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Kinetics and Mechanism of Electron Transfer in Proteins

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The results of studies on the kinetics of the oxidation-reduction reactions of individual proteins (electron transfer agents and enzymes) are described. Attention has been concentrated on the effect of the nature of the active centres in the protein molecules and of the modification of individual aminoacid residues on the rate of electron transfer in a homogeneous medium. Questions associated with the electrochemical reactions of proteins and with the effect of the state of the interface on the rate of this process are considered in detail. Ideas concerning the theoretical calculation of the rate constants for electron transfer in proteins are described.
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I. INTRODUCTION

The oxidation-reduction processes involving the synthesis and degradation of substances in living organisms are effected with participation of protein systems in which electron transfer takes place via the oxidation or reduction of active centres in the protein molecules.[1] The success of the practical employment of these reactions, which is now beginning to take shape, in the transformation of organic and inorganic compounds, in energy conversion, and in electrocatalytic systems[2-4] is to a large extent determined by the depth of our understanding of the principal characteristics of electron transfer in individual proteins. The quantitative estimation of the rate of electron transfer in proteins assumes no less importance in the modelling of enzyme catalysis and in the creation of effective low-molecular-weight catalysts.[5] The kinetics of the oxidation-reduction reactions of individual proteins are also of considerable theoretical interest for molecular biology and related fields, because they help in understanding the mechanism of the functioning of oxidation-reduction electron transfer chains in biological membranes.

Kinetic studies occupy at present a permanent place in the research into the mechanism of electron transfer in proteins. The development of preparative methods, which make it possible to obtain individual proteins in amounts necessary for chemical experiments, promoted the rapid accumulation of experimental data concerning the influence of the structure of the protein globule or even of individual aminoacids on the rate of electron transfer. A survey and discussion of these results appear to be extremely timely.

The present review is devoted to the analysis of the kinetic results of studies on the oxidation-reduction reactions of proteins—electron transfer agents and enzymes. It presents data on the chemical and electrochemical reactions of proteins and describes the principal ideas concerning the theoretical calculation of the rate of electron transfer.

II. OXIDATION-REDUCTION REACTIONS OF ELECTRON TRANSFER AGENTS

1. The Formal Kinetic Features of Electron Transfer in Proteins

Oxidation-reduction proteins which are components of the oxidation-reduction electron transfer chains or which participate in oxygen transport processes as a rule have low molecular weights and contain metals, iron–sulphur clusters, flavin-adenine dinucleotide (FAD), flavin mononucleotide (FMN), or other co-factors in the active centre. After isolation in the native state, these proteins are able to react with various compounds and such reactions are bimolecular.

Since protein molecules are charged species, the rate of the process depends on the electrostatic interactions. In order to eliminate this effect, the rate constant is referred to infinite ionic strength of the solution in accordance with the expression:[6]

$$\ln k^{\infty} = \ln k + 3.576 \left[\frac{\exp(-\kappa R_1)}{1+\kappa R_2} + \frac{\exp(-\kappa R_2)}{1+\kappa R_1}\right]\left[\frac{Z_1 Z_2}{R_1+R_2}\right],$$

in which Z_1, Z_2, R_1, and R_2 are respectively the charges and radii of the reacting molecules and $\kappa = 3.29\ \mu^{1/2}\ \text{nm}^{-1}$ (25 °C), where μ is the ionic strength of the solution expressed as a molarity.

The rate constants for the bimolecular reactions of electron transfer agents with complexes vary in the range 10^6–10^{-2} litre $\text{mol}^{-1}\ \text{s}^{-1}$ and depend on the pH, the ionic strength of the solution, and on the nature of the donor and acceptor. Analysis of the data presented in Table 1 shows that inorganic reactants containing aromatic ligands react at a higher rate than coordination compounds of the same metals without hydrophobic ligands. The reactivity of cobalt oxalate complexes is as a rule low. Low rate constants are also observed for proteins whose physiological function consists in transporting oxygen, namely for haemoglobin and myoglobin.

Table 1. The rate constants and certain thermodynamic parameters of the reactions of electron transferring agents with inorganic complexes at 25 °C.

