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pH-dependent redox potential: how to use it correctly in the activation energy analysis

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Abstract

The activation barrier (the activation free energy) for the reaction's elementary act proper does not depend on the presence of reactants outside the reaction complex. The barrier is determined directly by the concentration-independent configurational free energy. In the case of redox reactants with pH-dependent redox potential, only the pH-independent quantity, the configurational redox potential enters immediately into expression for activation energy. Some typical cases of such reactions have been discussed (e.g., simultaneous proton and electron detachment, acid dissociation followed by oxidation, dissociation after oxidation, and others). For these mechanisms, the algorithms for calculation of the configurational redox potential from the experimentally determined redox potentials have been described both for the data related to a dissolved reactant or to a prosthetic group of an enzyme. Some examples of pH-dependent enzymatic redox reactions, in particular for the Rieske iron–sulfur protein, have been discussed.

Keywords: Configurational free energy; Activation energy; Electron transfer; Proton transfer; Cytochrome bc_1 complex; Rieske protein

1. Introduction

Activation energy of any reaction depends necessarily on the equilibrium free energy gap between the state immediately preceding the activation barrier and the state immediately following it. For charge transfer reactions, this dependence is expressed by the well-known Marcus equation [1].

$$\Delta G^{\neq} = \frac{(\lambda + \Delta G)^2}{4\lambda} \quad (1)$$

where $\Delta G^{\neq}$ is the activation free energy, λ is the reorganization energy, and ΔG is the free energy gap described above. In Eq. (1), the energy of the reactants approach, and the lowering of the barrier due to adiabatic splitting of the energy levels are not included. An account of these factors will bring only some quantitative changes but will not affect the principal character of the $\Delta G^{\neq} - \Delta G$ dependency.

For electron transfer reactions, it is usual to find ΔG as the difference of redox potentials of the electron donor (D) and acceptor (A)

$$\Delta G = nF(E_D - E_A) \quad (2)$$

where n is the number of electrons transferred in the reaction elementary act, and F is Faraday constant. For multi-electron reactions, it is most often favorable to divide the total process into n one-electron steps, though in some cases, the multi-electron step seems to be preferable, for instance the two-electron step in the oxygen evolution reaction [2,3]. For the sake of simplicity, we will consider further only the one-electron transfer. The generalization to the multi-electron processes is straightforward.

In the case of the simplest redox reaction involving only one kind of each reduced and oxidized particles, for example,

$$D \leftrightarrow D^+ + e^-,\ A + e^- \leftrightarrow A^- \quad (I)$$

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the corresponding redox potentials are

$$E_D = E_D^0 + \frac{RT}{F}\ln\frac{[D^+]}{[D]} \quad (3)$$

$$E_A = E_A^0 + \frac{RT}{F}\ln\frac{[A]}{[A^-]} \quad (3a)$$

and the standard free energy gap is

$$\Delta G^0 = F(E_D^0 - E_A^0) \quad (4)$$

Experimentally, one can determine E^0 as corresponding middle point potentials (potentials of half transformation, $E_{1/2}$), and use these quantities for calculation of the free energy gap involved in Eq. (1).

As it will be shown below, the case of the reaction involving a change of the number of particles, for example, redox process accompanied by attachment or detachment of protons, is more complex

$$DXH \leftrightarrow DX + H^+ + e^- \quad (II)$$

The designation D is used here for the redox center, for example, metal ion, and X for its protonable ligand, but such a division is in principle not obligatory (see, e.g., quinol–semiquinone couple). Redox potential for reaction (II) is pH dependent

$$E = E_D^0 + \frac{RT}{F}\ln\frac{[DX]}{[DXH]} + \frac{RT}{F}\ln[H^+] \quad (5)$$

A similar relation (not considered here) may take also place for an acceptor.

For the proton-coupled redox reactions, the middle point potential determined experimentally depends on pH according to Eq. (5). So, the question arises, how to use these experimental data in the calculation of the free energy of an elementary act of reaction, and ultimately, in calculation of its activation energy. This problem is addressed in the present paper.

As will be discussed below, a correct calculation of the free energy gap involved in Eq. (1) demands for application of the notion of the configurational free energy as it was first done in our analysis of the oxygen evolution reaction [2]. Therefore, let us describe briefly the physical background of such an analysis.

2. Configurational free energy as the free energy of the reaction's elementary act

The free energy of any reaction ΔG can be considered as involving two constituents. One of them relates to contributions due to the intrinsic properties of reactants: their inner energy, including the energy of interaction with their surroundings, and the corresponding entropic components (vibrational and other internal modes of freedom, change of the solvent entropy under the action of the reactants' charges). This component can be called configurational free energy as it is determined by the configuration of reacting particles (ΔG_c). The second component is due to the change of entropy caused by mutual transposition of particles in solution: the place exchange of any two chemically different particles in the bulk of solution produces a new microstate increasing the system's entropy. This transpositional component (ΔG_t) is, naturally, strongly dependent on the solution concentration. In an ideal solution

$$\Delta G_t = \sum_1^m RT\ln X_f - \sum_1^n RT\ln X_i \quad (6)$$

where X_f and X_i are molar fractions of the m final and n initial reactants.

