidase incorporated into polymeric films prepared from polyvinylpyridine and polyethylene glycol can be used as amperometric sensors for detecting acetylcholine, choline and hydrogen peroxide.[8]

2. Enzymatic catalysis in reversed micelles and organic solvents

Many properties of biological membranes are efficiently mimicked by reversed micelles of surfactants in organic solvents, which can be regarded as biomembrane models.

In 1977, Martinek *et al.*[42] showed that enzymes solubilised in organic solvents with the aid of reversed micelles of synthetic surfactants retain their catalytic activities. Some hemoproteins, *e.g.*, cytochrome *c* and methemoglobin, induce the formation of reversed micelles by incorporating into bilayer membranes with simultaneous preservation of their enzymic activities.[43–45] Horseradish peroxidase solubilised in a sodium diisooctyl sulfosuccinate–water–octane system catalyses pyrogallol oxidation by hydrogen peroxide much more efficiently than the water-soluble enzyme.[46]

Efficient catalysis of redox reactions by hemoproteins incorporated into reversed micelles of surfactants was used for increasing the operational stability of catalase, superoxide dismutase and glutathione peroxidase.[47] Systems mimicking hemoproteins (albumin–hemin complexes and ferritin) were also incorporated into reversed micelles.[48]

Cytochrome P450 1B1 and cytochrome P450 reductase as a fusion protein was incorporated into oil-in-water macroemulsion, termed polyaphron, (the internal organic phase content was more than 0.74). The efficiency of such immobilisation exceeded 85%; the enzyme preparation remained active towards substrates (erythromycin, chlorotolurone) for 24 h at 15 °C.[49]

In order to decrease the water content in colloidal media, the substitution of a water-organic mixture for the water surrounding the enzyme in reversed micelles of surfactants was suggested.[50] The hemoglobin-catalysed oxidation of dibenzothiophene in various water-organic mixtures was studied by Klyachko and Klibanov.[51] The preparation of suspensions of lyophilised enzymes in organic solvents is an alternative procedure for excluding water. The enzymes suspended in organic solvents acquire new properties, which affects virtually all the aspects of their functioning, *viz.,* catalytic activity, substrate specificity, stereoselectivity, thermal stability, *etc.*[52, 53]

In addition to ordinary enzymic reactions, the reactions nonspecific for enzyme behaviour in aqueous media may occur either in nonaqueous organic solvents or in organic solvents with the H_2O content of 0.01 vol.%. The reasons for the alteration of the substrate specificities are different for individual enzymes. Nevertheless, enzymatic catalysis in organic solvents obeys the Michaelis–Menten kinetics, which suggests the formation of a classical enzyme–substrate complex in nonaqueous solutions.

The phenomenon of 'enzyme memory' has been discovered in experiments with enzyme suspensions in organic solvents. An enzyme lyophilised from an aqueous solution containing a specific ligand and then suspended in an organic solvent binds the ligand several tens of times more efficiently. This property disappears after repeated dissolution in water. A possible explanation of this phenomenon is that the enzyme retains an 'imprint' of the ligand even after its removal upon lyophilisation. In nonaqueous media where the conformation of the protein molecule becomes more rigid, the enzyme manifests higher affinity for the ligand.[54, 55] The ability of enzymes to retain the 'imprints' of ligands has a certain practical significance for the synthesis of chiral pharmacologically useful intermediates.

The effects of organic solvents on the properties of cytochrome P450 have been studied to a lesser degree. It was found that organic solvents favour the transition of cytochrome P450 into the inactive form, P420.[56] This transition is due to the changes in the nearest coordination sphere (sulfur atoms) around the iron atom. A similar transition is observed in the case of chloroperoxidase.[56] However, cytochrome *c* suspended in tetrahydrofuran containing 1% D_2O fully preserves its tertiary structure.[57] These conclusions were made on the basis of analysis of the tertiary structure of cytochrome *c* by two-dimensional NMR spectroscopy.

V. Chemical modification of proteins for triggering new catalytic functions

This section considers the methods used for enhancement of the enzymatic activity by introducing new catalytically active groups into the enzyme molecule.

Thus cytochrome P450 manifests monooxygenase activity only within a complex with the flavoprotein reductase. Here flavin nucleotides represent electron carriers which reduce the heme iron of cytochrome P450. The introduction of flavin nucleotides or their analogues into cytochrome P450 converts the latter into a self-competent enzyme which does not require the involvement of partner proteins in the catalytic cycle.