Oxidant	Reductant	pH	μ, M	k_{12}, litre mol^{-1} s^{-1}	$\Delta H^{\neq}$, kJ mol^{-1}	$\Delta S^{\neq}$, J mol^{-1} K^{-1}	Refs
$Ru(NH_3)(pyr)^{3+}$	Azurin	6.5	0.1	$2.0\cdot10^3$	41.1	−58.7	[7]
$Ru(pyr)_3^{2+*}$	"	7.0	0.1	$6.9\cdot10^8$	—	—	[8]
$Co(ox)_3^{3-}$	"	7.0	0.5	$2.9\cdot10^{-2}$	—	—	[9]
$Co(phen)_3^{3+}$	"	7.0	0.1	$3.2\cdot10^3$	59.9	20.9	[10]
$Co(5,6\text{-}Me_2\text{-}phen)_3^{3+}$	"	7.0	0.1	$1.5\cdot10^3$	48.6	−20.9	[10]
$Co(5\text{-}Cl\text{-}phen)_3^{3+}$	"	7.0	0.1	$4.2\cdot10^2$	37.3	−71.2	[10]
$Co(4,7\text{-}Me_2\text{-}phen)_3^{3+}$	"	7.0	0.1	$8.4\cdot10^1$	41.5	−71.2	[10]
Azurin	$Fe(EDTA)^{2-}$	7.0	0.2	$1.3\cdot10^3$	8.4	−155	[11]
$Co(phen)_3^{3+}$	Plastocyanin	7.0	0.1	$4.9\cdot10^3$	58.7	20.9	[10]
ditto	ditto	7.0	0.5	$1.0\cdot10^3$	—	—	[9]
"	"	7.0	0.1	$3.9\cdot10^2$	40.6	−61.2	[12]
$Ru(pyr)_3^{2+*}$	"	7.0	0.1	$1.6\cdot10^9$	—	—	[8]
$Co(5,6\text{-}Me_2\text{-}phen)_3^{3+}$	"	7.0	0.1	$8.0\cdot10^3$	57.0	4.2	[10]
$Co(5\text{-}Cl\text{-}phen)_3^{3+}$	"	7.0	0.1	$7.0\cdot10^2$	45.7	−33.5	[10]
$Co(4,7\text{-}Bs_2\text{-}phen)_3^{3-}$	"	7.0	0.1	$2.6\cdot10^1$	32.7	−10.9	[10]
$Co(ox)_3^{3-}$	"	7.0	0.5	$2.4\cdot10^{-1}$	—	—	[9]
$Ru(NH_3)_4(pyr)^{3+}$	"	6.5	0.1	$2.0\cdot10^3$	—	—	[7]
Plastocyanin	$Fe(EDTA)^{2-}$	7.5	0.2	$3.1\cdot10^4$	—	—	[11]
ditto	ditto	5.5	0.2	$5.5\cdot10^4$	—	—	[11]
"	$Ru(NH_3)_6^{2+}$	7.0	0.1	$2.4\cdot10^6$	—	—	[12]
$Ru(NH_3)_4(pyr)^{3+}$	Stellacyanin	6.5	0.1	$1.9\cdot10^5$	28.1	−50.3	[7]
$Ru(pyr)_3^{2+}$	ditto	7.0	0.1	$4.2\cdot10^8$	—	—	[8]
$Co(phen)_3^{3+}$	"	7.0	0.5	$1.8\cdot10^5$	—	—	[9]
$Co(ox)_3^{3-}$	"	7.0	0.5	$7.3\cdot10^2$	—	—	[9]
$Co(5,6\text{-}Me_2\text{-}phen)_3^{3+}$	"	7.0	0.1	$1.8\cdot10^4$	38.5	−29.3	[10]
$Co(4,7\text{-}Bs_2\text{-}phen)_3^{3-}$	"	7.0	0.1	$2.3\cdot10^6$	24.7	—	[10]
Stellacyanin	$Fe(EDTA)^{2-}$	7.5	0.5	$5.1\cdot10^5$	—	—	[11]
ditto	ditto	5.0	0.5	$5.7\cdot10^5$	—	—	[11]
"	$Fe(CN)_6^{4-}$	6.0	0.1	$1.6\cdot10^3$	—	—	[13]
$Ru(NH_3)_4(pyr)^{3+}$	Cytochrome *c*	5.3	0.5	$1.9\cdot10^4$	35.2	−46.1	[7]
$Co(ox)_3^{3-}$	ditto	7.0	0.5	5.5	—	—	[9]
$Co(pic)_2^{3+}$	"	7.0	0.2	$1.1\cdot10^4$	18.4	−10.5	[14]
$Co(phen)_3^{3+}$	"	7.0	0.1	$1.5\cdot10^3$	47.3	−25.1	[15]
$Co(5\text{-}Cl\text{-}phen)_3^{3+}$	"	7.0	0.1	$1.3\cdot10^2$	40.2	−67.0	[15]
$Co(5,6\text{-}Me_2\text{-}phen)_3^{3+}$	"	7.0	0.1	$2.7\cdot10^2$	51.9	−25.1	[15]
$Co(4,7\text{-}Me_2\text{-}phen)_3^{3+}$	"	7.0	0.1	$2.8\cdot10^1$	61.2	−12.6	[15]
$Co(4,7\text{-}Bs_2\text{-}phen)_3^{3-}$	"	7.0	0.1	$2.9\cdot10^3$	53.6	—	[15]
Cytochrome *c*	$Fe(pic)_2^{2-}$	7.0	0.2	$1.5\cdot10^4$	30.2	−64.5	[14]
ditto	$Ru(NH_3)_6^{2+}$	6.1	0.1	$3.8\cdot10^4$	—	—	[16]
"	$Fe(EDTA)^{2-}$	7.0	0.09	$9.0\cdot10^3$	—	—	[17]
$Fe(CN)_6^{3-}$	Cytochrome c_{551}	7.0	0.1	$1.0\cdot10^2$	—	—	[18]
$Co(phen)_3^{3+}$	ditto	7.0	0.1	$5.3\cdot10^4$	51.5	16.8	[15]
$Co(5\text{-}Cl\text{-}phen)_3^{3+}$	Cytochrome c_{551}	7.0	0.1	$4.4\cdot10^3$	39.4	−41.9	[15]
$Co(5,6\text{-}Me_2\text{-}phen)_3^{3+}$	ditto	7.0	0.1	$4.3\cdot10^3$	57.0	29.3	[15]
$Co(4,7\text{-}Me_2\text{-}phen)_3^{3+}$	"	7.0	0.1	$3.2\cdot10^3$	45.7	−25.1	[15]
$Co(4,7\text{-}Bs_2\text{-}phen)_3^{3-}$	"	7.0	0.1	$2.8\cdot10^4$	57.8	33.5	[14]
$Co(pic)_2$	"	7.0	0.2	$5.8\cdot10^2$	15.1	−142.5	[14]
Cytochrome c_{551}	$Fe(pic)_2^{2-}$	7.0	0.2	$3.2\cdot10^3$	40.2	−41.9	[19]
Cytochrome b_5	$Fe(EDTA)^{2-}$	7.0	0.5	$2.8\cdot10^2$	22.6	−122.3	[19]
Cytochrome *c* d_1 (haem *c*)	ditto	7.0	0.5	$2.0\cdot10^2$	—	—	[20]
Cytochrome *c* d_1 (haem d_1)	"	7.0	0.5	$0.6\cdot10^2$	—	—	[20]
Methaemoglobin	"	7.0	0.2	$2.9\cdot10^1$	23.0	−138	[21]
Methaemoglobin	"	7.0	0.2	$3.1\cdot10^1$	50.3	−54.3	[21]
ditto	"	6.3	0.15	$3.1\cdot10^1$	—	—	[22]
"	$Co(sep)^{2+}$	6.3	0.15	$3.5\cdot10^3$	—	—	[22]
"	SO_3^-	6.3	0.15	$4.5\cdot10^6$	—	—	[22]
$Co(ox)_3^{3-}$	HPIP	6.3	0.5	$8.0\cdot10^{-3}$	—	—	[9]
$Co(phen)_3^{3+}$	ditto	6.3	0.1	$2.8\cdot10^3$	62.4	30.2	[23]
$Fe(CN)_6^{4-}$	"	6.3	0.08	$(0.2\text{–}5)\cdot10^4$	—	—	[24]
HPIP	$Fe(EDTA)^{2-}$	7.0	0.1	$1.6\cdot10^3$	3.3	−171.8	[23]
Rubredoxin	$Ru(NH_3)_6^{2+}$	7.0	0.1	$9.5\cdot10^4$	5.9	−129.9	[25]
ditto	$V(H_2O)_6^{2+}$	4.0	0.1	$1.6\cdot10^4$	0.4	−167.6	[25]
"	$Cr(H_2O)_6^{2+}$	4.0	0.1	$1.2\cdot10^3$	0.4	−184.4	[25]
Ferredoxin	$V(EDTA)^{2-}$	7.3	0.05	$3.0\cdot10^4$	23.5	−88.0	[26]
	$Eu(PTDA)^{3-}$	7.3	0.05	$3.2\cdot10^5$	352.0	−29.3	[26]
$Fe(CN)_6^{3-}$	Cytochrome *f*	7.0	0.05	$1.7\cdot10^5$	3.6	−159.2	[27]

Notation: EDTA = ethylenediaminetetra-acetate; PDTA = 1,2-diaminopropanetetra-acetate; pyr = pyridine; ox = oxalate; pic = 2,6-dipicolinate; phen = 1,10-phenanthroline; 5-Cl-phen = 5-chloro-1,10-phenanthroline; $4,7\text{-}Me_2$-phen = 4,7-dimethyl-1,10-phenanthroline; $5,6\text{-}Me_2$-phen = 5,6-dimethyl-1,10-phenanthroline; 4,7-Bs-phen = 4,7-di-(benzenesulphonyl)-1,10-phenanthroline; sep = 1,3,6,8,10,13,16,19-octa-azabicyclo[6.6.6]eicosane; HPIP = high-potential iron-containing protein.

*Electronically excited.

Note. Numbers in square brackets denote references (Ed. of Translation).

The enthalpies and entropies of activation of electron transfer reactions vary from 0 to 352 kJ mol^{-1} and from −185 to 33 J mol^{-1} K^{-1} respectively, which indicates the possibility of appreciable conformational changes in the protein globules on electron transfer, because low activation enthalpies are typical for the formation of simple bimolecular collision complexes, while the activation entropy is −40 J mol^{-1} K^{-1}.

A species specificity is also manifested in electron transfer reactions. The high-potential iron-containing protein plastocyanin isolated from various sources reacts with different oxidants at unequal rates. At a low ionic strength, certain transfer agents form complexes with inorganic ions. The stability constant of the complex of azurin with the phenanthroline complex of cobalt is 457 litre mol^{-1}, while the rate constant for the reaction is $k = 21.3$ s^{-1}.[28] The reaction between the same copper-containing protein and hexacyanoferrate(III) proceeds in a more complex manner with formation of complexes of both oxidised and reduced azurin.[29] The oxidation of cytochrome *c* with hexacyanoferrate(III) also leads to the formation of a complex whose stability constant is 3.2×10^4 litre mol^{-1}, while the reaction rate constant is 1.9×10^3 s^{-1}.[30]

The effect of pH on the rate of the process is determined by the change in the degree of protonation of the aminoacid residues in the region of the centres of proteins. It has been shown that the deprotonated histidine residue His-83 in the azurin molecule decreases the electrostatic repulsion of positively charged ions, so that at pH's above the pK there is an appreciable increase of the reaction rate.[28] In reactions with hexacyanoferrate, His-35 is responsible for the binding of the substrate and its protonation in an acid medium appreciably accelerates the process. In the case of plastocyanin at pH < 5, the rate of reaction is appreciably reduced when the phenanthroline complex of cobalt is used, but is independent of pH when negatively charged complexes are employed.[10] This is because the phenanthroline complex binds to the deprotonated Tyr-83 residue located at a distance of 1 nm from the active centre, while hexacyanoferrate(III) forms a complex with deprotonated His-87 (pK = 5.7) located in the coordination sphere of copper.[31] Since the protonated Lys-72 and Lys-13 residues present near the haem group participate in the reactions of cytochrome *c* with hexacyanoferrate and other negatively charged reagents,[32] the association of cytochrome *c* with polyamines reduces the reaction rate. The rate of reaction is also influenced by the protonation of Tyr-87, which is responsible for conformational changes in the protein globule.[16]

Redox proteins exhibit overall an increased reactivity in relation to organic compounds (Table 2). Particularly large rate constants for the reduction of proteins have been observed in their reactions with the radical-cations of viologens and other radical species. The rate of reaction of flavodoxin, cytochrome *c*, and cytochrome c_3 with the methylviologen radical-cation approaches the rate of a diffusion-controlled process. Reduced flavin mononucleotide ($FMNH_2$) and its semiquinone ($FMNH^{\bullet}$) are also highly reactive owing to their low redox potentials.

It has been shown that the electron transfer in the reactions of cytochrome *c* with flavins takes place when the *N*-5-region in the flavin molecules is oriented towards the haem gap, while the *N*-pyrimidine component is oriented in the opposite direction.[34] The rigorous relative disposition of the reactants in the collision complex is responsible for the weak dependence of the reaction rate on the ionic strength of the solution despite the fact that $FMNH^{\bullet}$ and $FMNH_2$ have charges of −2 and −2.8 respectively. The results obtained are important for the theoretical analysis

of the dependence of the rate of electron transfer on the ionic strength of the solution.