In ideal solutions, configurational free energy does not depend on the solution concentration. In real solutions, some weak dependence on concentration can exist (due, for instance, to interaction with ionic atmosphere) but this effect is negligibly small as compared to dependence described by Eq. (6) (cf., e.g., activity coefficients of monovalent ions equal to about 0.8 at physiological ionic strengths and $X_{H+} \approx 2\times10^{-9}$ at pH = 7).

The configurational free energy can be calculated, if we know, for example, standard free energy ΔG^0 by the following formula

$$\Delta G_c = \Delta G^0 + \sum_1^n RT\ln X_i^0 - \sum_1^m RT\ln X_f^0 \quad (7)$$

Here X^0 denotes the mole fractions of the components at their standard states (they can be different, for example, for the pure solvent and one-molar solute).

From Eq. (7), we can see that configurational free energy corresponds to an imaginary case when all reactants are at their molar fractions equal to unity. This agrees with the physical sense of ΔG_c as the quantity not including the transpositional entropy (at $X = 1$, there is no possibility to exchange positions of two different molecules).

In the case when all standard states are chosen similarly, and the number of initial and final molecules is the same ($n = m$), $\Delta G_c = \Delta G^0$. Such a situation we meet in the simplest redox reaction (I) considered above: the standard free energy gap in the elementary act of reaction calculated by Eq. (4) is identical to the configurational free energy.

Let us consider some reaction complex, some cage, when all reactants have been gathered together. They transform in the product molecules remaining initially in the same complex. This can be imagined most easily for an enzymatic reaction with both reactants and products situated inside the active site and separated from the bulk of solution. The energy of the chemical transformation inside the reaction

complex depends on the properties of the reactants (initial and final ones) in this complex but evidently cannot depend on their presence and concentration in the surrounding solution. This concentration-independent energy is the configurational free energy.

The free energy profile is depicted in Fig. 1. The solid curve presents an activation barrier between two states, immediately preceding (i) and immediately following (f) the reaction. The height of the barrier gives the true activation energy, that is, the activation energy of the elementary act of reaction proper, not accounting for the possible pre-equilibria; this is precisely the quantity determined by Eq. (1). The energy gap between states (i) and (f) equals to ΔG_c. The free energies of (i) and (f) differ from the energies of the reactants in solution by the free energies of mixing of the corresponding substances with the solvent entirely due to the contribution of transpositional entropy, $\Delta G_t^i = \Sigma RT \ln X_i$ and $\Delta G_t^f = \Sigma RT \ln X_f$. We can present the total process as consisting of three steps: gathering of reactant from solution into the reaction complex with the energy expenditure $-\Delta G_t^i$, the chemical reaction proper (free energy change ΔG_c), and then mixing of the products with the solvent, ΔG_t^f. The total change of the free energy is concentration-dependent according to the usual thermodynamic expression. The total free energy change presents the driving force of the whole process, from the initial dissolved reactants to the final ones, determining the direction of the system's evolution and its final equilibrium state. The configurational free energy ΔG_c gives the driving force of each separate elementary act of reaction.

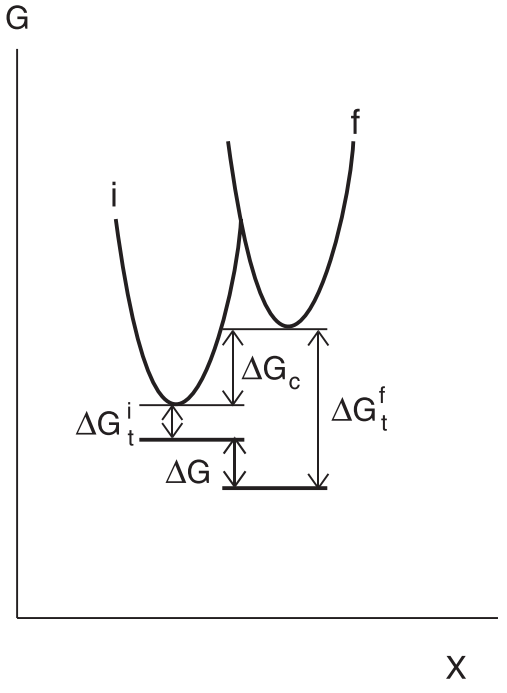

Fig. 1. Free energy diagram. Curves—configurational free energy profile for the reaction complex; bold horizontal lines—total free energy levels for the reactants in the bulk of solution. For designations, see text.