Semisynthetic flavohemoglobin was prepared by attachment of activated flavin derivatives [58] to the β-subunit of hemoglobin used in a 2:1 ratio.[59, 60] Hemoglobin catalyses monooxygenase reactions occurring in a system containing a hemoprotein, oxygen, NADPH and a flavoprotein (NADPH-dependent cytochrome P450 reductase). Covalent binding of flavin to hemoglobin makes it possible to conduct similar reactions in the absence of reductase. The rates of the catalytic reaction of aniline hydroxylation in the presence of flavohemoglobin and the microsomal monooxygenase system are comparable. The rate of aniline hydroxylation in the presence of semisynthetic flavohemoglobin increased in comparison with hydroxylation rates in the presence of hemoglobin and the hemoglobin–reductase system (7.7- and 1.3-fold, respectively).

The catalytic activity of the covalent complexes 'cytochrome P450–flavin adenine dinucleotide' (FAD) and 'cytochrome P450–flavin mononucleotide' (FMN) has been studied by Uvarov *et al.*[61] The complex in which cytochrome P450 is covalently bound to FMN (1:3) turned out to be more active. The catalytic rate constants for dimethylaniline and aminopyrine

N-demethylation and aniline hydroxylation were about 2 times lower than those of the analogous reactions catalysed by microsomes, which represent natural complexes of cytochrome P450 and reductase. The rates of reactions catalysed by covalent and noncovalent P450 – FAD complexes are 10 times lower than those of microsome-catalysed reactions.

A monomolecular monooxygenase system formed by covalent binding of the electron-accepting antibiotic bleomycin to a flavoprotein (NADPH-dependent cytochrome P450 reductase) has been described by the same authors.[61] The rates of reactions involving the reductase – bleomycin complex are 10 times lower than those of microsome-catalysed reactions.

Reductase does not possess its own catalytic activity in monooxygenase reactions; however, after incorporation of 1 to 10 hemin residues into the reductase molecule the semi-artificial hemoreductase manifested activity in NADPH-dependent N-demethylation of amidopyrine ($k_{cat} = 0.7$ min^{-1}). The k_{cat} values for the analogous reactions catalysed by the microsomal monooxygenase system and the semi-artificial flavocytochrome 2B4 are 2.4 and 0.98 min^{-1}, respectively.[11,12] A semi-artificial hemoreductase[62] which represents a covalent complex of hemin and NADPH-dependent cytochrome P450 reductase manifested reductase activity with respect to electron acceptors, such as potassium ferricyanide, cytochrome *c* and hemin.

The covalent binding of flavodoxin to ferredoxin-NADP$^+$ reductase[63] (1 : 1) gave an artificial enzyme which catalyses the reduction of cytochrome *c* with high efficiency. The transfer of electrons in this system occurs according to the same scheme as in NADPH-dependent cytochrome P450 reductase.

$$\text{NADPH} \longrightarrow \text{FAD (from ferredoxin-NADP}^+\text{ reductase)} \longrightarrow$$
$$\longrightarrow \text{FMN (flavodoxin)} \longrightarrow \text{cytochrome } c.$$

Ferredoxin-NADP$^+$ reductase modified with viologen in the presence of carbodiimide manifests oxidase activity, which is absent in the native enzyme.[64] In the absence of ferredoxin, the reductase does not reduce cytochrome *c*, whereas the modified enzyme displays high activity. In the presence of electron acceptors such as 2,6-dichloroindophenol and ferricyanide, the activity of the modified enzyme decreases twofold, whereas the rate of the cytochrome *c* reductase reactions increases 30-fold. The scheme of electron transport in a system with the nature enzyme appears as:

$$\text{NADH} \longrightarrow \text{reductase} \longrightarrow \text{ferredoxin} \longrightarrow \text{cytochrome } c.$$

When the reactions are carried out in the presence of superoxide dismutase, the viologen – reductase systems utilise the oxygen molecule reduced to the superoxide radical anion as the find electron acceptor. It is the presence of active oxygen species that accounts for the appearance of oxidase activity in the modified enzyme. Titration of the reduced viologen – reductase system revealed that the electron transfer to viologen occurs through the flavin group of FAD present in the reductase. The standard electrode potential of viologen covalently bound to ferredoxin-NADP$^+$ reductase is -365 mV, while that of soluble viologen is -420 mV. The electrode potentials of other reaction participants are -320 (NADPH), -344 (reductase) and -420 mV (ferredoxin). The electrochemical preparation of NADPH using the native enzyme requires an electron donor capable of reduction of ferredoxin, since in this case the scheme of electron transport appears as:

$$\text{ferredoxin} \longrightarrow \text{reductase} \longrightarrow \text{NADP}^+.$$

The electric current generated by the electrochemical system 'viologen – reductase' in the presence of NADP$^+$ points to the electron transfer between the electrodes and the system. The latter can therefore be used for regeneration of NADPH.