The rate of reaction of the solvated electron with cytochrome *c*, myoglobin, and methaemoglobin is determined by diffusion.[41,42] The redox state of the active centre has little influence on the rate of reduction of the protein and only the removal of haem from mylglobin retards the rate of reaction by a factor of two.

Table 2. The rate constants for the interaction of redox proteins with organic compounds (25 °C).

Protein	Reductant	pH	μ, M	k_{12}, litre mol^{-1} s^{-1}	Refs.
Cytochrome *c*	FMNH$^{\cdot}$	7.0	0.01	$6.0\cdot10^7$	[33]
	FMNH$^{\cdot}$	7.0	0.1	$7.0\cdot10^7$	[34]
	$FMHH_2$	7.0	0.1	$3.0\cdot10^7$	[34]
	2-Methyl-1,4-naphthohydroquinone	6.0	0.1	$8.5\cdot10^2$	[35]
	Tetrachloro-1,4-hydroquonone	7.4	0.02	$4.2\cdot10^5$*	[36]
	Tetramethylphenylenediamine	7.4	0.02	$2.6\cdot10^4$*	[36]
	N-Methylhydrophenazine	7.4	0.02	$6.0\cdot10^5$*	[36]
	MV^+	7.0	0.004	$7.2\cdot10^7$*	[37]
	$C_2H_5OH^{\cdot}$	7.0	0.004	$1.4\cdot10^8$*	[37]
	MV^+	7.0	0.2	$2.3\cdot10^8$*	[37]
	MV^+	7.2	0.07	$2.4\cdot10^7$	[38]
	BV^+	7.2	0.07	$3.3\cdot10^7$	[38]
Cytochrome c_3	MV^+	7.2	0.005	$4.5\cdot10^8$*	[37]
	$C_2H_5OH^{\cdot}$	7.2	0.005	$6.0\cdot10^8$*	[37]
	MV^+	7.2	0.3	$9.7\cdot10^8$*	[37]
Methaemoglobin	$FMNH_2$	7.0	0.1	$5.5\cdot10^6$	[39]
Metmyoglobin	MV^+	7.0	0.004	$2.3\cdot10^7$*	[37]
	$C_2H_5OH^{\cdot}$	7.0	0.004	$5.5\cdot10^7$*	[37]
	MV^+	7.0	0.2	$5.4\cdot10^7$*	[37]
	Ascorbate anion	7.2–8.1	0.3	$1.2\cdot10^{-2}$	[40]
	Ascorbate dianion	7.2–8.1	0.3	$1.2\cdot10^2$	[40]
Ferredoxin	PV^+	[illegible]	0.05	$1.2\cdot10^5$	[26]
Flavodoxin	MV^+	8.0	0.055	$2.4\cdot10^8$	[37]
Flavodoxin semiquinone	MV^+	8.0	0.055	$7.0\cdot10^8$	[37]

Notation: MV^+, BV^+, and PV^+ are respectively the radical-cations of methylviologen, benzylviologen, and propylviologen; $C_2H_5OH^{\cdot}$ = ethanol radical.

*At 20 °C.

Note. Numbers in square brackets denote references (Ed. of Translation).

The study of the reduction of the acetylated, succinylated, and carboxymethylated cytochrome *c* by solvated electrons and other reductants made it possible to elucidate the role of the electrostatic interaction and of the conformational changes in the redox reactions.[41] It was found that the rate of the reaction involving cytochrome *c* is determined mainly by the conformational change, while electron transfer proceeds in the region of the haem group.

2. Quantitative Characteristics of Electron Transfer

The results of studies on the redox reactions of electron transfer agents have been used in the investigation of the possibility of applying outer-sphere transfer theory to protein systems. According to Marcus's ideas,[43] the rate constant for outer-sphere electron transfer k_{12} is related to the reaction equilibrium constant K and the internal exchange rate constants k_{11} and k_{22} for each reactant by the following equation:

$$k_{12}^{\infty} = (k_{11}^{\infty} k_{22}^{\infty} K f)^{1/4},$$

where $\lg f = (\lg K)^2 / 4 \lg (k_{11}^{\infty}/k_{12}^{\infty}/P^2)$ and $P \cong 10^{11}$ litre^{-1} mol^{-1} s^{-1}. The above quantity is usually close to unity when K is small. It follows from this equation that the rate of electron transfer depends on the accessibility of the central atom of the complex to individual reactants and also on the difference between the potentials of the reacting compounds, i.e. on the equilibrium constant of the process. If electron transfer at the protein active centre proceeds in the same way as in low-molecular-weight redox compounds, then the internal exchange rate constants for the proteins, calculated for reactions with different reactants, should be identical within the limits of an order of magnitude. Under the conditions of kinetic selectivity, the constants differ by several orders of magnitude.

The study of the rate of reduction of cytochrome *c* by iron complexes containing EDTA derivatives as ligands revealed a satisfactory agreement between the experimental rates and the rates calculated in accordance with outer-sphere transfer theory.[44] A difference is observed only for coordination compounds with an incomplete coordination sphere, which may be due to the increase in the size of the complex owing to the formation of a hydrogen bond between the solvent and a hydroxyl incorporated in this sphere. In the oxidation of cytochrome *c* by substituted ferricenium ions, whose redox potentials ranged over 0.56 V, the calculated and experimental constants were also found to be similar.[45] The enthalpy of activation for the process reaches ~20 kJ mol^{-1} and the entropy varies between −24 and −48 J mol^{-1} K^{-1}. These data show that the process does indeed proceed via the formation of a simple bimolecular collision complex without appreciable structural changes in the protein globule.

When coordination compounds of other metals are used instead of isotypical iron complexes, an appreciable scatter of the calculated internal exchange rate constants is observed (Table 3). These constants are almost independent of the nature of the reactant only for two proteins—stellacyanin and rubredoxin. For the remaining proteins investigated, they can differ by more than five orders of magnitude when different reactants are used.

Anomalously high constants are observed in those cases where the interaction between the reactants leads to the formation of complexes, for example the complexes of cytochrome *f* with plastocyanin or cytochrome c_{551} with azurin. Higher and lower constants correspond respectively to reactants incorporating aromatic and hydrophilic ligands, which is associated with the ability of the former to penetrate the hydrophobic region of the active centre. Available data show that iron derivatives containing ethylenediaminetetraacetic acid or its analogues as ligands do not penetrate the protein globule. The constants obtained for these compounds can therefore be used as "reference" values in comparing the reactivities of different proteins. Evidently (Table 3) the internal exchange rate constants obtained using $Fe(EDTA)^{2-}$ are similar for different cytochromes. Stellacyanin and methaemoglobin have the highest and lowest internal exchange rate constants respectively.

In order to eliminate effects associated with the hydrophilic or hydrophobic properties of ligands, an electronically excited ruthenium complex was used (Table 1). Since the

excitation entails a marked increase of the oxidation-reduction capacity of the reactant, this is equivalent to an increase of the driving force of the reaction. The rate constants for bimolecular oxidation-reduction reactions of reduced copper-containing proteins agree well with the theoretical values calculated from the redox potential of the excited complex. This is an important confirmation of the applicability of the outer-sphere electron transfer theory to these metalloproteins. The possibility of applying this theory has been postulated also for other proteins, in particular for flavodoxin.[47]

Table 3. The internal exchange rate constants for metalloproteins at pH 7.0 and at 25 °C.

