Interaction-induced Redox Switch in the Electron Transfer Complex Rusticyanin-Cytochrome $c_4$

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The blue copper protein rusticyanin isolated from the acidophilic proteobacterium Thiobacillus ferroxidans displays a pH-dependent redox midpoint potential with a pK value of 7 on the oxidized form of the protein. The nature of the alterations of optical and EPR spectra observed above the pK value indicated that the redox-linked deprotonation occurs on the X-nitrogen of the histidine ligands to the copper ion. Complex formation between rusticyanin and its probable electron transfer partner, cytochrome $c_4$, induced a decrease of rusticyanin’s redox midpoint potential by more than 100 mV together with spectral changes similar to those observed above the pK value of the free form. Complex formation thus substantially modifies the pK value of the surface-exposed histidine ligand to the copper ion and thereby tunes the redox midpoint potential of the copper site. Comparisons with reports on other blue copper proteins suggest that the surface-exposed histidine ligand is employed as a redox tuning device by many members of this group of soluble electron carriers.

The vast majority of intramolecular biological electron transfer reactions are now well understood on the basis of Marcus’ theory of outer shell electron transfer (1–3). In the case of intermolecular electron transfer the situation is complicated by possible alterations of physico-chemical parameters of the individual redox proteins upon transient formation of an electron transfer complex. Modulations of redox midpoint potentials of either one or both of the involved redox centers upon complex formation have been reported (4–9), potentially affecting the driving force of the respective oxidation-reduction reactions.

Such effects are particularly conspicuous in complexes involving blue copper proteins (8–10). For the case of the complex between plastocyanin and photosystem 1, an involvement of the surface-exposed histidine ligand to the copper ion in the modulation of plastocyanin’s redox properties was discussed (8). The crucial role of the corresponding histidine residue in the amicyanin-methylamine dehydrogenase complex was re-addressed (11). In Cavazza et al. (12), respectively. The dissociation constant of the complex between both proteins was determined using the Biacore technique (13).

Optical Spectroscopy and Redox Titrations—Optical redox titrations were performed on a Kontron Uvikon 932 spectrophotometer according to Dutton (14) in the presence of ferrocene dicarboxylic acid, ferrocene monocarboxylic acid, and potassium ferricyanide (all at 5 mM). The ambient potential was adjusted with sodium dithionite or potassium hexachloroiridiate.

RESULTS AND DISCUSSION

Rusticyanin forms a tight electron transfer complex with cytochrome $c_4$. Despite several decades of research, the nature and sequence of electron carriers involved in the initial steps of Fe$^{3+}$ oxidation by $T$. ferroxidans are still uncertain. A number of soluble periplasmic redox proteins potentially participating in this electron transport chain have been purified and characterized and three of them have been studied in more detail, i.e. the blue copper protein rusticyanin (RCy; Ref. 15), a high potential iron sulfur protein (Refs. 16 and 17) and a diheme cytochrome of the $c_4$-type (12). Their organization into an electron transport chain bridging the span from Fe$^{3+}$ to the membrane-bound, energy-conserving electron transport pathways is still a matter of debate (13, 18–21).

In order to obtain information concerning the possible electron transfer partners of RCy, we have studied pairwise affinities within the set of soluble redox proteins using the Biacore technique. A particularly strong association constant ($K_D = 2.07 \times 10^{-7}$ M$^{-1}$) was found for the complex of rusticyanin with cytochrome $c_4$, indicating that electron transfer between these two redox components may represent a genuine segment of the Fe$^{3+}$-oxidizing pathway. While examining electron transfer re-
actions between these two redox proteins, we noticed (a) that partially reduced RCy was almost completely oxidized upon addition of ferricytochrome c₄ and (b) that cytochrome c₄ was reduced by excess of Fe³⁺ in the presence of RCy, whereas no reduction of cytochrome c₄ could be observed when RCy was absent (13). Considering the redox midpoint potentials of the free forms of RCy (+590 mV at pH 4.8) and of cytochrome c₄ (+385 mV, +480 mV), these findings strongly indicate significant changes in redox potentials of one or both of these electron transport proteins upon complex formation.

