Complete Thermodynamic Characterization of Reduction and Protonation of the bc_1-type Rieske [2Fe-2S] Center of *Thermus thermophilus*

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Rieske iron−sulfur (2Fe-2S) clusters play a central role in energy transduction by the quinone:cytochrome *c* oxidoreductases of the respiratory and photosynthetic chains (the bc_1 and b_6f complexes) and in the bacterial degradation of aromatic compounds.[1] Distinguished from "ferredoxin-type" 2Fe-2S clusters by reduction potentials up to 700 mV higher, Rieske centers have one iron atom coordinated by two nitrogenous (histidine, His) ligands rather than two thiolate (cysteine, Cys) ligands.[2,3] While the high, pH-dependent potentials of bc_1-type clusters are crucial for redox-coupled proton transfer, and for "bifurcation" of electron transfer within the bc_1-complex,[4] bacterial dioxygenase-type Rieske clusters, with the same 2Fe-2S Rieske core, display lower and pH-independent potentials.[5−8] Here we present the first complete thermodynamic description of a Rieske cluster, the soluble Rieske-fragment of the bc_1-complex from *Thermus thermophilus (Tt)*.[9] Using protein-film cyclic voltammetry,[10] which allows rapid transfer of a sample of protein molecules between different solutions, we have measured reduction potentials over a wide range of pH (up to pH 14). We have thus determined p*K* values for the His ligands in both redox states, and the reduction potential of each protonation state. Interpretation of these data now reveals how the His ligands dictate the unique physical properties of Rieske clusters.

Figure 1 shows how the reduction potential ($[2Fe\text{-}2S]^{2+/1+}$) of the *Tt* Rieske cluster varies between pH 3 and 14.[11] By defining this curve in three different ionic strengths (0.01, 0.1, and 2 M NaCl) we immediately identified the effect of a distant, ionizable amino acid residue in the low pH region. Since this weakly coupled residue can be "decoupled" by high salt concentrations, we do not consider it important to the intrinsic properties of the cluster and will therefore discuss only data recorded at the highest ionic strength.[12] The higher-pH data are uncomplicated by this distant charge, although *E* and p*K* values vary, as expected, with ionic-strength.[13] Figure 1 also shows fits to the data obtained using the thermodynamic scheme, Scheme 1, and eq 1 derived from it.[14]

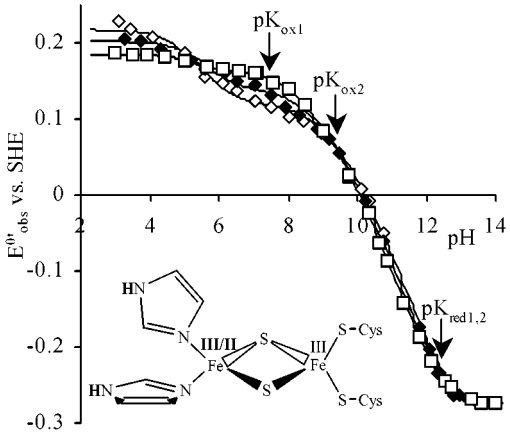

Figure 1. pH dependence of the Rieske-center potential, 20 °C, NaCl concentrations 2 M (□), 0.1 M (♦) and 0.01 M (◇).[11] The integrity of our high pH data was confirmed by switching the protein-film between solutions of high and low pH; no change in the low pH value resulted. Also shown are fits to the data obtained using Scheme 1. At low pH, the weakly coupled protonation was modeled using a simple square scheme; $pK_{ox} = 5.2, 5.1, 4.6$, $pK_{red} = 5.6, 6.2, 6.3$ for 2.0, 0.1, and 0.01 M NaCl, respectively. The Rieske cluster is also shown, identifying the centers of reduction and protonation.

$$E^{0'}_{obs} = E^{0'}_{alk} - \frac{RT}{F}\ln\left[\left(1 + \frac{a_{H+}}{K_{ox2}} + \frac{a^2_{H+}}{K_{ox1}K_{ox2}}\right)\Big/ \left(1 + \frac{a_{H+}}{K_{red2}} + \frac{a^2_{H+}}{K_{red1}K_{red2}}\right)\right] \quad (1)$$

