Influence of Chromium Binding on the Kinetics of Aqueous Chromium(II) Reduction of the Blue Copper Proteins Azurin and Stellacyanin[†,‡]

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The blue single copper proteins, azurin from *Pseudomonas aeruginosa* and stellacyanin from *Rhus vernicifera* can be reduced by Cr(II) aqua ions, and the Cr(III) ions formed as a result of the electron transfer to Cu(II) centers in the proteins are coordinated to specific sites on the protein surfaces. These sites have earlier been identified [Farver, O.; Pecht, I. *J. Isr. Chem.* **1981**, *21*, 13–17; Farver, O.; Licht, A.; Pecht, I. *Biochemistry* **1987**, *26*, 7317–7321]. In order to demonstrate that the site of coordination is the result of a trapping of the Cr(III) ion close to a redox active center on the protein rather than a migration of Cr(III) to a thermodynamically more advantageous site, we have now studied the Cr(II) reduction kinetics of the Cr(III)-labeled proteins and of the native proteins. For Cr(III)-labeled azurin we find $k = 300 \pm 10$ M^{-1} s^{-1} (298 K), $\Delta H^{\ddagger} = 0 \pm 2$ kJ/mol, and $\Delta S^{\ddagger} = -195 \pm 10$ J/(mol·K), at 0.1 M MES and pH 5 compared with $k = 450 \pm 15$ M^{-1} s^{-1}, $\Delta H^{\ddagger} = 8 \pm 1$ kJ/mol, and $\Delta S^{\ddagger} = -170 \pm 10$ J/(mol·K) for native azurin, under the same conditions. For Cr(III)-labeled stellacyanin in 0.1 M MES pH 5 the reduction is biphasic and we find $k_1 \approx 5 \times 10^3$ M^{-1} s^{-1} (298 K), $\Delta H^{\ddagger}_1 = 0 \pm 8$ kJ/mol, $\Delta S^{\ddagger}_1 = -170 \pm 30$ J/(mol·K), $k_2 \approx 800$ M^{-1} s^{-1} (298 K), $\Delta H^{\ddagger}_2 = 23 \pm 2$ kJ/mol, $\Delta S^{\ddagger}_2 = -107 \pm 7$ J/(mol·K), while for native stellacyanin the reaction is monophasic with $k \approx 1 \times 10^6$ M^{-1} s^{-1} (298 K), $\Delta H^{\ddagger} = 20 \pm 4$ kJ/mol, and $\Delta S^{\ddagger} = -60 \pm 14$ J/(mol·K). The large effect of coordinated Cr(III) on the reactions demonstrate that the binding centers on azurin and stellacyanin are indeed close to redox active centers on the protein surface. Electron transfer pathways from these centers to the Cu(II) ion in azurin and stellacyanin have been calculated and consist of relatively short through-bond interactions.

Introduction

Studies of reactions between small inorganic redox active ions and metalloproteins have given valuable information on protein reactivity and have helped to identify active redox sites on proteins.[1-3] The electron transfer (ET) reaction (1) between Cr(II) aqua ions and Cu(II) in blue copper proteins (P[Cu(II)]), yielding colorless P[Cu(I)] and Cr(III), has been examined in several studies.[4-10]

$$\text{P[Cu(II)]} + \text{Cr(II)} \rightarrow \text{Cr(III)–P[Cu(I)]} \qquad (1)$$

The Cr(II) ion is a probe particularly well suited in this respect. In addition to the kinetics of the redox reaction, it may provide information of the location of the ET site on the proteins. The substitution inert Cr(III) ions,[11] formed as a result of the ET reaction, are expected to bind tightly and close to the ET site of the protein. Thus, by identifying the Cr(III) coordination site on the protein, the nature of the redox active site may be inferred. This method has been used previously in studies of ET centers on the blue single copper proteins azurin,[7] plastocyanin[8] and stellacyanin.[9,10] Also the effect of Cr(III) binding in azurin and plastocyanin on ET reactions with biological partners have been reported.[12-14] In order to demonstrate whether the unique Cr(III) binding sites on these copper proteins result from trapping of the substitution inert label close to the ET center or from binding at a thermodynamically more advantageous coordination center, we have examined the reduction kinetics of Cr(III)-labeled Cu(II) proteins (Cr(III)–P[Cu(II)]) with Cr(II) ions.

$$\text{Cr(III)–P[Cu(II)]} + \text{Cr(II)} \rightarrow \text{Cr(III)–P[Cu(I)]} + \text{Cr(III)} \qquad (2)$$