Protein	Reagent	k_{11}^{∞}, litre mol^{-1} s^{-1}	Refs.
Cytochrome c_{551}	$Co(phen)_3^{3+}$	$2.2 \cdot 10^5$	[15]
ditto	Stellacyanin	$6.0 \cdot 10^{3*}$	[46]
"	$Co(ox)_3^{3-}$	1.4**	[9]
"	$Fe(pic)_2^{2-}$	$4.5 \cdot 10^{-1}$	[14]
"	$Fe(EDTA)^{2-}$	2.0	[14]
"	$Co(pic)_2^{-}$	$2.9 \cdot 10^3$	[14]
Cytochrome *c*	$Fe(EDTA)^{2-}$	$1.2 \cdot 10^1$	[15]
ditto	$Ru(NH_3)_6^{2+}$	$1.6 \cdot 10^1$	[7]
"	$Co(phen)_3^{3+}$	$7.1 \cdot 10^2$	[7]
"	$Ru(NH_3)_5(pyr)^{3+}$	$2.0 \cdot 10^4$	[7]
"	Ferrocenium ions	$(0.5 - 7.0) \cdot 10^3$	[45]
"	$Co(ox)_3^{3-}$	$4.6 \cdot 10^{1**}$	[9]
Cytochrome b_5	$Fe(EDTA)^{2-}$	4.3	[19]
Stellacyanin	$Co(ox)_3^{3-}$	$2.5 \cdot 10^{4**}$	[9]
ditto	$Fe(EDTA)^{2-}$	$2.3 \cdot 10^5$	[7]
"	Cytochrome c_{551}	$2.9 \cdot 10^{4*}$	[46]
"	$Ru(NH_3)_5(pyr)^{3+}$	$1.7 \cdot 10^5$	[7]
"	$Co(phen)_3^{3+}$	$3.0 \cdot 10^5$	[7]
Plastocyanin	ditto	$1.1 \cdot 10^4$	[10]
ditto	$Fe(EDTA)^{2-}$	$7.3 \cdot 10^1$	[10]
"	$Ru(NH_3)_6(pyr)^{3+}$	$4.9 \cdot 10^4$	[10]
"	Cytochrome *f*	$4.0 \cdot 10^{1*}$	[11]
Azurin	$Co(ox)_3^{3-}$	$2.7 \cdot 10^{-3**}$	[9]
ditto	$Fe(EDTA)^{2-}$	$2.8 \cdot 10^{-2}$	[15]
"	$Co(phen)_3^{3+}$	$1.6 \cdot 10^1$	[14]
"	Cytochrome c_{551}	$8.0 \cdot 10^{2*}$	[14]
HPIP	$Co(phen)_3^{3+}$	$1.8 \cdot 10^4$	[15]
ditto	$Fe(CN)_6^{3-}$	$1.0 \cdot 10^{1*}$	[23]
"	$Fe(EDTA)^{2-}$	$1.5 \cdot 10^{-2}$	[15]
"	$Co(ox)_3^{3-}$	$1.1 \cdot 10^{-3**}$	[23]
"	$Ru(NH_3)(pyr)^{3+}$	$2.1 \cdot 10^3$	[7]
	$Ru(NH_3)(pyr)_5^{2+}$	2.0	[15]
Rubredoxin	$Ru(NH_3)_6^{2+}$	$1.0 \cdot 10^{0*}$	[25]
ditto	$V(H_2O)_6^{2+}$	$1.7 \cdot 10^{0*}$	[25]
Methaemoglobin	$Fe(EDTA)^{2-}$	$2.8 \cdot 10^{-3}$	[24]
Metmyoglobin	ditto	$1.3 \cdot 10^{-1}$	[24]

*μ = 0.1 M. **μ = 0.5 M.

Note. Numbers in square brackets denote references (Ed. of Translation).

The relations arising from the outer-sphere transfer theory have been observed also when organic reactants were employed. Thus for cytochrome *c* or cytochrome *f* a linear dependence of the logarithm of the rate constant on the standard potentials of quinones has been observed experimentally with the slope of the resulting linear plot close to the theoretical value.[48] These experimental data show that the rate-limiting stage of the process is a one-electron transfer and that the internal exchange rate constants for the quinones employed do not differ greatly.

In the reactions of electron transferring proteins with organic reactants, the rate of electron transfer was found to approach a limiting value with increase of the free energy of reaction.[49] This is apparently due to the fact that the energy of the reorganisation of the solvent in these processes is lower than in reactions with inorganic charged complexes and the system approaches the "inversion" region,[43] in which the rate diminishes with increase of the free energy of reaction. It is essential to note that the attainment of a limiting rate for a large free energy change has been observed also in reactions involving low-molecular-weight compounds. In order to account for the experimental data, an empirical expression has been put forward[50] for the free energy of activation, which is equal to the free energy of reaction when the reaction is highly exothermic. This expression has been justified theoretically by Scandola and Balzani.[51]

Together with outer-sphere electron transfer theory, the thermally activated tunnel transfer theory has been used to calculate the rate of the process in proteins.[52–54] According to Chien,[55] the tunnel electron transfer rate constant is given by

$$k_1^{\infty} = 0.6023 \cdot k_{\mu} (2\pi\lambda_0^3 r / R_1)$$

$$k_{\mu} = (4\pi^2/h) |T(r)|^2 (4\pi k_B T\Delta)^{-1/2} \exp[-(E_a - E_b - \Delta)^2/4 k_B T\Delta],$$

$$|T(r)| = 2.7 (N_a N_b)^{-1/2} \exp(-7.2\, r),$$

where λ_0 is the decay constant, r the electron transfer distance, R_1 the radius of the protein molecule, h Planck's constant, k_B Boltzmann's constant, T the absolute temperature, Δ the vibrational synchronisation parameter, E_a and E_b are the standard potentials of the protein and the mediator, N_a and N_b the numbers of distribution sites for the initial and final wave functions, and $T(r)$ is the electron exchange matrix element. The decay constant for protein molecules is usually assumed to be 0.07 nm and the vibrational synchronisation parameter does not exceed 1 eV.

According to the tunnel transfer theory, the enthalpy and entropy of activation are expressed by the equations

$$\Delta H^{\neq} = (E_a - E_b - \Delta)^2/4\Delta - 3RT/2,$$

$$\Delta S^{\neq} = R \ln \left[\frac{23.8}{k_B T} \left(\frac{2\pi\lambda_0^3 r}{R_1} \right) \left(\frac{1}{4\pi k_B T\Delta} \right)^{1/2} |T(r)|^2 \right] - \frac{3R}{2}.$$

These equations are used to calculate the vibrational synchronisation parameters and decay constants.

For cytochrome *c*, the rate constants calculated within the framework of the outer-sphere and tunnel transfer theories are similar.[44] However, when ferredoxin is reduced by vanadium and europium complexes or the methylviologen radical-cation, the mutual exchange rate constants calculated using the outer-sphere transfer theory are smaller by four orders of magnitude than the constants determined experimentally for model compounds. At the same time, the calculation performed within the framework of the tunnel transfer theory yields values close to the experimental data.[26] It is essential to note that the calculated constants are extremely sensitive to the vibrational synchronisation parameter and the decay constant. The latter can be calculated from thermodynamic data, but in most cases their use in subsequent calculations does not lead to a satisfactory agreement with experimental quantities.

Assuming that the decay constant is invariant for different metalloproteins, Mauk and co-workers[15] proposed a semi-empirical formula relating the electron transfer distance to the mutual exchange rate constant:

$$r = 0.62 - 0.035 \ln k_{11}^{\infty}.$$

After being reduced by the van der Waals radius, this distance corresponds to the depth of the active centre, which is 0.34–0.69 for cytochromes, 0.28 for plastocyanin, and 0.56 for azurin, and 0.58 nm for high-potential iron-containing proteins. The active centre of stellacyanin is located on the surface of the globule and for this reason the mutual exchange rate constant for this protein has been used in the derivation of the last equation. Its application to

electron transfer agents which do not contain metals leads to results which are satisfactorily correlated with X-ray diffraction data.[56]

3. Experimental Data for Electron Transfer in Protein Complexes and Structural Ideas

The rate constants for reactions in protein complexes assume values in the range 10^3–10^7 litre mol^{-1} s^{-1} (Table 4). The pairs of proteins forming complexes in electron transport chains (cytochrome *c* oxidase–cytochrome *c*, cytochrome *c*–cytochrome b_5, plastocyanin–cytochrome *f*, azurin–cytochrome c_{551}, etc.) react much faster than proteins which do not give rise to such complexes. Similar pairs are produced as a result of the electrostatic interaction of the positive and negative charges on the aminoacid residues, which has been established in relation to complexes of cytochrome *c* with cytochrome c_1 and cytochrome *c* oxidase,[68] of cytochrome b_5 with methaemoglobin,[69] and of cytochrome b_2 with cytochrome *c*.[70] The formation of complexes between proteins which are not physiological partners is possible on interaction of sections bearing both unlike and like charges.[57,70] The hydrophobic interaction forces apparently play an important role in such cases.

Table 4. The rate constants for electron transfer between proteins (pH 6.1–7.6).

