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Redox Thermodynamics of the Fe^{3+}/Fe^{2+} Couple in Horseradish Peroxidase and Its Cyanide Complex

Gianantonio Battistuzzi, Marco Borsari, Antonio Ranieri, and Marco Sola*,[1]

Department of Chemistry, University of Modena and Reggio Emilia, via Campi 183, 41100 Modena, Italy

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The polypeptide matrix is known to remarkably affect the electronic structure and the reduction potential ($E^{\circ\prime}$) of metal redox centers in proteins.[2] A valuable insight into the mechanism of $E^{\circ\prime}$ modulation in electron-transfer (ET) metalloproteins has been gained from the factorization of the corresponding enthalpic ($\Delta H^{\circ\prime}_{rc}$) and entropic ($\Delta S^{\circ\prime}_{rc}$) components, determined from the analysis of the temperature dependence of the reduction potential.[3] It has been shown that $\Delta H^{\circ\prime}_{rc}$ is determined primarily by metal−ligand binding interactions and the electrostatics at the interface between the metal and the protein environment and the solvent, whereas $\Delta S^{\circ\prime}_{rc}$ is mainly linked to oxidation state-dependent changes in conformational degrees of freedom of the polypeptide chain and solvent reorganization effects.[3]

Here, we have extended this approach to redox metalloenzymes. In particular, we have measured the thermodynamics of Fe^{3+} reduction for horseradish peroxidase (HRP-C), an enzyme containing a five-coordinate high-spin heme which catalyzes the oxidation of a wide variety of substrates by H_2O_2 or other organic peroxides and is the best known example of secretory plant heme-peroxidases.[4] We have also measured the reduction enthalpy and entropy for the six-coordinate low-spin cyanide adduct. The $E^{\circ\prime}$ values of these species at various temperatures have been obtained with a UV−vis spectroelectrochemical approach.[5,6]

At 25 °C and pH 7 the reduction potential of native HRP-C is found to be somewhat lower than previous literature values (−0.306 V vs −0.266 V).[7] This is probably due to differences in the ionic composition of the solutions employed. In fact, the $E^{\circ\prime}$ of HPR-C is likely to be sensitive to salt effects, analogously to what has been found previously for ET metalloproteins.[3a,8] In the same conditions, the reduction potential for the cyanide adduct is −0.430 V.

The $\Delta H^{\circ\prime}_{rc}$ and $\Delta S^{\circ\prime}_{rc}$ values for HRP-C and its cyanide complex, determined from the variable temperature experiments (Figure 1), are reported in Table 1, along with the corresponding enthalpic and entropic contributions to $E^{\circ\prime}$ ($-\Delta H^{\circ\prime}_{rc}/F$ and $T\Delta S^{\circ\prime}_{rc}/F$, respectively).

In both cases, reduction enthalpies and entropies are positive (Table 1). The negative reduction potentials turn out to be the result of two opposing contributions: a large enthalpic term, which disfavors Fe^{3+} reduction and is the determinant of the negative $E^{\circ\prime}$ values for both species, and a smaller, yet relevant, entropic contribution, which instead favors Fe^{3+} reduction. The enthalpy and entropy changes thus partially compensate. An analogous behavior was found previously for ET metalloproteins with positive reduction potentials (cytochromes *c*, blue-copper proteins, and HiPIPs) for which both terms were found to be negative.[3] However, this compensation is not a general thermodynamic feature of electron transport metalloproteins. In fact, both the enthalpic and entropic term concur to determine the negative $E^{\circ\prime}$ values for several ferredoxins.[3e] Instead, what does appear to be a common property

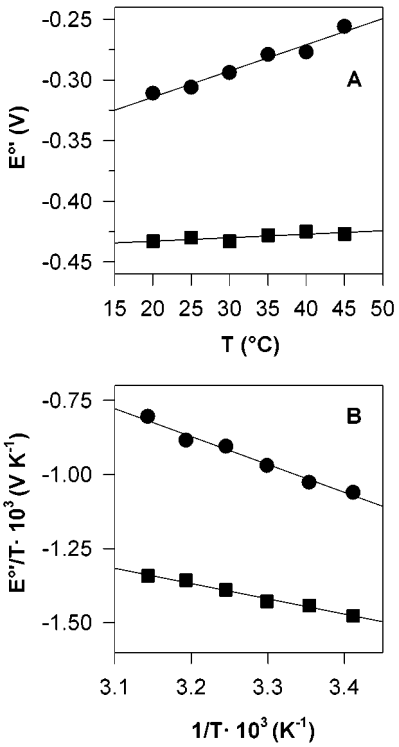

Figure 1. Temperature dependence of the reduction potential (A) and $E^{\circ\prime}/T$ vs $1/T$ plots (B) for native HRP-C (●) and its cyanide adduct (■). The slope of the plots yields the $\Delta S^{\circ\prime}_{rc}/F$ and $-\Delta H^{\circ\prime}_{rc}/F$ values, respectively. Solid lines are least-squares fit to the data points. Protein concentration, 2×10^{-5} M. Cyanide concentration, 1×10^{-3} M. Error bars have the same dimensions of the symbols.