The proteins studied are *Pseudomonas aeruginosa* azurin (Az) and *Rhus vernicifera* stellacyanin (St). Az and St are blue single copper (type 1) proteins. Az consists of 128 amino acid residues ($M_w \approx 14\,600$), while St is a glycoprotein with 107 amino acid residues ($M_w \approx 20\,000$) containing 40 % w/w carbohydrate. The kinetics of the reactions (1) of the native proteins with Cr(II) have been examined before.[4,5] By repeating these experiments and comparing with the reduction reaction (2) of the Cr(III)-

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[‡] Abbreviations: Az, azurin; St, stellacyanin; MES, 2-morpholinoethanesulfonic acid; Cr–Az, chromium(III)-modified azurin; Cr–St, chromium(III)-modified stellacyanin; ET, electron transfer.

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labeled proteins, we have now shown that binding of Cr(III) alters the kinetics of reduction and have thereby demonstrated that Cr(III) indeed binds at or close to the site of ET on the proteins.

Experimental Section

Stellacyanin (St) was isolated from the acetone extract of the sap from the Japanese *Rhus vernicifera* lacquer tree and purified according to Reinhammar.[15] The final A_{604}/A_{280} was 0.16. The concentration of oxidized St was determined from A_{604} and $\epsilon_{604} = 4090\ M^{-1}\ cm^{-1}$.[16] Azurin (Az) was isolated from *Pseudomonas aeruginosa*, according to Ambler and Wynn.[17] A_{625}/A_{280} was 0.55. The concentration of oxidized Az was determined from A_{625} and $\epsilon_{625} = 5700\ M^{-1}\ cm^{-1}$.[18] Cr(III)-labeled proteins were produced essentially as described by Farver *et al.*[7,8,10] Reduction of the native Cu(II) protein (150 μM in 0.1 M MES, pH 7) was performed with a stoichiometric amount of Cr(II) perchlorate (3 mM in 0.1 M MES, pH 4) under anaerobic conditions. This was followed by dialysis with 0.1 M MES pH 5, reoxidation of the protein with $IrCl_6^{2-}$, and finally concentration by ultrafiltration on a YM10 membrane. λ_{max} ($\approx$ 600 nm) of the electronic absorbance spectra and $A_{\lambda(max)}/A_{280}$ were the same for the Cr-modified proteins as for the native proteins. The chromium and copper contents were determined by atomic absorption spectroscopy on a Perkin-Elmer Zeeman 5000 instrument.

The stopped-flow kinetic experiments were performed under anaerobic conditions in a Hi-Tech Scientific PQ/SF-53 thermostated spectrophotometer. Dilute protein ([Az] 20–30 μM, [Cr–Az] 25–30 μM, [St] 45–65 μM, [Cr–St] 40–50 μM, 0.1 M MES pH 5) and [Cr(II)] (1–20 mM in 0.1 M MES pH 4) solutions continuously purged with argon were used. Dilute Cr(II) solutions were prepared from a concentrated stock solution (made from high purity Cr metal and a slight excess of warm deoxygenated 0.5 M perchloric acid) and 0.1 M MES, pH 5. The Cr(II) concentrations were determined by reacting aliquots with excess standard $KMnO_4$ in 0.5 M H_2SO_4 and determining the residual MnO_4^- spectrophotometrically ($\epsilon_{545} = 2340\ M^{-1}\ cm^{-1}$). To obtain pseudo-first-order conditions, a 50- to 100-fold excess of reducing agent over protein was used in most experiments. Mixing of the reagent solutions were achieved in $\approx$1 ms, and in each experiment 400 pairs of data were recorded by monitoring the disappearance of the blue color ($\lambda \approx 600$ nm). To obtain the rate dependence on the concentration of Cr(II), alterable amounts of the solutions were mixed (using a variable ratio mode device calibrated on the reaction[19] between 2,6-dichlorophenolindophenol and ascorbic acid). The temperature was lowered, in $\approx$5° steps, from 25 to 8 °C. For Az, Cr–Az, and St at least two sets of stopped-flow data were averaged and fitted to single-exponential and multiexponential decay functions using the Hi-Tech HS-1 Data Pro software. In case of Cr–St, however, each set of data was analyzed individually. The pH of the resulting mixture was measured and was in the range 4.5–5.0.