Oxidant	Reductant	*T*, °C	μ, M	*k*, litre mol^{-1} s^{-1}	Refs.
Azurin	Cytochrome *c*	25	0.1	$6.6\cdot10^3$	[57]
ditto	Cytochrome c_{551}	25	0.1	$3.0\cdot10^6$	[58]
"	Cytochrome c_{553}	25	0.05	$(2–0.5)\cdot10^6$	[59]
HPIP	Cytochrome c_2	20	0.02	$(2.4–1.5)\cdot10^5$	[47]
ditto	Cytochrome *c*	20	0.02	$(8.7–2.6)\cdot10^4$	[46]
"	ditto	—	0.21	$(12–3.8)\cdot10^4$	[60]
Plastocyanin	Cytochrome *f*	25	0.1	$3.6\cdot10^7$	[61]
ditto	Cytochrome c_{553}	25	0.1	$5.0\cdot10^5$	[61]
"	Cytochrome c_{551}	25	0.1	$7.5\cdot10^5$	[61]
"	Cytochrome *c*	25	0.1	$1.0\cdot10^6$	[61]
"	Cytochrome c_{553}	25	0.05	$4.0\cdot10^5$	[59]
"	Cytochrome *c*	25	0.10	$1.5\cdot10^6$	[57]
Cytochrome *c*	Cytochrome c_{553}	25	0.05	$4.0\cdot10^4$	[59]
"	Cytochrome c_{551}	20	0.02	$4.9\cdot10^4$	[62]
"	Azurin	25	0.05	$1.6\cdot10^3$	[59]
"	Stellacyanin	25	0.1	$1.9\cdot10^3$	[46]
"	Cytochrome b_5	25	0.1	$3.0\cdot10^7$	[63]
"	Flavodoxin semiquinone	23,5	0.18	$1.3\cdot10^5$	[64]
"	Cytochrome c_1	10	1.02	$2.4\cdot10^5$	[65]
Cytochrome c_1	Cytochrome *c*	10	1.02	$3.3\cdot10^5$	[65]
Cytochrome c_{551}	Stellacyanin	25	0.1	$1.7\cdot10^6$	[46]
Cytochrome *c* oxidase	Cytochrome *c*	25	0.1	$1.0\cdot10^8$	[66]
ditto	ditto	8	0.05	$3.5\cdot10^7$	[67]

Note. Numbers in square brackets denote references (Ed. of Translation).

The groups involved in the formation of the complexes have been discovered by selective modification. In cytochrome *c*, the Lys-13, Lys-25, Lys-27, Lys-72, and Lys-79 residues are in the vicinity of haem and interact with the negatively charged Asp-48, Asp-60, Glu-43, Glu-44, and haem propionate in cytochrome b_5 in a complementary manner.[63] The carboxy-groups of Asp-112, Glu-114, and Glu-198 participate in the interaction of cytochrome *c* with cytochrome *c* oxidase.[71] The electrostatic interaction between the two physiological partners in the cytochrome b_5-NADH–cytochrome b_5 reductase system takes place between the complementary Glu-47, Glu-48, and Glu-52 residues, haem propionate, and the sixth carboxy-group of cytochrome b_5, whose position has not been established.[72] The lysine residues at a distance of 3 nm from the redox centres also participate in the reactions of cytochrome c_3 with hydrogenase or ferredoxin.[73]
cytochrome c_3 with hydrogenase or ferredoxin.[73]

X-Ray diffraction data for individual proteins made it possible to construct models of the complexes and to put forward a hypothetical electron transfer pathway. In the complex of cytochrome *c* with cytochrome b_5, the prosthetic groups are almost coplanar and the distance between the nearest atoms of the porphyrin ring is 0.85 nm.[74] The structure of the cytochrome *c* peroxidase–cytochrome *c* complex has been analysed in greater detail.[75] In this complex the His-13, His-27, His-72, His-86, His-87, Gln-12, and Gln-16 residues of cytochrome c interact with the Asp-79, Asp-216, Asp-37, Asp-37, Asp-34, Asn-87, and Gln-86 residues of the peroxidase. In the complex two haem moieties are parallel, but their planes are separated in the vertical direction by a gap of 0.68 nm. The distance between the iron atoms is 2.46 nm and the minimum distance between the haem moieties is 1.65 nm. It has been assumed[75] that the electron transfer in the complex takes place with migration of the free valence, since it has been suggested[76] that this process proceeds via a tunnel mechanism. The experimental detection of the charge-transfer complex between haem and hexacyanoferrate and also of charge transfer in the cytochrome c–P_{870} complex constitutes an important confirmation of tunnel transfer.[77]

Hypothetical models of the cytochrome b_5–methaemoglobin [78] and flavodoxin–cytochrome *c* complexes have also been constructed.[64] In the complex of cytochrome b_5 with methaemoglobin subunits, the negatively charged groups of cytochrome b_5 interact with the positively charged groups of methaemoglobin. The haem groups in the complex are arranged in parallel at a distance of 0.7–0.8 nm from one another, while the distance between the iron atoms is 1.6 nm. Since the latter is close to the corresponding distance in the cytochrome *c* peroxidase–cytochrome *c* complex, presumably the electron transfer between the haem groups proceeds via tunnelling with active participation of the propionate group of the haem in cytochrome b_5.[79]

The Lys-13, Lys-25, Lys-27, and Lys-29 residues of cytochrome *c* and the Glu-120, Glu-65, Glu-62, and Asp-28 groups in flavodoxin take part in the formation of the flavodoxin–cytochrome *c* complex.[64] The shortest distance between the sulphide bond of Cys-17 in cytochrome *c* and the methyl group at C(7) of flavin in flavodoxin is 0.34 nm. The distance between the carbon atoms in the conjugate systems is 0.57 nm. The haem and flavin planes are inclined at an angle of 30°, which ensures a satisfactory overlap of the π orbitals and a high rate of electron transfer within the complex. Analysis of X-ray diffraction data for cytochrome c_3 [80] and of kinetic results for cytochrome cd_1 [81] confirmed the conclusion that a parallel arrangement of the haem groups is essential in order to ensure a high rate of intramolecular electron transfer.

Experimental rates of electron transfer in protein molecules have been obtained using proteins labelled with ruthenium pentammine.[82–84] It has been found that the rates of electron transfer in cytochrome *c* 20 and 82 s^{-1} over distances of 1.4 and 1.6 nm respectively. In the azurin molecule the electron transfer to the active centre at a distance of 1.2 nm from the label is slower (1.9 s^{-1}).[84] An even greater difference between the rates has been observed for electron transfer between the haemoglobin subunits.[85] In this protein the distance between the metal ions is 2.5 nm and the rate of electron transfer is 60 s^{-1}. All these results indicate the specificity of the protein globule and the influence of the nature of the active centre. The spatial orientation of both metal atoms and the ligands is extremely

important. Electron transfer between active centres apparently takes place via tunnelling, but one cannot rule out the possibility of valence transfer and also the involvement in this process of intermediate covalent bonds and aromatic aminoacid residues.

III. QUANTITATIVE CHARACTERISTICS OF THE OXIDATION–REDUCTION REACTIONS OF ENZYMES

Few quantitative data are available for electron transfer with participation of enzymes, which can be accounted for by the high specificity of enzymes and the complexity of the mechanism of their action. Reaction series have been selected for a few enzymes, which made it possible to obtain kinetic data for the redox reactions of catalytically active proteins.

1. Oxidation of the Active Centre of Glucose Oxidase

The oxidation of the active centre of glucose oxidase by quinonoid electron acceptors proceeds at rates ranging from 3.5×10^3 to 1.5×10^6 litre mol^{-1} s^{-1}.[56] The rate of reaction for compounds having the *p*-quinone structure increases with increase of the redox potential of the electron acceptor. There is a linear relation between the logarithms of the rate constants and the potentials of the acceptors, which is described by a correlation straight line with a slope of 8.4 V^{-1}.

Inorganic complexes react with reduced glucose oxidase more slowly despite their high potential, but the rate of oxidation by hexacyanoferrate(III) greatly increases in an acid medium. At a high ionic strength of the solution the phenanthroline or bipyridyl complexes of cobalt react with the above substrate at a rate which is close to the rate of its oxidation by hexacyanoferrate(III), but, when the pH is diminished (to 4), the rate changes much less.

The data obtained led to the following conclusions: (1) the rate-limiting stage of the process is one-electron transfer, because the rates of reactions with participation of one-electron acceptors (tetracyano-*p*-quinodimethane and its radical-anion) obey the Brønsted relation; (2) electron transfer takes place via the outer-sphere mechanism; (3) derivatives of *o*-quinones form an independent group of acceptors, which have a higher reactivity than the *para*-derivatives owing to the *ortho*-effect; (4) the low rate of oxidation of glucose oxidase by inorganic compounds is caused by the specificity of the action of one-electron acceptors, which is determined by the presence of the charge.

The mutual exchange rate constants for glucose oxidase calculated on the basis of the outer-sphere transfer theory are 10^{-2}–10^{-9} litre mol^{-1} s^{-1} at a high ionic strength and the electron transfer distance for inorganic reagents is 1.1 to 1.3 nm in a neutral medium and 0.8–0.9 nm in the acid region. In the case of hydroquinone the distance is 0.88 nm. These differences in the calculated distances are determined by the degree of penetration of the reagents into the active centre zone, which entails structural changes in the protein globule.