Table 1. Thermodynamic Parameters for $Fe^{3+} \rightarrow Fe^{2+}$ Reduction in Native HRP-C and in Its Cyanide Adduct[a]

protein	$E^{\circ\prime}$ (V)[b]	$\Delta H^{\circ\prime}_{rc}$ (kJ mol^{-1})	$\Delta S^{\circ\prime}_{rc}$ (J K^{-1} mol^{-1})	$-\Delta H^{\circ\prime}_{rc}/F$ (V)	$T\Delta S^{\circ\prime}_{rc}/F$ (V)[b]
HRP	−0.306	+91	+210	−0.943	+0.648
HRP-CN	−0.430	+50	+30	−0.517	+0.093

[a] Average errors on $E^{\circ\prime}$, $\Delta H^{\circ\prime}_{rc}$, and $\Delta S^{\circ\prime}_{rc}$ values, are ±0.005 V, ±5 kJ mol^{-1}, and ±10 J K^{-1} mol^{-1}, respectively. [b] At 25 °C.

of the vast majority of ET metalloproteins, irrespective of the nature of the metal center, is the entropy loss on reduction. On the contrary, we have found here that reduction of the ferriheme in HRP-C results in a remarkable increase in entropy. Further investigations would tell if this is a distinctive property of the metallo-oxidoreductases and, if this is the case, how these opposite entropy changes on metal reduction are related to the different functional roles, ET or chemical catalysis, of metal redox centers in proteins.

However, the importance of entropic effects in determining the $E^{\circ\prime}$ values of redox metal centers is unequivocally demonstrated

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by the effect of cyanide binding to HRP. Indeed, the decrease in $E^{\circ\prime}$ of the Fe^{3+}/Fe^{2+} couple on cyanide binding turns out to be a fully entropic effect (see Table 1 and discussion below): $\Delta E^{\circ\prime} = -\Delta\Delta H^{\circ\prime}_{rc}/F + T\Delta\Delta S^{\circ\prime}_{rc}/F = +0.426 + (-0.555) = -0.129$ V (the small difference with the experimental $\Delta E^{\circ\prime}$ value of -0.124 V is due to the fact that the $\Delta H^{\circ\prime}_{rc}$ and $\Delta S^{\circ\prime}_{rc}$ values are rounded to the closest integer, because of the experimental error).

The Enthalpic Term. The oxidized state is enthalpically stabilized over the reduced state in both free and cyanide-bound HRP-C. In the free enzyme, such a stabilization is particularly large, likely due to coordinative and noncoordinative contributions. In particular, the significant anionic character conferred to the proximal histidine (His170) by the strong hydrogen bond between its imidazolic $N_\delta H$ and the side chain of the nearby Asp235,[9] greatly increases its basicity, thereby selectively stabilizing the higher oxidation states of the heme iron.[4,10] Consistently, heme-peroxidases experiencing a decrease in the basicity of the proximal histidine as a consequence of the weakening of the His170-Asp235 hydrogen bond show greater $E^{\circ\prime}$ values.[10] Moreover, the polarity of the heme pocket, which is characterized by the presence of two water molecules close to the heme in the distal site and by an extended network of hydrogen bonds, involving the solvent molecules and the catalytically relevant Arg38 and His42,[9] electrostatically stabilizes the Fe(III) form of the enzyme, in which the heme is positively charged (+1).

Upon cyanide binding, the hydrogen bonding network in the distal site is known to undergo a deep reorganization[11] due to (i) the displacement of the water molecules close to the heme, (ii) the protonation of the N_ϵ of the imidazole ring of the distal histine, which now forms an hydrogen bond with the nitrogen atom of the bound cyanide and (iii) the breaking of the hydrogen bond involving the $N_\delta H$ of the proximal histidine, which thereby assumes a fully anionic character. The first effect should favor the reduced state of the iron as compared to the free enzyme, inducing a decrease in reduction enthalpy, whereas the breaking of the hydrogen bond involving the $N_\delta H$ of the proximal histidine should further increase the enthalpic stabilization of the Fe(III) as compared to free HRP-C. The significant decrease in the reduction enthalpy measured for the cyanide derivative as compared to the free enzyme implies that the effects of water exclusion from the immediate vicinity of the heme and π-back-bonding from Fe^{2+} to cyanide, assisted by the decrease of the negative charge of cyanide owing to the hydrogen bonds formed with the distal histidine and arginine, prevail on other coordinative and noncoordinative effects, thus favoring the ferrous heme. Hence, as noted above, the decrease in $E^{\circ\prime}$ of HRP-C on cyanide binding is totally entropic in origin.