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(11) Reduction potentials were measured using protein-film voltammetry (ref 10). Protein solution (200 μM, pH 7.5) was applied directly to a freshly polished pyrolytic graphite edge electrode and then placed into solution in an all-glass electrochemical cell. pH was controlled by mixtures of four buffers: 10 mM sodium acetate, HEPES, MES, TAPS, CAPS and sodium phosphate, depending on the pH; volumetric solutions of NaOH were used above pH 13. Potentials were independent of buffer composition. Upon cycling the potential, clearly defined oxidation and reduction waves were observed; in the absence of protein no relevant peaks were apparent. At slow scan rate the response was reversible: peak half-height widths and separations were close to their Nernstian values (typically 110 mV and 20 mV respectively), and protein coverage was maximally 2×10^{-11} mol cm^{-2}, corresponding to a (sub)-monolayer (ref 22). Several measurements were also made using diffusional voltammetry; small discrepancies in $E^{0'}_{obs}$(<25 mV) reflect small redox-dependent changes in adsorption. The pH of each solution was checked immediately following measurement, at the experimental temperature, by using a standard glass electrode, calibrated at temperature according to known standards (Lide, D. R. *Handbook of Chemistry and Physics*, 81st ed.; CRC Press: Florida, 2000). NaOH (1 M) was used as a pH 14 standard. The effects of high Na^+ concentration were corrected by standard formulae (Bard, A. J.; Faulkner, L. R. *Electrochemical Methods: Fundamentals and Applications*; Wiley: New York, 1980).
(12) Schröter et al. have also noted a p*K* value < 7 affecting the potential of the Rieske cluster in *Paracoccus denitrificans* bc_1 complex; however, this was not ascribed to a residue on the Rieske protein itself (Schröter, T.; Hatzfeld, O. M.; Gemeinhardt, S.; Korn, M.; Friedrich, T.; Ludwig, B.; Link, T. A. *Eur. J. Biochem.* **1998**, *255*, 100−106).
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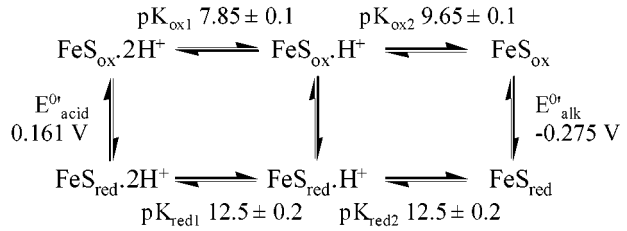
Scheme 1. Thermodynamic Scheme Relating the Different States of the Rieske Cluster (20 °C, 2 M NaCl).



Between pH 10 and 12 the gradient of the curve is −116 mV per decade [−2RT(ln 10)/F], showing that two protons must be transferred for every electron. While gradients above −60 mV per decade, at high pH, have been reported previously, the strongly alkaline conditions necessary precluded unequivocal identification of the 1 e$^-$:2 H$^+$ process.[1] Between pH 8.2 and 9.2 the gradient is close to −58 mV per decade, reflecting stability of the singly protonated oxidized state (Figure 2).

The thermodynamics for deprotonation of each redox state are reported in Scheme 1. Values for pK_{ox1} and pK_{ox2} have been reported previously for a number of Rieske clusters,[1] and circular dichroism[15] and resonance Raman spectroscopy[16] were used to assign these values (7.7 and 9.1 for the bovine Rieske fragment) to the "imidazole-imidazolate" transitions of the two His ligands. In the oxidized state, the two imidazoles are both structurally[3] and magnetically inequivalent,[5] with different solution mobilities,[2] and thus may adopt different pK values. It is likely that this difference in pK is increased by the less-favorable electrostatics imposed on deprotonation of the second imidazole by the proximity of the first imidazolate.

This communication reports the first measurement of the high pK values of the reduced form (Scheme 1). For the Fe^{II} state the His pK's have decreased by 1.7 units, from 14.2 for free imidazole,[17] and for Fe^{III} by 6.35 or 4.55 units, reflecting the more positive electrostatic potential from Fe^{III} than Fe^{II}. While we cannot exclude the possibility that the two values differ slightly (<0.5 units) we have modeled our data with pK_{red1} = pK_{red2}, implying the two protonations are cooperative and that the singly protonated reduced state is never stable (Figure 2). Our analyses cannot distinguish equal pK values from "crossed" values (pK_{red1} > pK_{red2}). Cooperative proton transfer is difficult to rationalize without invoking a change in conformation upon reduction and/or the first protonation. However, experiments using fast-scan protein-film voltammetry to switch between FeS$_{red}$.2H$^+$ and FeS$_{ox}$ (pH 11) or FeS$_{ox}$.H$^+$ (pH 9) as many as 2000 times per second, failed to detect any evidence of limiting coupled kinetics.[18]