2. Reactions of Cytochrome b_2 with Electron Acceptors

The rate constants for the oxidation of cytochrome b_2 by organic electron acceptors depend on the redox potentials of the compounds and vary in the range 1.2×10^4–4.7×10^7 litre mol^{-1} s^{-1}.[86] A linear relation is observed between the logarithm of the rate constant and the redox potential, the slope of the corresponding plot being 8.6 V^{-1}. Reduced cytochrome b_1 also reacts with inorganic complexes, haem being oxidised, while organic electron acceptors interact with another prosthetic group of the enzyme (FMN). The rate constants for mutual exchange with the complexes, calculated from kinetic data, vary in the range 1×10^1 to 6.6×10^6 litre mol^{-1} s^{-1}, while the constant for *p*-benzoquinone is 1.6×10^3 litre mol^{-1} s^{-1}. This constant is particularly large for the bipyridyl complex of cobalt, which reacts in the cytochrome *c* binding zone. The electron transfer distance for non-penetrating complexes is 0.31–0.54 nm, while for *p*-benzoquinone it is 0.36 nm. These values agree with those obtained for other cytochromes.

3, Characteristics of Electron Transfer in the Peroxidase Process

The formation of active intermediate states of the enzyme (the complexes E_1 and E_2) is characteristic of the catalysis by peroxidase.[87] It has been suggested that the iron in compound E_2 is in the ferryl [FeO^{2+}] state. In E_1 one of the oxidation equivalents exists in the form of the porphyrin π-radical-cation, while the other is analogous to the E_2 equivalent. In cytochrome *c* peroxidase the free radical is localised at the Trp-51 residue and not on the porphyrin ring.[88] The oxidation-reduction potentials of the two forms of enzymes depend little on the pH and amount to 0.963 V for the E_2/E transition and 0.94 V for the E_1/E_2 transition.[87]

Table 5. The rate constants for the reduction of peroxidases at 25 °C.[89–92]

Peroxidase	Reagent*	pH	μ, M	k_{12}, litre mol^{-1} s^{-1}
Cytochrome *c* peroxidase (E_2 complex)	$Co(tpyr)_2^{2+}$	7.0	0.1	$1.1 \cdot 10^5$
ditto	$Ru(NH_3)_5(pyr)^{2+}$	7.0	0.1	$9.5 \cdot 10^5$
"	$Fe(CN)_6^{4-}$	7.0	0.1	$7.6 \cdot 10^3$
"	$Fe(CN)_6^{4-}$	5.26	0.1	$8.1 \cdot 10^3$
"	$Fe(bpyr)_3^{2+}$	5.26	0.1	$3.6 \cdot 10^2$
"	$Fe(dmbpyr)_3^{2+}$	5.26	0.5*	$4.2 \cdot 10^3$
"	$Fe(tmbpyr)_3^{2+}$	5.26	1.0	$1.2 \cdot 10^4$
"	$Fe(phen)_2(CN)_2$	5.26	2.0	$3.4 \cdot 10^4$
"	$Fe(phen)(CN)_4^{2-}$	5.26	1.0	$8.0 \cdot 10^5$
Horseradish peroxidase (E_1 complex)	$Fe(CN)_6^{4-}$	6.93	0.11	$8.5 \cdot 10^5$
Horseradish peroxidase (E_2 complex)	$Fe(CN)_6^{4-}$	6.83	0.11	$1.8 \cdot 10^4$
Horseradish peroxidase	$Co(sep)^{2+}$	6.3	0.15	$7.0 \cdot 10^4$
ditto		6.3	0.15	$5.0 \cdot 10^5$
"	MV^+	7.0	∞	$4.9 \cdot 10^5$

Notation: tpyr = terpyridyl; dmbpyr = dimethylbipyridyl; tmbpyr = trimethylbipyridyl.

*At 20 °C.

The reduction of the complex E_1 by inorganic compounds proceeds in most cases via a one-electron mechanism with formation of the complex E_2. The rate constants for the reduction of the complex E_1 are in the range 10^4–10^7 litre mol^{-1} s^{-1} (Table 5). Ruthenium derivatives containing an aromatic compound as the ligand reacts at an exceptionally high rate.[89] Ferriperoxidase is also reduced by powerful reductants to the ferro-form. The standard potential of this transition is −0.25 V.[93] Cobalt sepulchrate, whose

standard potential is −0.3 V, reacts at a rate of 7×10^4 litre mol^{-1} s^{-1}, while dithionite reacts faster almost by an order of magnitude.[92] In the reactions of ferriperoxidase with MV^+, a marked dependence of the rate of reduction on the ionic strength of the solution is observed. The charge of peroxidase calculated from these results is −5. It has been shown[90] that, in the reduction of cytochrome *c* peroxidase in the E_2 state by iron complexes, a linear relation is observed between the free energies of reaction and activation, which agrees with the outer sphere transfer theory. The mutual exchange rate constant calculated for hexacyanoferrate(II) is 7.5×10^{-5} litre mol^{-1} s^{-1} while that calculated for the bis-(terpyridyl)cobalt(II) is 1.3×10^{-4} litre mol^{-1} s^{-1}. The mutual exchange rate constant for the complex E_2 of horseradish peroxidase is almost an order of magnitude smaller (9×10^{-7} litre mol^{-1} s^{-1}) when hexacyanoferrate(II) is used. The electron transfer distances calculated from these data are 0.93, 0.95, and 1.1 nm respectively for the above three cases.

The free energies of the reactions of ferriperoxidase with reductants are smaller than for the reactions involving the complexes E_1 or E_2 and the probability that the system enters the inversion region diminishes. The present authors determined[4] the mutual exchange rate constant for the interaction of MV^+ with ferriperoxidase. It amounts to 3.6 litre mol^{-1} s^{-1} and the electron transfer distance is 0.58 nm. Calculation within the framework of tunnel transfer with $\Delta = 0.8$ eV and a decay constant of 0.07 nm, adopted by analogy with ferricytochrome *c*, also yielded an electron transfer distance of 0.58 nm.

4. The Oxidation of Electron Donors in the Laccase Reaction

Laccase is one of the few oxidases which reduce molecular oxygen to water in a four-electron process. The active centre of laccase includes copper ions of three types which are in the bivalent state and all participate in the catalytic reduction of oxygen. Laccase is a two-substrate enzyme catalysing the oxidation of the hydroxy-derivatives of benzene or inorganic ions by molecular oxygen. The rate constant for the bimolecular interaction of the laccase active centre with oxygen is 1.6×10^6 litre mol^{-1} s^{-1} and the rates of oxidation of substrates vary in the range 1.0×10^5–2.5×10^6 litre mol^{-1} s^{-1} and depend on the nature of the enzyme.[94,95] The rate constants for the reduction of the active centre are not correlated with the redox potentials of the donors. However, for one of the laccases investigated,[95] an increased bimolecular constant has been observed for a series of hydroquinones containing substituents with an unshared electron pair despite the fact that the introduction of the substituent increases the redox potential of the electron donor. A similar effect of substituents has likewise been observed in the study of prestationary kinetics of the reduction of copper of the first type in laccase.[96] It has been suggested that this is caused by the additional coordination of the nucleophilic substituent to copper atoms of the third type. This in fact accounts for the appreciable increase of the constant for the inhibition by the fluoride ion of the oxidation of 2,3-dicyanohydroquinone, because the affinity of fluoride for copper of the second type also diminishes on coordination of copper of the third type to the azide.[97]

Kinetic data for the reduction of the active centre of laccase by hydroquinone and hexacyanoferrate(II), obtained by the stopped flow method,[98] permit the conclusion that the reaction proceeds via the outer sphere transfer mechanism. The absence of the Brønsted relation in oxygen reduction processes is due to the complexity of the mechanism and the four-electron reduction of oxygen.[98]

An important question in catalysis by laccase is that of the nature of the high potential of the active centre of the enzyme. Spectroelectrochemical measurements of the redox potential of laccase in the range 7–41 °C yielded the following changes in the thermodynamic parameters and the reduction potential of the active centre: $\Delta S = -58.2$ J mol^{-1} K^{-1}, $\Delta H = -92.6$ kJ mol^{-1}, and E (25 °C) = 780 mV.[99] Comparison of the entropy and enthalpy parameters with the values known for one-centre metalloproteins permits the conclusion that the high potential of the blue copper is due mainly to stabilisation by ligands, while protein solvation effects play an appreciably smaller role.