Results

The reduction of the blue copper proteins (native or Cr(III)-modified) by aqueous Cr(II) was examined by stopped-flow techniques under pseudo-first-order conditions with a typical 50-fold excess of reductant. The Cr contents of the modified proteins were determined by atomic absorption. The Cr:Cu ratios were as follows: Cr–Az (0.9–1.2):1 and Cr–St (0.7–1.1):1. Typical kinetic traces are shown in Figures 1–4. Az, Cr–Az, and St showed single exponential decays (Figures 1–3), while analysis of the Cr–St data required a double exponential decay (Figure 4). The reactions between Az, Cr–Az, or Cr–St and Cr(II) were followed to completion on a 1–5-s time scale, while the faster reaction of St with Cr(II) was measured on a 10-ms time scale. Rate dependencies on the Cr(II) concentration were linear under the conditions examined (1–10 mM Cr(II) for Az and Cr–Az, and 1–4 mM for St and Cr–St). A plot of the observed first-order rate constant, k_{obs}, against [Cr(II)] for Az and Cr–Az is shown in Figure 5. The calculated second-order rate constants and thermal activation parameters are summarized in Table I.

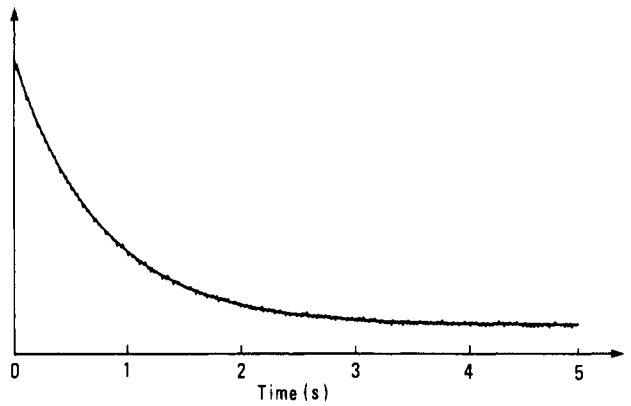

Figure 1. Time-resolved changes in the absorption (AU) at 625 nm obtained after mixing azurin and Cr(II). The conditions were [Az] = 13.2 μM, [Cr(II)] = 3.5 mM, 0.1 M MES, pH 5.0, and 298 K. The smooth curve represents a monophasic fit.

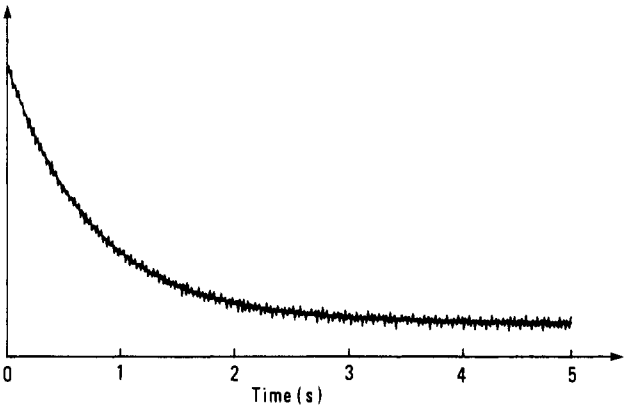

Figure 2. Time-resolved changes in the absorption (AU) at 625 nm obtained after mixing chromium(III)-modified azurin and Cr(II). The conditions were [Cr–Az] = 14.1 μM, [Cr(II)] = 4.7 mM, 0.1 M MES, pH 5.0, and 298 K. The smooth curve represents a monophasic fit.

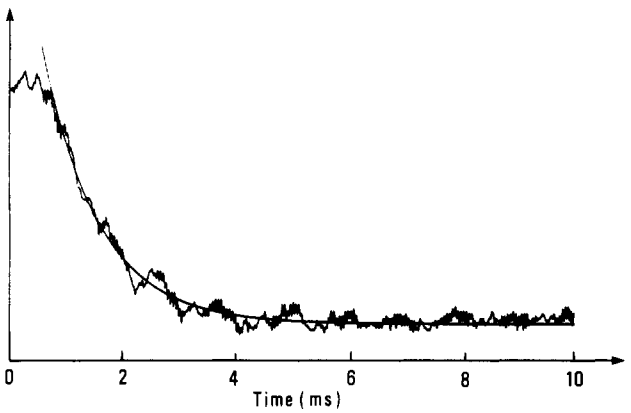

Figure 3. Time-resolved changes in the absorption (AU) at 604 nm obtained after mixing stellacyanin and Cr(II). The conditions were [St] = 33.0 μM, [Cr(II)] = 1.1 mM, 0.1 M MES, pH 4.9, and 281 K. The smooth curve represents a monophasic fit.