Ferrocene and its derivatives are known to be efficient electron carriers. Modification of glucose oxidase (GOx) with ferrocenecarboxylic acids (FCA) has been described by Badia *et al.*[65] Covalent derivatisation of the GOx molecule containing two FAD molecules with additional electron carrier groups imparts the ability to be involved in the electrochemical reaction. The optimum number of FCA groups is 13 residues per GOx molecule. In native glucose oxidase, reduced $FADH_2$ is oxidised with atmospheric oxygen, whereas the role of oxygen in ferroceneglucose oxidase is played by FCA responsible for 'internal' electron transport. Ferroceneglucose oxidase undergoes electrochemical conversions on the electrodes according to the scheme:

$$\text{FADH}_2\text{–GOx–(FCA)}_n \leftrightarrow \text{FADH}_2\text{–GOx–(FCA}^+)_n + n\text{e} \leftrightarrow$$
$$\leftrightarrow \text{FAD–GOx–(FCA)}_2\text{(FCA}^+)_{n-2} + 2\text{H}^+ + n\text{e}.$$

The arrangement of bound FCA residues relative to the FAD molecule is more important for electron transport than their number. The electrode potential of the $FADH_2/FADH^{\bullet}$ couple is -219 mV, that of the FCA^+/FCA couple is $+773$ mV. Hence, the motive force of the electron transport in the given system is about 1 V.

Modification of cytochrome $P450_{cam}$ and β-lactamase with a new electron-active reagent, *N*-(2-ferrocenylethyl)maleimide, was described by Gleria *et al.*[66–68] who showed that modified enzymes are electrochemically active. These authors suggest using *N*-(2-ferrocenylethyl)maleimide as a promising reagent for the conversion of electropassive enzymes into electroactive ones as well as for the design of electrochemical sensors and for studies of mechanisms of intraprotein electron transport.

The covalent binding of glutathione reductase to the photosensitive dye eosin (1 : 2) was used to obtain a photoactivated system which can be used for glutathione reduction.[69] Eosin-modified glutathione reductase retains 50% of activity of the native enzyme in a NADPH-dependent reaction. However, the photosystem thus obtained was 1000 times less active in glutathione reduction than the glutathione reductase with NADPH as the electron source.

VI. Effect of temperature on hemoprotein-catalysed reactions

Studies of reactions catalysed by cytochrome P450 2B4 have shown that an increase in temperature causes an increase in the rate of substrate hydroxylation. For example, with a rise in temperature from 20 to 37 °C, the catalytic constant, k_{cat}, for peroxide-dependent O-dealkylation of *p*-nitroanisole increases twofold.[70]

The catalytic cycle of cytochrome P450 is rather complex and comprises at least seven steps.[1,71] The effect of temperature on the binding of various substrates (*e.g.*, benzphetamine) to cytochrome P450 and on the spin equilibrium between low- and high-spin forms of the hemoprotein was studied in most detail. It was shown[72] that a change in temperature from 12 to 27 °C has no effect on the absorption spectra of cytochrome P450 2B4. The spectra are not changed after addition of benzphetamine (0.8 mol litre^{-1}) either. The dissociation constants (K_d) and concentration $[ES]_{max}$ of enzyme – substrate complexes show the following temperature dependence:

Temperature /°C	K_d /μmol litre^{-1}	$[ES]_{max}$ /μmol litre^{-1}
13.8	189 ± 5	0.085 ± 0.002
23.8	86 ± 5	0.087 ± 0.004

As can be seen, the concentration of enzyme – substrate complexes is practically constant at different temperatures. However, the use of the 'temperature jump' technique, which consists of instantaneous increase in the temperature in the reaction cell upon high-power electric discharge revealed enhanced binding of the substrate to the enzyme.[72]

The substrate-induced spin equilibrium obeys a bimolecular mechanism:

$$\text{P450(l.s.)} + \text{S} \leftrightarrow \text{P450(h.s.)–S},$$

where l.s. and h.s. are the concentrations of the low-spin and high-spin forms of the enzyme, respectively. The catalytic constant of this reaction can be written as:

$$k_{cat} = \frac{1}{\tau_f} = k_1([E]_{eq} + [S]_{eq}) + k_{-1},$$

where τ_f is the fast relaxation time, *i.e.,* the time during which the system passes into a new state and $[E]_{eq}$ and $[S]_{eq}$ are the equilibrium concentrations of the enzyme and the substrate, respectively. In the absence of the substrate, the relaxation does not occur, which is consistent with the experimental data concerning the effect of temperature on the absorption spectra of cytochrome P450 not bound to the substrate.