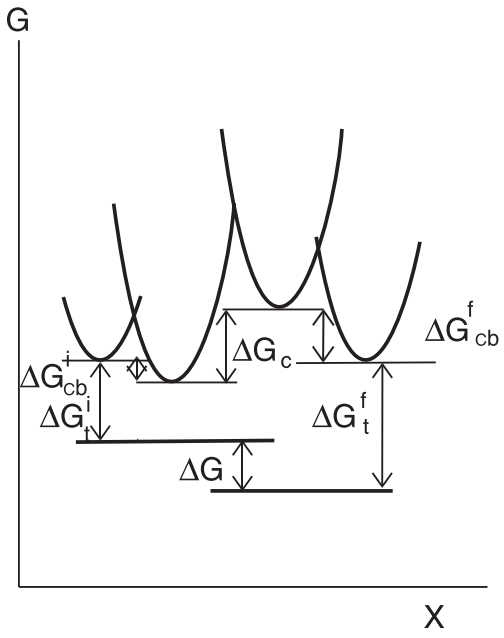

Fig. 2. Free energy diagram. Curves—configurational free energy profile for the pre-reaction and reaction complexes; bold horizontal lines—total free energy levels for the reactants in the bulk of solution. For designations, see text.

The configurational free energy ΔG_c determines the height of the barrier for the reaction proper that is independent on the energies of demixing and mixing processes taking place before and after the reaction elementary act. As was mentioned above, in the case of equal transpositional components for initial and final reactants, the standard free energy equals the configurational one. However, in the opposite case, they can differ substantially, and even the situation depicted in Fig. 1 becomes possible: the total process is exergonic due to much larger mixing entropy of the products while the elementary act of reaction is endergonic.

In the scheme of Fig. 1, the free energy differences of the substances in the reaction complex and in the bulk of solution are due only to the corresponding transpositional entropies, so the intrinsic properties of each molecule in complex and in solution are supposed to be the same. In a more general case, some energy change due to complex formation may exist. This is shown in Fig. 2 where configurational binding energies ΔG_{cb}^i and ΔG_{cb}^f are included (as it is shown in the figure, these binding energies may be both positive and negative). Such a situation is typical of enzymatic reactions.

3. pH-dependent redox potential in the calculation of the redox reaction activation energy

From the foregoing, we see that in calculation of activation energy we shall substitute in Eq. (1) the concentration-independent configurational free energy ΔG_c. When the

oxidation (e.g., of DXH) is accompanied by detachment of proton (reaction (II)), the energy of the elementary act should not be dependent on the subsequent (after the oxidation proper) mixing of this proton with the surrounding solvent.

Let us consider several possible mechanisms of reaction (II) (for sake of clarity, we will suppose that acceptor's reduction is not accompanied by a change of particles' number, as in reaction (I); if this is not the case, the generalization is self-evident).

The first mechanism is the simultaneous (concerted) electron and proton transfer

$$DXH \rightarrow DX + H^+ + e^- \qquad (IIa)$$

The electrode potential obeys Eq. (5), and

$$E_c = E^0_{1/2} + \frac{RT}{F} \ln 55.5 \qquad (5a)$$

Here $E^0_{1/2}$ is the middle point potential ([DX]/[DXH] = 1) at pH = 0, E_c is the "configurational" electrode potential that should be employed for ΔG_c calculation, and the molar fraction of H^+ at pH = 0 (1 mol/l) is 1/55.5. As a matter of fact, to calculate the configurational, that is, concentration-independent potential, we do not need the middle point potential exactly at pH = 0. The same result will be obtained if using another standard, for example, at pH = 7; in this case, the mole fraction of H^+ at this new standard state, viz. $10^{-7}/55.5$ should be employed.

For mechanism (IIa), at pH < pK_{DXH}, the observed reaction rate is pH independent: the activation energy determined by configurational redox potential do not depend on pH by definition, and the reactant's concentration, [DXH], is given (in the case when not [DXH] but only the sum of [DXH] and [DX^-] is fixed, [DXH] depends on pH at pH values close or higher than the corresponding pK).

Three versions of reaction (IIa) should be considered. First, all the reactants are dissolved. In that case, we can find from the experimentally determined pH-dependent redox potential the configurational potential (Eq. (5a)) and use it immediately for calculation of the activation free energy. The same algorithm should be employed in the analysis of other mechanisms discussed below (reactions (IIb) and (IIc)) for the case of the reactants dissolved.

The second case is when DXH presents a prosthetic group inside the enzyme but only the redox potential of this group in aqueous solution is known. The redox potential in enzymatic reaction differs from that in solution due to interaction of components with the proteinaceous surroundings: binding energies at the scheme of Fig. 2. These energies for the neutral particles DXH and DX could be expected to be rather similar; to the contrary, the effect of surroundings on the energy of charged H^+ ion could be very substantial. The latter depends on details of the mechanism of reaction (IIa). If DXH is situated in such a way that it borders the aqueous surroundings, the hydrated proton can form immediately in the elementary act of reaction, and its energy will be not very strongly different to the hydronium energy in the bulk of solution that determines the redox potential of the dissolved couple. If the hydrogen ion appears initially inside the protein, the energy of the process may differ substantially. A low dielectric constant of protein makes formation of the charged particle unfavorable (ΔG^f_{cb} strongly positive), the effect that is necessary to compensate, at least partly, by the intraprotein permanent electric field. This problem deserves a special analysis in each particular case: calculation of the intraprotein electric field and of the ion charging energy [4].