**Redox Properties of the Complex**—Fig. 1 shows the redox potential dependence at pH 4.8 of rusticyanin's optical band in the vicinity of 600 nm in the presence of a stoichiometric amount of cytochrome c₄. An Eₘ (redox midpoint potential) value of +490 mV was observed, i.e. a value that is by about 100 mV lower than that obtained in free rusticyanin at the same pH value (indicated by the dotted line in Fig. 1). The Eₘ values of both cytochrome c₄ hemes remained constant within experimental precision (not shown).

**Electronic Alterations of the Copper Site Induced by Complex Formation**—In addition to the drop in electrochemical potential, alterations of optical and EPR parameters of the copper site in rusticyanin were observed in the complex. The peak wavelength of the optical band in the vicinity of 600 nm, typical for blue copper proteins in the oxidized state, was found to be shifted in the complex by 14 nm toward shorter wavelengths (Fig. 2, continuous versus dashed trace). This spectral band arises from a charge transfer transition between the copper ion and the sulfur atom of the cysteine ligand (22), and modifications of this band are therefore indicative of substantial rearrangements of the electron density distribution between the copper site and its ligands. Fig. 3, trace b, shows the X-band EPR spectrum recorded on oxidized free RCy at a concentration of 20 µM. The obtained spectrum corresponded to those previously reported for oxidized RCy at low pH values (Ref. 23, see also Fig. 3, trace c). Addition of a stoichiometric amount of oxidized cytochrome c₄ to the sample used for recording the spectrum shown in Fig. 3, trace b, resulted in dramatically altered EPR spectral properties (Fig. 3, trace a) involving an increase in g₉ as well as modifications in apparent hyperfine parameters on all g values. These EPR spectra thus provided evidence for redistributions of the unpaired electron density at the copper site upon complex formation corroborating the significantly altered electronic structure of the copper-ligand sphere in the complex.

**RCy in the Complex with Cytochrome c₄ Resembles Free RCy at High pH Values**—The changes in the optical spectrum induced by complex formation was suggestively reminiscent of previously reported data on rusticyanin obtained at pH values above 7, i.e. a blueshift of the charge transfer band from 597 to 577 nm (11). Apart from the blueshift, the charge transfer band was seen to persist up to pH values of about 10, demonstrating that the overall distorted tetrahedral ligand geometry (involving one cysteine-, one methionine, and two histidine residues (24, 25), see Fig. 6) was maintained up to this pH value. Above pH 10, an essentially irreversible loss of the Cu-S₅₋ charge transfer transition was observed indicating denaturing of the protein. The copper center in rusticyanin thus retains its ligand scheme up to high pH values, whereas the copper-ligand interactions undergo noticeable alterations in the pH range between 7 and 10 reflected by the blueshift of the charge transfer band.

The similarity of the spectral changes observed at high pH and in the complex suggested a possible correlation of both
phenomena. To substantiate such a correlation, we examined the pH dependence of rusticyanin’s EPR spectral parameters and of its reduction potential in the alkaline region. EPR spectra recorded at several pH values between pH 4 and pH 11 (Fig. 3, traces c–e) indeed showed that new spectral forms with significantly altered g and hyperfine tensors appear above pH 7. It is of note that comparable spectral transitions at high pH values have already been reported previously (23); the much lower signal-to-noise ratio, however, precluded an interpretation of the EPR parameters in the alkaline region. The spectra obtained in the region between pH 7 and pH 10 (Fig. 3, traces c–e) were heterogeneous, i.e. reflected the sequential deprotonations of more than one group. The general spectral features of the high pH paramagnetic species, however, involved a shift of g to toward higher values and the loss of the hyperfine structure on g, i.e. intriguingly resembling those observed in the complex. At pH 9.5, the new paramagnetic species have become dominant, whereas the low pH spectrum has essentially vanished (as judged, for instance, from the disappearance of the trough at g = 1.96 in Fig. 3). Fig. 4 shows a pH titration of signals characteristic for the low and the high pH spectra, respectively. As can be seen from this figure, the low pH spectrum converted into the high pH spectra in the pH range between 7 and 9. Above pH 9.5, a progressive loss of the signal was found most probably corresponding to the loss of the copper site as discussed above.