The reduction potential of the Rieske cluster falls nearly 0.450 V from the "acid limit" of $E^{0\prime}_{acid}$ = +0.161 V, to $E^{0\prime}_{alk}$ = −0.275 V in the "alkaline limit".[19] The formal charge on the cluster is clearly the key to elevating the reduction potentials of Rieske-type clusters above those of ferredoxin-type clusters, which have only Cys (thiolate) ligation. At the acid limit the imidazole ligands are neutral, and the charge on the oxidized Rieske cluster is 0. The charge on an oxidized ferredoxin cluster is −2. However at the alkaline limit, with the imidazoles ionized, the charge on the oxidized Rieske cluster is also −2, and the reduction potential now lies comfortably within the range of ferredoxin cluster potentials.[20] Thus, the *protonated* state of the His ligands is crucial for the raised reduction potential, imidazolate and thiolate having approximately equivalent effects. In addition, imidazole is capable of acting as π-acceptor, as well as σ-donor. This would further stabilize Fe^{II} and raise the reduction potential.[17]

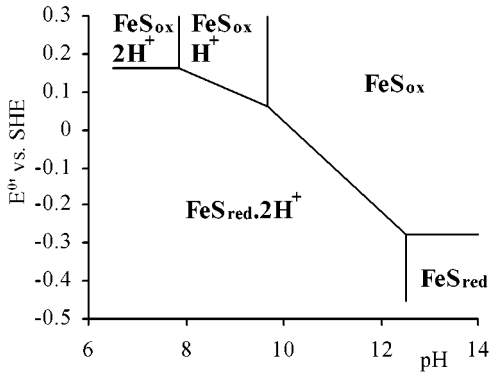


Figure 2. Regions of stability for the different redox and protonation states of the Rieske cluster (20 °C, 2 M NaCl).

Alternatively, from Figure 2, it is clear that the high potentials of Rieske bc_1-type clusters are a direct consequence of the *separation* of pK_{ox} and pK_{red}. Indeed, the lower potentials of Rieske clusters in bacterial dioxygenase systems (~ −0.15 V)[6−8,16] can be considered a result of their *lack* of pH dependence, and we estimate, taking −300 mV as an "origin", that p$K_{ox1,2}$ and p$K_{red1,2}$ cannot be separated by more than ~2 pH units. Consequently, for p$K_{red1,2}$ ≈ 14, no pH dependence is expected until the pH is raised to at least 12. This key difference between bc_1/b_6f and bacterial dioxygenase clusters has been suggested to result from the more positive electrostatic potential at high-potential (bc_1-type) clusters,[8] which increases the reduction potential, but also decreases the histidine pK values, in accordance with our reasoning. However, we also note the presence of a disulfide bridge in the immediate vicinity of only the bc_1/b_6f-type clusters which may significantly perturb the properties of the cluster.

Finally, curves similar to that in Figure 1 were obtained at five different temperatures from 0 to 40 °C, and could be fit using eq 1. First, pK values were extrapolated to the physiological temperature of *Tt* (70 °C): pK_{ox1} = 7.10 ± 0.1, pK_{ox2} = 9.44 ± 0.03, p$K_{red1,2}$ = 11.42 ± 0.05. All the pK values are decreased, but notably, a significant proportion of the protein may now exist in the *singly* protonated (FeS$_{ox}$.H$^+$) form at physiological pH, as required if the Rieske center is to accept both e$^-$ and H$^+$ from quinol during turnover of the bc_1-complex.[4] Second, $E^{0\prime}_{acid}$ and $E^{0\prime}_{alk}$ have very similar temperature dependencies; $\Delta S^{\ominus}$ values for reduction are -45 ± 25 and −50 ± 20 J K^{-1} mol^{-1} at the acid and alkaline limits, respectively.[21] Thus, we conclude that the difference in $E^{0\prime}_{acid}$ and $E^{0\prime}_{alk}$ originates from differences in enthalpy: $\Delta H^{\ominus}$ values for reduction are −28.7 ± 7.3 and +11.9 ± 5.9 kJ mol^{-1} (20 °C), respectively. By extension, $\Delta(\Delta H^{\ominus})$ = −40.6 ± 9.4 kJ mol^{-1}, clearly identifying the large difference in reduction potential with stronger N−H bonding in the reduced state.

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(18) The average position of the oxidative and reductive peaks shifted by <40 mV over experimental time scales differing by 6 orders of magnitude. This is typical for an "simple" electron-transfer process observed using a pyrolytic graphite-edge electrode (ref 22).
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