In aqueous solutions, a water molecule is the common proton acceptor (formation of H_3O^+), and this is implied in Eq. (II). If in active site this role is played by another base B, one should take into account the pK difference of H_3O^+ (pK = − 1.44 in aqueous solution) and BH^+ (this difference reflects the intrinsic difference in proton affinities of the corresponding bases). The pK difference in aqueous solution should be corrected by the corresponding electrostatic contributions for two ions in the protein. In the same site, the effects of the intraprotein field on pK's are identical (this practically does not matter, if B is the neutral base, e.g., Lys, or, say, a carboxylate anion). The charging energies (dielectric response energies) for H_3O^+ and ammonium ion of Lys are quite similar due to their practically equal radii. For hydronium and carboxylates, they should differ markedly, but what is more important is that for cationic and neutral acids, the effect of charging energy on pK has the opposite sign. Here, again, the electrostatic calculations based on the known protein structure are necessary.

The third, and what is very often the case when the redox potential is determined experimentally for the redox prosthetic group inside the protein. In this case, the binding energies of DXH and DX are accounted for automatically. However, the pH-dependent potential is determined ultimately by H^+ ions in solution, so the problem of their energy immediately in the active site (in form of H_3O^+ or BH^+) remains important. In conclusion, we can formulate the following algorithm. Having an experimentally determined pH-dependent redox potential for a prosthetic group in enzyme, we should recalculate it to a configurational potential according to Eq. (5a), and then correct it, if necessary, for the change of the acceptor's pK and the difference of electrostatic energies as discussed above.

The second mechanism is a two-step process with electrolytic dissociation preceding the rate-determining oxidation

$$DXH \leftrightarrow DX^- + H^+ \rightarrow DX + H^+ + e^- \qquad (IIb)$$

The dissociation constant of the reduced form

$$K_r = [DX^-][H^+]/[DXH] \qquad (8a)$$

The equilibrium redox potential for the second step of reaction (IIb)

$$E = E^{0b}_{1/2} + \frac{RT}{F}\ln\frac{[DX]}{[DX^-]} = E^{0b}_{1/2} + \frac{RT}{F}\ln\frac{[DX][H^+]}{[DXH]K_r} \quad (8b)$$

It coincides, of course, with the redox potential of the total reaction (Eq. (5)), and hence

$$E^{0b}_{1/2} = E^0_{1/2} + \frac{RT}{F}\ln K_r \quad (8c)$$

Concentration-independent potential $E^{0b}_{1/2}$ is nothing else as the configurational electrode potential for the second step of reaction (IIb). It coincides with the middle point potential determined experimentally at $pH \gg pK_r$. The activation energy of this reaction is pH independent while the reaction rate, at given [DXH] and $pH < pK_r$, is inversely proportional to H^+ concentration because the dependence of the concentration of the redox-active species (Eq. (8a)). In practice, usually only the sum of [DXH] and [DX^-] is kept constant, and [DXH] is approximately constant when the dissociation degree of DXH is low ($pH \ll pK_r$). In the opposite limiting case ($pH \gg pK_r$), DXH is fully dissociated, and the measured redox potential becomes pH independent.

Let us consider the case when we know the configurational redox potential for dissolved reactants, and we need to find it for the enzyme's prosthetic group. The only charged particle involved in the rate-determining oxidation step is DX^-. The electrostatic contributions to its energy can be analyzed in the common way, giving the correction to the experimentally determined redox potential of DXH in aqueous solution. Hydrogen ion does not participate in this step of the reaction, so difficulties with estimates of its energy discussed above for the first mechanism (electrostatic contributions, properties of the primary proton acceptor) do not impede the theoretical analysis of this mechanism.

The next case of mechanism (IIb) is when we have an experimentally determined redox potential of enzyme in an aqueous solution, and its pH dependence (giving the pK_r value). The binding energies of the oxidized and two reduced forms are included automatically, and the proton acceptor is water, that is, it lies outside the protein. Therefore, the configurational redox potential for the prosthetic group in enzyme can be evaluated from the experimental data strictly quantitatively using Eq. (8c).

The third possible mechanism is the two-step process with the rate-determining oxidation preceding the electrolytic dissociation

$$DXH \rightarrow DXH^+ + e^- \leftrightarrow DX + H^+ + e^- \quad (IIc)$$

Redox potential for the first step

$$E = E^{0c}_{1/2} + \frac{RT}{F}\ln\frac{[DXH^+]}{[DXH]} = E^{0c}_{1/2} + \frac{RT}{F}\ln\frac{[DX][H^+]}{[DXH]K_o} \quad (9a)$$

where K_o is the dissociation constant of the oxidized form

$$K_o = \frac{[DX][H^+]}{[DXH^+]} \quad (9b)$$

Equalizing Eqs. (5) and (9a), we find the configurational redox potential for the first step of reaction (IIc)

$$E^{0c}_{1/2} = E^0_{1/2} + \frac{RT}{F}\ln K_o \quad (9c)$$

This potential coincides with the middle point potential determined experimentally at $pH \ll pK_o$.