A Redox-linked Deprotonation Results in Drastic Drop of the \( E_m \) Value at High pH Values—The redox midpoint potential of rusticyanin has previously been reported to show only a weak pH dependence up to pH 6.2 (26). Stimulated by the above described phenomena observed at high pH values, we extended the determination of rusticyanin’s \( E_m \) values up to pH 9. Fig. 5 shows the results of equilibrium redox titrations of free RCy at pH 3 and pH 9. The weak pH dependence seen at low pH values ceased at about pH 7 when replaced by a strongly pH-dependent curve with a slope of about 60 mV/pH above this pH value. The obtained dependence at high pH values is characteristic of the involvement of a dissociable proton on the oxidized form of the protein in the redox transition. At pH 9, the \( E_m \) value of free RCy corresponds roughly to that observed at pH 4.8 in the complex with cytochrome \( c_4 \) (see Fig. 5). Since the slope of the \( E_m \) versus pH curve was still close to the theoretical value for a single redox-linked proton (59 mV/pH) at pH 9, the pH of the dissociable proton on the reduced form of the protein must be significantly above this pH value.

A strikingly similar pH dependence of redox properties is observed in the [2Fe-2S] protein from cytochrome bc complexes, the so-called Rieske proteins. The [2Fe-2S] cluster in this class of proteins is ligated by two cysteine and two histidine residues. The Rieske clusters are characterized by a pH-dependent redox midpoint potential above a pH value that varies in the range of pH 6 to pH 8 between species (27, 28). The redox-linked proton is generally believed to correspond to the nitrogen proton of the imidazole moiety of the histidine ligands (27). A similar situation appears to apply to RCy. The only residues that are liable to have pK values in the observed range, and the deprotonation of which will induce significant modifications of the electronic structure at the copper ion, are the two histidine ligands His-85 and His-143 (see Fig. 6). The additional negative charges on the histidinates above the respective pK values would be expected to render the copper ion more difficult to reduce and thus to lower its redox midpoint potential. The sequential deprotonation of the two histidine ligands at high pH values also rationalizes the observed heterogeneity of EPR spectra recorded in the alkaline region. The spectra shown in Fig. 3, traces d and e, represent superpositions of spectra arising from RCy molecules with either one or both histidine ligands in the deprotonated state.

Molecular Correlation between the High pH Phenomena and Complex Formation—The ensemble of the similarities between RCy at high pH and in the complex with cytochrome \( c_4 \) demonstrates that docking of RCy to cytochrome \( c_4 \) induced changes at the copper site related to the redox-linked deprotonation at high pH values. Since the respective effects in the complex were observed at pH 4.8 and below, complex formation must have decreased the pK value of the redox-linked proton by about 4 pH units (see above). From an inspection of the three-dimensional structure of RCy (24) (see Fig. 6), the N* proton on the exposed copper-ligand histidine 143 appears predestined to form hydrogen bridges with suitable residues on its partner in complex formation. Such an interaction would weaken the H–N* (His-143) bond (i.e. decrease the pK value of the protonation site) and thereby induce electronic changes in the imidazole ring resembling deprotonation (Fig. 6). Attempts to co-crystallize RCy and cytochrome \( c_4 \) in order to obtain a detailed picture of the interaction surface in the complex are presently under way in our laboratory.