IV. THE RATES OF ELECTROCHEMICAL REACTIONS OF PROTEINS

There exists a profound analogy between electron transfer processes in solution and electrode reactions. Within the framework of the simple isotropic medium models, the outer-sphere transfer theory yields the following expression relating the rates of the processes in the bulk of the solution and on the electrode surface:[43]

$$(k_h/P_h)^{1/2} \geqslant k_s/P_s ,$$

where k_h and k_s are the rate constants for the bimolecular and heterogeneous processes respectively and P_h and P_s are the numbers of the maximum possible collisions in the bulk (~10^{11} litre mol^{-1} s^{-1}) and on the surface (~10^4 cm s^{-1}). With the aid of this equation, it is possible to estimate the rates of electrochemical reactions of proteins on the basis of kinetic results, while the concept of tunnel transfer[100] makes it possible to calculate the rate from structural data for protein molecules.

1. The Redox Reactions of Proteins at the Mercury Electrode

In the early investigations the electrochemical properties of proteins were most often studied at dropping and stationary mercury electrodes. Cytochrome *c* was the first protein to be investigated and it has been studied in greatest detail.[101] In the numerous investigations devoted to this protein, different degrees of reversibility of the electrochemical reactions of cytochrome *c* at mercury electrodes were observed. For example, Betso et al.[102] and Scheller et al.[103] believe that the reduction of cytochrome c at low concentrations is reversible. However, other investigators[104,105] have shown that, in contrast to haemin, native cytochrome *c* is reduced with a high overpotential, reaching 0.3 V, while the heterogeneous rate constant for this process is extremely low (10^{-10}–10^{-11} cm s^{-1}). The observed difference in the electrochemical behaviour of cytochrome c is apparently caused by the different compositions of the medium and by the presence of impurities. Indeed it has been shown recently[101] that, even in highly purified protein preparations, there is an admixture which influences significantly the rate of the electrochemical reaction.

The study of electrochemical properties of cytochrome *c* at the mercury electrode made it possible to formulate the problem of the state of the protein globule on its surface. It has been suggested that the first adsorbed portions of the protein "are flattened" and are irreversibly denatured on the electrode surface.[106,107] According to Kuznetsov,[108] the cytochrome *c* molecules are discharged within the pores of the monolayer. The adsorption of native globules in such

pores is reversible. The area of the pore is 4 nm^2 for cytochrome *c*, which is smaller by a factor of 2.25 than the cross-sectional area of the native protein molecule. It is essential to note that the adsorption of cytochrome *c* on the second and subsequent layers is slower compared with the adsorption on the free mercury surface. For this reason, at a high overpotential, electron transfer may be limited by the exchange of the adsorbed and freely diffusing molecules. As a result, the limiting currents do not reach the values obtained from the Ilkovič equation.

Cytochrome c_7, whose structure resembles that of cytochrome *c*, is a three-haem protein. This low-potential haemoprotein is reduced reversibly at −0.17 and −0.22 V.[109] The rate of the process is determined by diffusion and electron transfer not retarded by the adsorption of the protein. Another cytochrome, namely cytochrome c_{553}, has a low molecular weight, but its haem environment differs from that in cytochrome *c*. The electrochemical reaction of cytochrome c_{553} is quasi-reversible.[110] The cathodic reduction overpotential of this cytochrome does not exceed 40 mV and the redox potential of the system is 0.02 V. The electrochemical reduction of the four-haem cytochrome c_3 is reversible with a low rate constant (>0.1 cm s^{-1}) but takes place stepwise owing to the non-equivalence of the haem groups in the protein molecule.[111,112]

Cytochrome b_5 partly cleaved by trypsin gives rise to distinct waves under the conditions of classical polarography and the half-wave potential is near its redox potential.[113] The native cytochrome b_5, isolated using detergents, is reduced at the mercury electrode with much more difficulty and a much higher overpotential, while cytochrome P-450 is not reduced at all.[113] Other haem-containing proteins, whose natural functions consist in electron transfer, namely metmyoglobin and methaemoglobin, are reduced at the electrode with a high overpotential and irreversibility.[114,115]

Comparison of the electrochemical properties of haem-containing electron transferring proteins shows that only low-molecular proteins with low redox potentials, which are correlated with the degree of accessibility of haem to water, undergo rapid electrochemical reactions.[116] Consequently the structural features of the protein molecule play an appreciable role in electrochemical reactions. There is no doubt that the adsorption and orientation factors also affect the rate of the process. The weak adsorption and (or) aggregation of cytochrome b_5 is apparently the cause of the slowness of the reaction, although the active centre has an appreciable area of contact with the aqueous medium.

The electrochemical properties of other proteins have also been investigated at the mercury electrode. The reduction of ferredoxin from higher plants takes place at −0.33 V and that of bacterial ferredoxin occurs at −0.325 V and protons participate in the electrode reaction.[117] The available literature data show that ferredoxins of plant and microbial origin behave differently at the electrode.[118]

Comparative electrochemical studies of five proteins, namely ferredoxin, rubredoxin, flavodoxin, cytochrome c_3, and cytochrome *c*, have revealed the following features:[119]

(1) The positively charged cytochrome c_3 is reduced readily at the mercury electrode, because the process takes place below the zero-charge potential of mercury, and poly-L-lysine inhibits the process.

(2) Bacterial and plant ferredoxins as well as flavodoxin are negatively charged. Their redox potentials are identical with the zero-charge potential of the electrode. For this reason, a high rate of electrochemical reaction is observed only in the presence of positively charged surfactants.

(3) The positively charged cytochrome c is slowly reduced on the positively charged electrode in the region of the redox reaction of the protein.

All these factors permit the conclusion that electrostatic interaction plays a significant role in the electrochemical reactions of proteins and that the reduction of positively charged proteins is slow at a positive potential higher than the zero-change potential. Similar difficulties arise also in the reduction of negatively charged proteins if the electrode potential is more negative than the charge of the protein [the electrode potential corresponds to a charge more negative than that of the protein?]. A non-uniform distribution of charges on the surfaces of the protein globules can, however, lead to the observation of the effective interaction of like charges on the electrode and the protein or, conversely, the process may not occur despite the fact that they have opposite charges. An example is provided by data for the reduction of the negatively charged rubredoxin and flavodoxin, whose redox reactions do not occur when the mercury electrode is positively charged.[119] Therefore, as in cytochrome *c*, the orientation of the dipole in these proteins is such that it forms an angle with the haem group. Consequently, as the protein molecule approaches the positively charged surface, the side groups interact preferentially and the active centre does not come into direct contact with the electrode.

The electrochemical reduction of enzymes whose molecular weight is higher than that of the electron-transferring proteins proceeds as a rule with dissociation of the co-factor.[114] Even in the case of cholesterol oxidase, in which FAD is bound to the protein by a covalent bond, adsorption leads to the partial liberation of the cofactor and the electrochemical properties of the system are determined by the redox reactions of FAD in the presence of the denatured protein.[120] The catalytically active fragments of glucose oxidase are formed on partial cleavage of the native structure of the enzyme and are reduced at a potential higher by 0.1 V than the reduction potential of FAD.[121] Furthermore, the enzymically reduced enzyme is oxidised electrochemically with a low overpotential. The agreement of the half-wave potential $E_{1/2}$ with the redox potential of the enzyme and the constancy of $E_{1/2}$ as the enzyme concentration is varied show that the glucose oxidase fragment reacts reversibly. Native glucose oxidase reacts quasi-reversibly on platinum electrodes.[122]

Other proteins investigated, including flavoproteins from egg yolk, are denatured when adsorbed on the mercury electrode with liberation of the co-factor, which is reduced in the region of the redox potential.[121] When peroxidase is adsorbed on an amalgamated gold electrode, an oxidation-reduction process is observed, but it is associated with redox reactions of the disulphide bonds.[123]

2. Electrochemical Reactions of Proteins on Solid Electrodes

It was shown in early investigations that the electrochemical reaction of cytochrome *c* on gold, platinum, nickel, silver, and other metallic electrodes and on the silicon *p*-type semiconductor electrode is irreversible.[101] A quasi-reversible reaction of cytochrome *c* has been observed on optically transparent electrodes.[124] It has been shown[101] that thoroughly purified cytochrome *c* reacts quasi-reversibly on a tin-doped indium oxide electrode with a heterogeneous rate constant of 4.9×10^{-3} cm s^{-1}. Detailed investigation of the electrochemical reaction of cytochrome *c* on solid electrodes showed that the rate of the process depends not only on the degree of purification of the protein but also on the method used to prepare the electrode and on the composition of the buffer solution.[125] The available data demonstrate convincingly that cytochrome *c* can exchange electrons with

various electrodes. The rate of the process is then close to that calculated from the mutual exchange rate constant, which amounts to 0.1 cm s^{-1} when account is taken of the steric factor (0.025).[126]

The reduction and oxidation of ferredoxin on pyrolytic graphite electrodes proceeds reversibly only in the presence of magnesium ions.[127,128] The electrochemical reactions of rubredoxin, flavodoxin, and ferredoxin on the same electrodes in the presence of positively charged chromium hexammine ions or magnesium ions are either quasi-reversible or reversible.[129] Ions are not required for the rapid reaction of azurin. The observed phenomena can be accounted for by the fact that all the proteins investigated are negatively charged and have redox potentials in the region of which the graphite surface has a negative capacitive charge. The positively charged ions are concentrated in the outer plane of the Helmholtz layer and thereby diminish the repelling effect of the electrode on rubredoxin, flavodoxin, and ferredoxin. The weak influence of the positively charged ions in the case of azurin is caused by the high degree of charge pairing between side groups.