For the third mechanism, both activation energy and preexponential factor (concentration of the reacting species) are pH independent (the latter at $pH \ll pK_r$), hence, so is the reaction rate. The quantitative evaluation of the configurational redox potential from the experimental data is, in general, similar to that described for the second mechanism: correction for electrostatic energy of DXH^+ inside the protein if we know the redox potential of the dissolved reactants, and a direct usage of Eq. (9c) when redox potential and its pH dependence were determined for the enzyme.

We have considered different versions of reduction by DXH. A quite similar analysis can be applied to the case of different mechanisms, say, of oxidation by DXH^+:

$$DXH^+ + e^- \rightarrow DX^- + H^+ \quad (IIIa)$$

$$DXH^+ + e^- \leftrightarrow DX + H^+ + e^- \rightarrow DX^- + H^+ \quad (IIIb)$$

$$DXH^+ + e^- \rightarrow DXH \leftrightarrow DX^- + H^+ \quad (IIIc)$$

Many other mechanisms can be imagined, and the general approach to their analysis should be similar to that described above. As an example, let us consider oxidation accompanied by absorption of protons in the reduced form

$$DXH^+ \leftrightarrow DX + H^+ + e^- \rightarrow DXH$$

For the redox potential, we can write

$$E = E_m + \frac{RT}{F}\ln\frac{[DXH^+]}{[DXH]} = E_m - \frac{RT}{F}\ln K_o + \frac{RT}{F}\ln\frac{[DX]}{[DXH]} + \frac{RT}{F}\ln[H^+] \quad (9d)$$

Here E_m is the middle point redox potential of the DXH^+/DXH couple (determined at $pH \ll pK_o$) and $E_m - (RT/F)\ln K_o + (RT/F)\ln 55.5$ is the configurational potential for the $(DX + H^+)$/DXH couple (cf. Eqs. (5) and (5a)).

It is expedient to stress here again the difference between the total driving force of the whole process, from the very initial to the very final state, and the driving force of the elementary act of reaction that determines, according to Eq. (1), its true activation energy. Most mechanisms of reaction (II) (and reactions (IIIa–c)) result, ultimately, in liberation of one proton, and hence, the free energy of the whole process, which can be measured experimentally, is pH dependent. However, the true activation energy, related to the elementary act of reaction proper and not accounting for the processes preceding and following the electron transfer, is not affected by hydrogen ions' concentration.

As described above, the configurational potentials are determined from the experimental data on the pH-dependent redox potentials measured under equilibrium conditions. The quantities obtained in such a way are the pH-independent constants characteristic of the elementary act of the electron transfer. Therefore, they do not change if, in kinetic experiments, the equilibrium of proton exchange between enzyme and surrounding solution has not been established. Correspondingly, the true activation energy of electron transfer does not depend on the rate of proton equilibration. However, the rate of the redox reaction may depend on the proton exchange kinetics. That will be the case for mechanism (IIb) where the concentration of the redox reactant DX^- depends on the degree of the preceding electrolytic dissociation, and hence, if the dissociation equilibrium is not achieved, depends on the rate of proton transfer at this step. For mechanisms (IIa) and (IIc), proton liberates into solution after the electron transfer step, and hence, the rate of the latter does not depend on the proton exchange kinetics (except in the case of a so slow proton removal that the reversal of the redox reaction becomes possible).

4. Application of the configurational redox potential to an analysis of enzymatic reactions: the redox reactions of the Rieske protein

Let us now consider some examples of enzymatic redox reactions with the pH-dependent redox potential. An interesting case presents the Rieske protein whose redox potential varies due to, most probably, protonation–deprotonation of a His liganding the Fe_2S_2 cluster [5,6]. Corresponding $pK_o = 7.6$ and $pK_r \sim 10$. The Rieske protein is a subunit of cytochrome bc_1 (or, in chloroplasts, b_6f) complex. It transfers electron from quinol to cyt c_1 (cyt f). In this process, it shuttles from the position in vicinity of cyt b (b state), where it contacts quinol bound in the Q_o site, to the direct contact with the heme of cyt c_1 (c_1 state) (see, e.g., Refs. [7,8]). The mechanisms of these reactions are rather complex, and there are controversial opinions on them. The goal of this section is not to discuss details of these mechanisms but to illustrate the possibility of using the configurational electrode potential in consideration of such processes, and to point to some questions arising as a result of that consideration.