Role of the Unique Redox Complex in Electron Transport of Thiobacilli—The observed complex-induced alterations of RCy’s redox properties make sense with regard to the thermodynamic constraints of electron transfer in Thiobacilli. These...
organisms use the relatively weak reducing power of the iron(II) \( (E_{m,2} \approx 700 \text{ mV, Ref. 29}) \), abundant in the growth medium, to drive electron transport toward molecular oxygen \( (E_{m,2} \approx 1150 \text{ mV, Ref. 29}) \) via a membrane-bound cytochrome oxidase (Fig. 7). From comparisons to other organisms (30, 31) and from the operon structure of the cytochrome \( c_4 \)/oxidase genes (32), it appears likely that cytochrome \( c_4 \) serves as the immediate donor to cytochrome oxidase in Thiobacilli. Since the involvement of RCy in the early steps of Fe(II) oxidation has been shown, electron transport would be expected to proceed from the copper protein toward the cytochrome. Such an electron transport, however, would be strongly hampered by the unfavorable redox equilibrium if there were not the above shown complex-induced drop in reduction potential of RCy (see Fig. 7). The extent of the observed drop in \( E_m \) value, on the other side, is thermodynamically equivalent to a more than 100-fold preferential binding of the oxidized form of Fe(II) to cytochrome \( c_4 \), i.e. seemingly in contradiction to electron transfer from the copper protein to the cytochrome. RCy, however, is by far the most abundant soluble redox protein in Thiobacilli accounting for up to 5% of the total soluble protein. This large pool of RCy molecules will be held at a very high degree of reduction by the abundant Fe(II) in the medium, assuring saturation of the binding sites on cytochrome \( c_4 \) by reduced RCy and thus allowing efficient electron transfer from RCy to cytochrome \( c_4 \).

Blue Copper Proteins Use the Surface-exposed Histidine as a Redox-tuning Device—As shown in Fig. 6, the likely key residue mediating the interaction-induced changes in electrochemical potential is located in a strikingly strategic position on the surface of the protein. This histidine residue in its privileged position is conserved in the large majority of blue copper proteins (33). Modifications of redox properties reminiscent of those seen for RCy have furthermore been observed for the electron transfer complexes between amicyanin and methyamine dehydrogenase (7, 10) and between plastocyanin and photosystem I (8).

The crucial role of the surface-exposed histidine in complex-induced alterations of redox midpoint potential of the copper site has recently been demonstrated for the electron transfer complex between amicyanin and methyamine dehydrogenase (10, 34). Free amicyanin shows a pH-dependent redox potential below pH 7.5 and constant \( E_m \) values above this pK value, indicating a redox-linked protonation on the reduced form of the protein. The presence of such a pK value on the reduced protein is common to the large majority of blue copper proteins (35). Already more than a decade ago, the protonation of the \( \delta \)-nitrogen on the exposed histidine ligand has been identified as the molecular basis for the respective redox phenomenon in plastocyanin (36). This protonation entails breakage of this histidine-copper ligation and subsequent rotation of the histidine side chain (36). The recent study of the amicyanin-methyamine dehydrogenase complex has shown that a corresponding rotation of the exposed histidine in amicyanin is hampered by the presence of methyamine dehydrogenase at the interaction surface of the complex (10). The resulting stabilization of the copper-histidine bond is equivalent to a decreased pK value of the protonation site on N\(^{\delta^-}\) and therefore translates into a decrease of amicyanin’s redox midpoint potential at pH values below the pK of the free form (10).

Rusticyanin, by contrast, is an acid-stable protein and the pK value of the N\(^{\delta^-}\) protonation site therefore needs to be shifted to pH values significantly below pH 2 in order to assure integrity of the copper site at the physiological pH values of the organism (24, 25). Consequently, the above described pK value on the reduced form of the protein was not observed in RCy (26). The drastically decreased pK value on N\(^{\delta^-}\) (His-143) most probably results from the presence of the two aromatic amino acid residues Phe-83 and Phe-51 in the immediate vicinity of the surface-exposed histidine 143 (see Fig. 6), rendering the environment of this residue significantly more hydrophobic in RCy than in other blue copper proteins. However, the reduced solubility of the surface-exposed histidine obviously also affects the pK-value of the proton on N\(^{\delta^-}\). This pK is found slightly above pH 7 in RCy (Fig. 5), whereas it is beyond the experimentally accessible range of pH values in other blue copper proteins. In RCy, it is, therefore, a pK shift of the N\(^{\delta^-}\) proton that lends itself for inducing changes in redox midpoint potential upon complex formation rather than a respective shift of N\(^{\delta^-}\) as described for the amicyanin-methyamine dehydrogenase complex (10).

It therefore appears likely to us that the surface-exposed histidine ligand to the copper ion is employed as a redox tuning device in blue copper proteins in general.
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