The Entropic Term. Reduction of native HRP-C is accompanied by a significant increase in entropy. The absence of structural information on the Fe(II) form of HRP-C does not allow interpretation of the entropic data. However, reduction-induced solvent reorganization effects in the catalytic site are likely to be important effectors of $\Delta S^{\circ\prime}_{rc}$ in HRP-C due to the solvent accessibility and the complicated array of hydrogen bonds present in the heme pocket.[9,11] In particular, the decreased electrostatic interaction of the metal ion in its reduced form with the water molecules in the cavity should lead to a decrease in ordering and may be responsible for the observed positive $\Delta S^{\circ\prime}_{rc}$. This hypothesis is supported by the remarkable lowering of the reduction entropy upon cyanide binding, which indeed diminishes the accessibility of the heme group to the solvent. Further contributions to the positive $\Delta S^{\circ\prime}_{rc}$ values arising from a greater flexibility of the polypeptide chain in the reduced as compared to the oxidized form in both free and cyanide-bound HRP-C cannot be excluded.

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(1) Fax: +39-059373543. Telephone: +39-0592055037. E-mail: sola.marco@unimo.it. URL: http://www.chimica.unimo.it/websola/bioichemgroup.html.

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(6) All experiments were carried out in an homemade OTTLE cell. The three-electrode configuration consisted of a gold minigrid working electrode (Buckbee-Mears, Chicago, IL), an homemade $Ag/AgCl/KCl_{sat}$ micro-reference electrode, separated from the working solution by a Vycor set, and a platinum wire as counter electrode. The reference electrode was calibrated against a saturated calomel electrode before each set of measurements. All potentials are referenced to the NHE. Potentials were applied across the OTTLE cell with an Amel model 533 potentiostat/galvanostat. Constant temperature was maintained by a circulating water bath, and the OTTLE cell temperature was monitored with a Cu-costan microthermocouple. UV−vis spectra were recorded using a diode-array Hewlett-Packard HP 8452-A spectrophotometer. The variable temperature experiments were performed using a "nonisothermal" cell configuration. The temperature of the reference electrode was kept constant, while that of the working electrode was varied. For this experimental configuration $\Delta S^{\circ\prime}_{rc}$ is calculated from the slope of the $E^{\circ\prime}$ vs temperature plot, whereas $\Delta H^{\circ\prime}_{rc}$ is obtained form the Gibbs−Helmoltz equation, namely from the slope of the $E^{\circ\prime}/T$ vs $1/T$ plot (see Yee, E. L.; Cave, R. J.; Guyer, K. L.; Tyma, P. D.; Weaver, M. J. *J. Am. Chem. Soc.* **1979**, *101*, 1131−1137). HRP was purchased from Sigma and used without further purification, since the absorption spectrum gave an RZ value (A_{403}/A_{280} ratio) above 3.1 (see Chattopadhyay, K.; Mazumdar, S. *New J. Chem.* **1998**, 137−139). All chemicals were reagent grade. All experiments were carried out under argon over the 20−45 °C range, using 1 mL samples containing 2×10^{-5} M HRP made up in 25 mM phosphate buffer plus 10 mM NaCl at pH 7, in the presence of 0.2 mM methyl viologen and 2×10^{-6} M lumiflavine-3-acetate, indigo disulfonate, phenazine methosulfate and methylene blue used as mediators. Nernst plots consisted of at least six points and were invariably linear, with a slope consistent with a one electron reduction process. The same mediators were used previously for lignin and Mn-peroxidases from *Phanerochaete crisosporium* (see Millis, C. V.; Cai, D.; Stankovic, M. T.; Tien, M. *Biochemistry* **1989**, *28*, 8484−8489). Application of conventional diffusion-controlled voltammetric techniques to HRP is hampered by its relatively high molecular mass (MW = 42 kDa) (see Bond, A. *Inorg. Chim. Acta* **1994**, *226*, 293−340; Chattopadhyay, K.; Mazumdar, S. *New J. Chem.* **1998**, 137−139; Santucci, R.; Bongiovanni, C.; Marini, S.; Del Conte, R.; Tien, M.; Banci, L.; Coletta, M. *Biochem J.* **2000**, *349*, 85−90).

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