The kinetics of reaction of quinol oxidation were found to be inversely proportional to H^+ concentration in pH region of 5.5–7.0, that is, in the region where the dissociation degree of Fe_2S_2 $HisH^+$ is low and increases sharply with increasing pH [9–11]. Such a behavior was ascribed to the involvement in the reaction of only deprotonated form (necessary to form a hydrogen bond between Nε of His and OH of quinol) [9–11] (see also Ref. [12]). At the same time, the activation energy of reaction reveals to be independent of pH [9–11]. This fully corroborates with our analysis: the total driving force of the reaction is pH dependent while the driving force of the elementary act of electron transfer is determined by the pH-independent conformational redox potential of the deprotonated form of the oxidized redox center.[1] The whole picture corresponds to mechanism (IIIb). Unfortunately, the configurational potential for the DX/DX^- couple is experimentally inaccessible: for this purpose, one needs the redox potential measurements at $pH \gg 10$ [5]; in the case of an concerted electron–proton transfer (see below), the configurational potential of the $(DX + H^+)$/DXH couple could be employed.

The consideration above was based on the assumption that the rate-determining step presents a pure electron transfer. However, Crofts et al. [14] (see also Refs. [9–11]) have supposed that the process is a coupled (concerted) electron–proton transfer, with the deprotonated His playing the role of an acceptor for the proton detached from quinol. This mechanism is very probable because the acidity of $HisH^+$ is much less than that of the oxidized quinol QH_2^+. In that case, the driving force for the elementary act of this coupled reaction should depend also on the difference of pK's of the proton donor and acceptor (or, the same, on pK of the proton donor and the configurational potential of the $(DX + H^+)$/DXH couple; the latter includes pK of the proton acceptor).

Comparing kinetics for the wild type and four mutants at equal degree of His deprotonation, Guergova-Kuras et al. [10] have found the slope for the $\ln(k)$–E_m plot equal to 0.011 mV^{-1}. However, this slope is not characteristic for comparison with the calculations according to Marcus equation (Eq. (1)). For the concerted electron–proton transfer, this equation involves the free energy gap ΔG that depends not only on difference of redox potentials E_m but

[1] The pH-dependent activation energy was observed in Ref. [13]. However, as it was stressed in Ref. [11], the measurements of Ref. [13] were performed for a steady-state process rather than for a partial reaction, and hence, they reflect a complex interplay of many catalytic steps that can be pH dependent. It should be noted here that for the deprotonation step (which was supposed in Ref. [13] as the rate-determining step), only the total driving force is pH dependent while the activation energy should be determined by the configurational free energy gap that, as it was explained above, is pH independent.

also on the difference of pK's. For three mutants as well as for WT, pK's are practically identical but for the most diverging Y156W mutant, pK is by 0.9 units higher. Therefore, in this case, the coupled electron–proton transfer is more favorable as one could judge from the difference of redox potentials: a stronger base favors proton transfer, and the total ΔG is by 53 meV more negative. Replotting $\ln(k)$ as a function of $E_m + pK(2.3\ RT/F)$, that is, practically, as a function of the configurational potential of the $(DX + H^+)/DXH$ couple (see Eq. (9d) and comments to it), we obtain the slope of about 0.02 mV^{-1}. It should be noted that practically the same slope was found when plotting $\ln(k)$ at constant pH against E_m [10]. However, this result is due to compensation of two opposite errors: comparison of the rates at different concentrations of the reacting, that is, deprotonated form of the iron–histidine center, and the usage of E_m (i.e., configurational potential for DXH^+/DXH couple) instead of E_c of the real redox couple involved in the process. The same compensation (and similar slope) took place for a series of mutants from yeast [15]. Such a compensation is possible only at pH values sufficiently lower than pK's of all mutants providing, at constant pH, $\Delta \lg C \approx -\Delta pK$ (C is the concentration of the deprotonated form).

For the partial reaction of quinol oxidation, a high activation energy, approximately 65 kJ/mol, has been found [11]. To explain this large value, Crofts et al. [14] have accepted an unusually large value of reorganization energy, $\lambda \sim 2.0–2.5$ eV. However, this seems to be hardly probable. We can estimate λ using Marcus formula for reorganization energy

$$\lambda = e^2\left(\frac{1}{\varepsilon_o} - \frac{1}{\varepsilon_s}\right)\left(\frac{1}{2a_1} + \frac{1}{2a_2} - \frac{1}{R}\right) \tag{10}$$

Here e is the charge to be transferred, ε_o and ε_s the optical and static dielectric constants, a_1 and a_2 the radii of reacting ions, and R the distance between their centers. Accepting radii of proton donor and acceptor equal to radii of O and N atoms (1.6 Å), R equal to their sum, $\varepsilon_o = 2.5$, typical of proteinaceous medium [16], and ε_s lying between 4 (experimental value averaged over the whole protein globule) and 10 (the upper limit for the active site [16]), we obtain, for the proton transfer, $\lambda = 0.7–1.3$ eV. This is definitely an overestimate because the charge associated with the proton is not localized on one atom only but is delocalized markedly over aromatic moieties connected with those atoms (imidazole, phenyl rings) making the effective radii substantially larger. For electron transfer, due to much larger radii of reactants (the whole phenolic group and the iron–sulfur center), λ should be substantially lower. It is necessary to stress here that for the coupled electron–proton transfer, the reorganization energy does not equal the sum of these two quantities: in the case of charge transfers in opposite directions (or, the same, transfer of two opposite charges in the same direction), it should be substantially lower, even lower than the largest λ for one of the processes [17]. In our case, electron and proton are transferred in the same direction, practically along the same straight line. The shifts of positive and a negative charge demand for opposite changes of the medium polarization, these changes largely compensating each other. This should result in a substantial decrease in reorganization energy. This conclusion is easy to understand if we imagine a limiting case: the centers of gravity of the charges of proton and electron coinciding in both the initial and final states. Under such conditions, there is no charge density redistribution, and hence, the medium reorganization energy is zero.[2]