The electrochemical oxidation of cytochrome b_5 at platinum gauze electrodes takes place at 0.9 V, while the reduction of the enzyme is masked by the reduction of water.[130] The data obtained indicate the irreversibility of the electrochemical reaction of the enzyme. However, spectroelectrochemical studies have shown that both cofactors (FMN and haem) exchange electrons via an independent pathway.

The electrochemical reactions of oxygen transporting agents are extremely low. It has been shown[4] that the electrochemical reaction of methaemoglobin at the SnO_2 electrode proceeds irreversibly with a heterogeneous constant of 5.2×10^{-6} cm s^{-1} and a transport coefficient of 0.29. The adsorption of the tetraethylammonium ion increases the constant by a factor of 3. This may be caused by the change in the potential of the diffuse layer of the electrode following the adsorption of the surface-active cation.

3. The Oxidation-Reduction Reactions of Proteins on Modified Electrodes

The results for the electrochemical reactions of proteins on solid electrodes discussed above show that the rate of the process is greatly influenced by the state of the electrode–buffer solution interface. Modifying agents are used to alter it in a specific manner (Table 6). The electrochemical reactions of cytochromes on gold, carbon, or platinum electrodes modified with 4,4′-bipyridyl or its sulphur-containing analogues take place at the equilibrium potential with a high rate constant, which is close to the constant for the thoroughly investigated hexacyanoferrate(III)/hexacyanoferrate(II) pair. Together with these four analogues of 4,4′-bipyridyl, several compounds capable of accelerating the redox reaction of cytochrome *c* are known: 4,4′-azopyridyl, 2,5-di(4-pyridyl)-1,3,4-thiadiazole, and purine and its derivatives.[133,145] The modifying agents each contain as a rule one nitrogen atom at the ends of the molecule, have a definite length, and possess a conjugated electron system. Other compounds which do not meet these requirements but whose structure is close to that of 4,4′-bipyridyl, such as pyridine, pyrazine, 4-phenylpyridine, 2,2′-bipyridyl, and 1,2-di(4-pyridyl)ethane, do not increase the electrochemical activity of cytochromes.

The mechanism of the action of the modifying agents consists in their adsorption on the electrode surface and the formation of a layer on which proteins are absorbed.[146] Positively charged lysine residues participate in the interaction of the protein with the adsorption layer, which promotes the orientation of haem towards the electrode surface.

The mechanism of the action of another modifying agent, polyethyleneimine, apparently also consists in orienting the protein globules.[134] However, the concentration dependence of the potential of the redox reaction of the protein and the dependence of the reaction current on the electrode modification time have shown that polyethyleneimine does not form a continuous coating on the electrode.

Table 6. Electrochemical parameters of proteins determined on chemically modified electrodes.

Protein	Electrode	Modifying agent	Electrochemical parameters		Refs.
			k_s, cm s^{-1}	α	
Cytochrome *c*	Au	4,4′-Bipyridyl; 1,2-di-(4-pyridyl)ethylene	$1.9 \cdot 10^{-2}$	—	[131]
ditto	Au	di(4-pyridyl) disulphide; di(4-pyridyl) sulphide	$(2.1-8.9) \cdot 10^{-3}$	—	[132]
»	Au	Purine	$1.0 \cdot 10^{-3}$	—	[133]
»	SnO_2	Polyethyleneimide	—	0.3	[134]
»	C	4,4′-Bipyridyl	$4.4 \cdot 10^{-4}$	—	[135]
Cytochrome c_3	Au	ditto	$>10^{-2}$	—	[131]
Cytochrome $c\,d_1$	C	»	$4.0 \cdot 10^{-4}$		[135]
Cytochrome *c*	Au	Methylviologen	$3.4 \cdot 10^{-5}$	0.25	[136]
Myoglobin	Au	ditto	$7.7 \cdot 10^{-5}$	0.18	[136]
ditto	Au	»	$3.9 \cdot 10^{-11}$	0.18	[137]
Ferredoxin	Au	»	$(59-6.5) \cdot 10^{-5}$	0.4—0.6	[138,139]
Peroxidase	Au	*NN*′-Di(2-aminoethyl)bipyridyl; methylviologen; benzylviologen	$(84-1.3) \cdot 10^{-5}$	0.31—0.71	[140]
Cytochrome *c*	Au, Pt	*NN*′-Bis[3](trimethoxysilyl)propyl] bipyridyl	<0.06	—	[141]
ditto	C	*N*-Vinyl-4,4′-bipyridyl	—	—	[142]
»	Pt	Polyalkylated ferrocene	$(2.4-8.7) \cdot 10^{-4}$	—	[143]
»	*n*-Si		$(1.8-3.5) \cdot 10^{-4}$	—	[144]
Ferredoxin	C	*NN*′-Di(3-aminopropyl)-bipyridyl	$(1.6-4.0) \cdot 10^{-3}$* $>10^{-2}$**	—	[145]

*For bacterial ferredoxin. **For plant ferredoxin.

Note. Numbers in square brackets denote references (Ed. of Translation).

Viologens polymerised on gold electrodes are also used as modifying agents.[136–140] The constants for the electrochemical reactions of proteins on such electrodes are, however, small and the transfer coefficient α in certain systems is close to unity (Table 6). The action of polyviologens as agents promoting the electrochemical reactions of proteins has a mechanism analogous to that of the action of 4,4′-bipyridyl, which has been confirmed by the electrochemical inertness of the modifying agents as regards the redox reactions of proteins. Furthermore, polyviologens are conjugated molecules whose end groups contain nitrogen atoms, so that they resemble bipyridyl.

Electrodes modified with *NN'*-bis[3-(trimethoxysilyl)-propyl]bipyridyl and related compounds (Table 6) contain immobilised viologens or ferrocene. The electrochemical reactions of proteins on these electrodes proceed via a mediator mechanism.[147] For cytochrome *c*, the process can be represented by the following equations:

$$MV^{2+} + e \xrightarrow{k_S} MV^{+},$$

$$MV^{+} + \text{cytochrome } c \text{ (oxidised)} \rightarrow MV^{2+} + \text{cytochrome } c \text{ (reduced)}.$$

The rate constant for the bimolecular reaction in this process exceeds 10^8 litre mol^{-1} s^{-1}. The rate of the process is limited by the reaction of the protein on the electrode surface and the current is therefore directly proportional to the square root of the rate of potential scan.[141,144]

4. Electrochemical Reactions of Covalently Immobilised Enzymes

When glucose oxidase is immobilised on graphite electrodes via cyanuric chloride, the differential pulse voltamperogram shows a peak at −0.31 V.[148] The potential of this peak is displaced by 20 mV towards the cathodic region compared with the enzyme adsorbed on the graphite electrode and by 100 mV into the anodic region compared with the redox reaction of the enzyme in solution. Immobilised L-amino-acid oxidase and xanthine oxidase react at the same potentials.[149] These results demonstrate the possibility of direct electron transfer to flavin oxidases. However, the native state of the immobilised enzymes, i.e. their ability to catalyse the reactions of substrates, remains open to doubt. The point is that the enzymes investigated readily dissociate into the apoenzyme and the co-factor. The fact that the proteins are reduced at a potential differing from the reduction potential of the adsorbed FAD does not fully demonstrate the native state of the enzyme, because it can be due to the reduction of the FAD, which is associated [with the protein? (Ed.of Translation)] in a region other than that of the active centre. Such effects are observed, for example, in the association of FAD within an inert protein.[106]

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Thus the electron transfer proteins and oxidation-reduction enzymes react similarly to non-protein redox systems. The specificity of the action of the polypeptide chain consists in regulating the potential of the active centre and its orientation in complexes where electron transfer takes place. One cannot rule out the possibility that the side groups of the aminoacids are directly involved in electron transfer over long distances, as shown for the cytochrome *c* peroxidase–cytochrome *c* complex.[150] Further progress in the study of the mechansim of electron transfer in proteins depends to a large extent on the elucidation of the contribution of the polypeptide chain to the activation energy and the adiabatic nature of the transfer. Extremely interesting results on these lines have been obtained in a number of studies.[151–154]

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