A study of temperature-dependent electrochemical reduction of the fusion protein ‡ (within the temperature range of 4–36 °C) revealed that the rate of reduction is a maximum at 22 °C.[6] The reason should be sought in the simultaneous occurrence of several parallel reactions in electrochemical systems that are differently affected by temperature.

VII. Effect of high pressure on the catalytic activity of hemoproteins

The effect of high pressure on the kinetics and dynamics of conformational conversions of proteins has been studied by Morild.[73] High pressures are used for shifting chemical and biochemical equilibria as well as for changing the rates of individual steps of enzymatic reactions.

The changes in the reaction and activation (ΔV^* and ΔV^{**}) volumes calculated from the experimental data on the effect of high pressure can be utilised in the study of molecular mechanisms of different enzymes and their transition states during the formation of new bonds. If ΔV^* and ΔV^{**} values are negative, an increase in pressure will shift the equilibrium towards the formation of enzymatic reaction products in accordance with the Le Châtelier principle.[74, 75]

The effect of high pressure on bacterial camphor-metabolising cytochrome $P450_{cam}$ was extensively studied by several authors.[76–79] It was found that the pressure affects the equilibrium between low-spin and high-spin states. An increase in pressure to 2.2 kbar induces the transition of cytochrome $P450_{cam}$ into the inactive form, P420. Chloroperoxidase is also converted into C420 at high pressure.[75] The effect of high pressure on the redox potential of cytochrome *c* was studied by Tschirret-Guth *et al.*[80] By measuring changes in the standard potential (E^0) as the function of pressure (P), one can calculate the reaction volume, ΔV^0:

$$\left(\frac{\partial E^0}{\partial P}\right)_t = \frac{\Delta V^0}{nF},$$

where F is the Faraday constant, $\Delta V^0 = \sum V_p^0 - \sum V_r^0$ ($\sum V_p^0$ and $\sum V_r^0$ are the sums of standard molar volumes of the products and reactants, respectively). As the pressure is increased, the reaction accompanied by a decrease in the molar volume will be preferred. In the case of a redox process, this will induce a positive shift of the redox potential. According to Tschirret-Guth *et al.*,[80] the increase in pressure to 5 kbar results in the changes of the potential from −5 mV to +76 mV; the molar volume also changes upon transition from the oxidised to the reduced form of cytochrome *c:*

$$V[\text{cyt } c(Fe^{2+})] - V[\text{cyt } c(Fe^{3+})] = -24 \text{ ml mol}^{-1}.$$

These findings are consistent with the results of structural and physicochemical studies, *viz.,* ferrocytochrome *c* has a more compact structure than ferricytochrome *c*. By changing the pressure on the enzymic system, one can shift the chemical equilibrium in redox hemoproteins towards the formation of the reduced form.

‡ The fusion protein is a gene-engineered protein containing both reductase and cytochrome P450 4A1 in the same polypeptide chain.

VIII. Conclusion

Most enzymes, including hemoproteins, are rather sensitive to physical and chemical influences which can induce complete or partial loss of their activities. This necessitates the design of artificial enzymes based on proteins with higher resistance to such influences. In some studies, artificial hemoproteins were prepared as a result of the formation of noncovalent specific complexes of hemin [32, 81] or its analogues (cobalt octaethylbisporphyrinate, cobalt octapyridinemethylphthalocyanine and lutecium diphthalocyanine) [82] with human or bovine serum albumin and synthetic peptides.[83, 84] These complexes manifest oxygenase-like catalytic activity in aminopyrine N-demethylation and aniline hydroxylation. Yet another trend, *viz.,* the design and *de novo* synthesis of artificial proteins with predetermined structure and functional properties by gene engineering methods is also in progress.[85, 86]

This review summarises the data on targeted action of various physicochemical factors on individual steps of enzymic reactions catalysed by hemoproteins. A rational combination of physicochemical approaches to the study of natural, semi-artificial and artificial enzymic systems can be useful in the design of new effective biosensors, test systems and bioreactors.

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