At rather low reorganization energy, the question arises of how to explain the high activation energy. The simplest idea is to suppose a very endergonic reaction, with ΔG close to $\Delta G^{\neq}$. The estimates done in Ref. [14] show that it is not very probable. A more definite conclusion can be drawn on the basis of the $\ln(k)–E_c$ slope. This slope, expressed in mV^{-1}, is nothing else as $\alpha F/RT$, where α is the so-called Brønsted transfer coefficient $\alpha = \partial\Delta G^{\neq}/\partial\Delta G$. Differentiating Eq. (1), we obtain

$$\alpha = \frac{1}{2} + \frac{\Delta G}{2\lambda} \tag{11}$$

The slope 0.02 mV^{-1} corresponds to $\alpha = 0.5$. Therefore, $\Delta G \ll 2\lambda$, and hence, $\Delta G \ll \Delta G^{\neq}$ (due to some scatter of points and a probable error in determination of the slope, one cannot assert that ΔG equals strictly to zero, but it seems probable that it does not exceed, say, ~ 10 kJ/mol). So the oxidation process is not too endergonic.

By the way, the low slope of the $\ln(k)–E_m$ line can be interpreted as an argument against the pure electronic transfer (without a coupled movement of proton). When we suppose that the changes in the hydrogen bonding in different mutants similarly affect the acidities of both oxidized and reduced forms (i.e., $pK_o - pK_r \approx$ constant), then E_m parallels the configurational potential of the DX/DX^- couple, and the slope may be characteristic of ΔG of reaction. At slope 0.011 mV^{-1}, $\alpha = 0.28$, and hence, the reaction is strongly exergonic, ΔG of order of −40 kJ/mol. This is improbable because, in this case, the intermediate semiquinone should be very stable, and would be detected easily.

A possible explanation of a high activation energy could be proposed if we remember that Eq. (1) refers to the true activation energy, that is, the true barrier in the reaction complex, but not to the apparent, that is, experimentally determined activation energy. The latter includes the com-

[2] This limiting case can be considered as an idealized case of the H atom transfer (in real H transfer, dipoles of X–H bonds change their position and directions, so some medium reorganization should take place). The reaction of quinol oxidation should be better termed "the coupled electron–proton transfer" rather than "the H atom transfer" to stress the fact that the centers of gravity of electron and proton are separated by a rather large distance.

ponents due to the work necessary for an approach of reactants from their equilibrium distance to the distance optimal for the charge transfer

$$\Delta G_{\mathrm{app}}^{\neq} = W_{\mathrm{i}} + \frac{(\lambda + \Delta G_{\mathrm{eq}} + W_{\mathrm{f}} - W_{\mathrm{i}})^2}{4\lambda} \qquad (12)$$

Here, W_{i} and W_{f} are the works of approach of reactants from their initial and final states corresponding to the optimal distance, and ΔG_{eq} denotes the free energy gap at equilibrium distance (this is the quantity that is determined from the difference of the equilibrium redox potentials).

In electron transfer reactions, where the probability of electron transfer is usually already high enough at the equilibrium van der Waals' contacts, the work terms are often neglected (except the case of two similarly charged ions where their electrostatic repulsion plays a substantial role). The situation is different for proton transfer (for the review on the proton transfer mechanism, see, e.g., Ref. [18]). The probability of the proton underbarrier transition (tunneling) depends sharply on the inter-reactant distance. Therefore, it is usually favorable to approach the reactants to some nonequilibrium distance against the repulsion forces to provide better conditions for proton tunneling: increase in activation energy (an additional work W) is overcompensated by higher tunneling probability. Hence, the work term W_{i} can be large, increasing the activation energy substantially. At the same time, one could expect the term W_{f} to be of the same order of magnitude, and so the difference of these two works will not bring a large contribution into activation energy (as a matter of fact, in the last term of Eq. (11), ΔG means $\Delta G_{\mathrm{eq}} - W_{\mathrm{i}} + W_{\mathrm{f}}$). A large repulsion energy would be not very probable when determined only by the potential barrier along the hydrogen bond O—H. . .N. However, a substantial contribution to W_{i} can be brought by repulsion of surrounding side chains.