The kinetics of the Cr(II) reduction of the oxidized native proteins are quite different from those of the ET reactions of the Cr(III)-labeled proteins. The results on the native proteins are in reasonable accordance with those reported in refs 4 and 5 considering the somewhat different experimental conditions. At room temperature the rate constants are rather similar for Az and Cr–Az although the activation parameters differ considerably. For Cr–St, the two rate constants for the biphasic reduction are approximately 3 orders of magnitude smaller than that of reduction of oxidized native St. The activation enthalpy, $\Delta H^{\ddagger}$, is smaller for Cr–Az than for Az and this seems also to be the case for Cr–St compared with the native protein, although the

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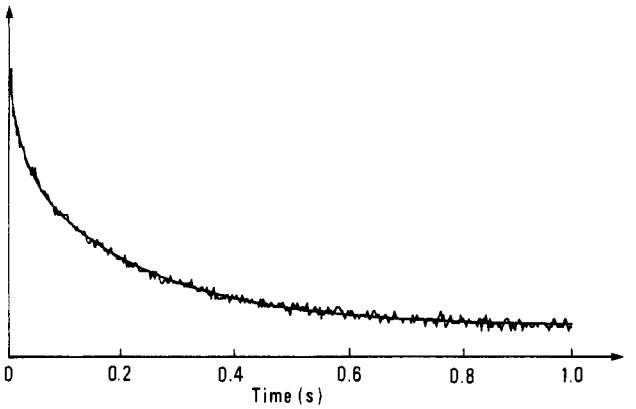

Figure 4. Time-resolved changes in the absorption (AU) at 604 nm obtained after mixing chromium(III)-modified stellacyanin and Cr(II). The conditions were [Cr-St] = 33.0 μM, [Cr(II)] = 2.1 mM, 0.1 M MES, pH 4.6, and 298 K. The smooth curve represents a biphasic fit.

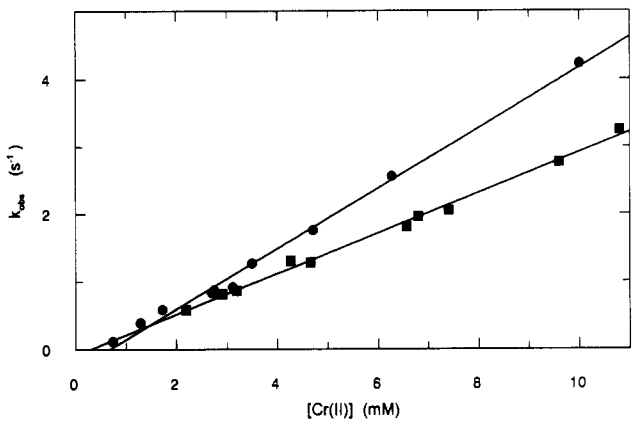

Figure 5. Cr(II) concentration dependence of the reduction of azurin (●) and Cr(III)-modified azurin (■) in 0.1 MES (pH ≈5), 298 K.

Table I. Kinetic Parameters for the Reduction of Cu(II) in Azurin, Stallacyanin, and the Cr(III)-Modified Proteins in 0.1 M MES, pH 4.5–5.0[a]

	k (M^{-1} s^{-1})	$\Delta H^{\ddagger}$ (kJ/mol)	$\Delta S^{\ddagger}$ (J/(mol·K))
azurin	450 ± 15	8 ± 1	−170 ± 10
	1.6×10^3 [b]	10 ± 2[c]	−142 ● 8[c]
Cr(III)–azurin	300 ± 10	0 ± 2	−195 ± 10
stellacyanin	≈1×10^6	20 ± 4	−60 ± 4
	≈6×10^5 [d]		
Cr(III)–stellacyanin[e]	≈5×10^3	0 ± 8	−170 ± 30
	≈800	23 ± 2	−107 ± 7

[a] The second order rate constants at 298 K are calculated from Cr(II) dependencies. [b] Reference 5 (0.12 M acetate, pH 4, 298 K). [c] Reference 5 (0.4 M acetate, pH 4.3). [d] Reference 4 (0.1 M NaCl, pH 4.2, 277 K). [e] Double exponential decay.