The hypothesis presented can be tested by studying the kinetic H/D isotope effect: it should be rather large (usually at least ~ 2–3), and activation energy for D should be higher than for H (lower tunneling probability for D demands for a shorter tunneling distance, and hence, for a larger W_{i}; for details, see Ref. [18]). A low kinetic isotope effect was observed in Ref. [13] but those data refer not to the partial reaction of quinol oxidation but to a rather complex steady-state process [11]. A detailed experimental study of kinetic isotope effect seems to be very desirable.

A large repulsion work may have another consequence. Immediately after completing the concerted electron and proton tunneling, the system is in a nonequilibrium configuration, with a shortened N—O distance. It possesses an excess repulsion energy W_{f}, and this energy could be used to give the Rieske protein an impetus to move away, in a direction to its c_1 position (and/or to push the semiquinone toward cyt b_{L}). It would be interesting to explore these possibilities by molecular dynamics simulations.

One more point is to be mentioned here. The proton tunneling probability is rather sensitive to details of the structure of reacting complex, especially to the tunneling distance in the optimal configuration. Therefore, this probability, and hence, the pre-exponential factor can be somewhat different for different mutants causing some scatter in the $\ln(k)$–E_{m} plot.

The next step in the whole process is the very fast reduction of cyt c_1 by the Rieske protein in the c_1 state. To measure the rate of this reaction, Millet et al. [19] started with the fully reduced state (both for the Rieske and cyt c_1), then oxidized cyt c_1 in 1 μs by the flash-excited Ru complex, and then observed the kinetics of cyt c_1 re-reduction by the Rieske protein. The kinetics were shown to be independent of the presence of myxothiazole; this suggests that in the reaction studied, the complex was initially not in the b state but in the c_1 state or an intermediate one. The electron transfer kinetics is practically pH independent in the pH range 7–10 where the redox potential of the Rieske center decreases significantly. When this pH-dependent potential is substituted in Marcus equation (Eq. (1)), a strong pH dependence of kinetics is predicted that contradicts the experimental data. On the other hand, if a single value of redox potential corresponding to low pH is used (essentially, the configurational potential of the DXH^{+}/DXH couple), then the calculated kinetics are pH independent (see, e.g., mechanism (IIc)).

In spite of the good agreement with the experiment described above, Millet et al. [19] prefer the mechanism of "conformationally gated" process when the rate-determining step is not the electron transfer proper but the preceding change of the bc_1 complex conformation from some intermediate one to the true c_1 state. This conclusion is based on the fact that the observed reaction rate is nearly the same for the wild type and three mutants studied, while their redox potentials (and hence, the calculated electron transfer rates) were different. The authors used redox potentials determined at pH 8 while the configurational potential determined at low pH should be employed (see above). Such a potential was determined in Ref. [10] for Y156W mutant, and it was found to be 114 mV more negative than for the wild type, so the shift is stronger than at pH 8 (-62 mV). A similar situation can be expected with the other mutants: the values -90, -130, and -180 mV were found at pH 7 (most probably, at this pH, the potentials are practically pH independent) for Y185W, S183A, and Y185F/S183A mutants of yeast protein [15] analogous to Y156W, S154A, and Y156F/S154A mutants of *Rhodobacter sphaeroides* studied in Ref. [19] (for the latter two, shift at pH 8 equals to -109 and -156 mV). These larger potential shifts (especially for Y156W) increase the discrepancy between the calculated and observed reaction rates giving more strength to the conclusions of the paper [19].

Another mechanism, namely, the coupled electron–proton transfer in the Rieske–cyt c_1 reaction, was supposed in Ref. [6]. The acidity of the cytochromes' propionate is higher

than for $HisH^+$, and hence, such a mechanism would demand a substantial change of p*K*'s upon the Rieske protein docking to cyt c_1. Such a possibility could be considered by a detailed study of the protein complex's electrostatics. In any case, the involvement of proton transfer in this reaction can be tested by measurements of the H/D kinetic isotope effect.

We have considered here different possible rate-determining electron-transfer steps accompanied by proton transfer. The method of calculation of their configurational free energy depends on whether the reactants are dissolved or constitute a part of an enzyme, and on the conditions of the experimental determination of redox potentials. The algorithms proposed can be extended to other, more complex cases, for example, both electron donor and acceptor involved in protonation–deprotonation reactions, the same or different proton acceptors for two redox partners, a multistep redox process, etc. An example of analysis of such a multistep process as the oxygen evolution reaction (water oxidation) was given in our previous work [2,3]. It was shown that the configurational potential for this four-electron reaction is 0.17 V higher than its standard potential, and hence, substantially higher than the redox potential of P680, the electron acceptor in the photosynthetic reaction center. The energy deficiency can be decreased substantially if proton(s) detached from water bind immediately to a sufficiently strong base (see the discussion of mechanism (IIa)). Different combinations of one- and two-electron steps have been considered (for some of them, correction to the standard potential exceeded 0.3 V). It was shown that, from the energetic point of view, the most favorable pathway is the two-electron oxidation of water to peroxide with two subsequent one-electron steps.

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