double exponential decay makes the analysis more difficult. For both pairs the activation entropies, $\Delta S^{\ddagger}$, decrease going from the native to the Cr(III)-labeled proteins. The reduction potentials at pH 7 for Az[20] and St[21] are 304 and 184 mV, respectively, and the redox potentials of the modified copper proteins was found to change less than 15 mV upon Cr(III) labeling.[22] For the $Cr(OH)^{2+}/Cr^{2+}$ couple the standard reduction potential is −182 mV.[23]

Discussion

Azurin. Previous studies show that reduction of native *P. aeruginosa* azurin (Az[Cu(II)]) with Cr(II) results in a substitution inert 1:1 Cr(III)–Az[Cu(I)] complex. The coordinated Cr(III) was located in one specific peptide stretch containing Glu91[7] (Figures 6 and Figure 7). The three dimensional structure of *P. aeruginosa* azurin is known at high resolution,[24] and potential tunneling pathways for ET from the Cr(III) site to the Cu(II) center (direct O-ε(Glu91) to Cu distance ≈ 13 Å) may be examined using the procedure of Beratan and Onuchic.[25,26] The electronic coupling, EC, from electron donor to acceptor is calculated according to the expression

$$EC = \Pi\epsilon_i$$

where ϵ_i are (semi-empirical) decay factors for all the links in a given ET pathway. Three types of decay factors are included, those of (a) covalent bonds, ϵ_C, (b) hydrogen bonds, ϵ_H, and (c) through-space jumps, ϵ_S. The rate constant of a given pathway is proportional to its EC term squared and the model may therefore predict favorable pathways for ET in the protein. According to this model the carboxylate group of Glu91 is a "hot spot" of ET, since the coupling between the copper atom and O-ε of Glu91 is a factor of 10 larger than that of a through-space exponential decay. The pathway of optimal coupling from O-ε of Glu91 to N-δ of His46 is shown in Figure 7. The calculated overall electron decay factor is EC = 6.2×10^{-3}. This very efficient pathway consists of six covalent bonds and two hydrogen bonds between O-ε(Glu91) and N(Gly88) and between O(Ile87) and N(His46), respectively, but since the carboxylate group of Glu91 is at the surface of the protein, it could be quite flexible in solution. The

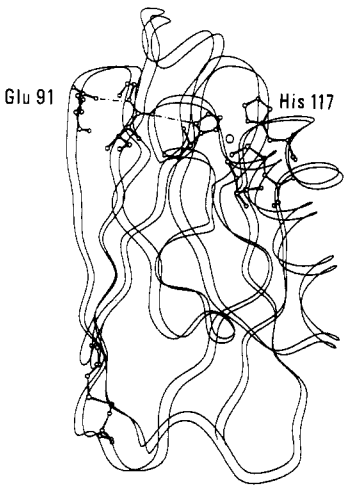

Figure 6. Schematic drawing of the structure of *P. aeruginosa* azurin[24] showing the copper center with the ligands His46, Cys112, His117, and Met121, the disulfide bridge Cys3–Cys26, the apparent Cr(III) binding site Glu91, and the residues Gly88 and Ile87.

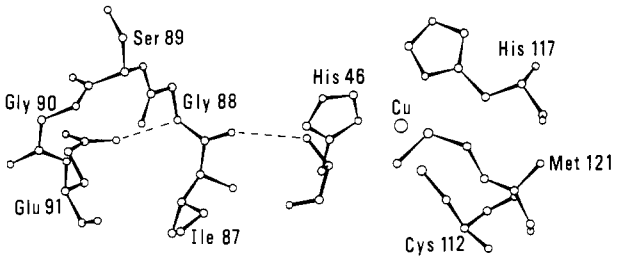

Figure 7. The apparent binding site of Cr(III) in azurin (O-ε of Glu91) and the optimal electron transfer pathway from Glu91 to Cu(II) including two hydrogen bonds between O-ε(Glu91) and N(Gly88) and between O(Ile87) and N(His46), calculated by using the method of Beratan *et al.*[25,26]

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formation of this hydrogen bond might very well be the rate limiting step in the reaction of Az with Cr(II). An alternative pathway involves two backbone hydrogen bonds (N(Glu91) to O(Gly88) and O(Ile87) to N(His46)) and 14 covalent bonds and has an overall decay factor EC = 5.0×10^{-5}.

The reaction center for reduction of Cr–Az with Cr(II) is as yet unknown. From previous studies it is known that the original Cr(III) label is retained on the protein even after reduction of Cr–Az with a large excess of $^{51}Cr(II)$ while the Cr(III) ion produced does not get bound to the protein.[7] These observations combined with the present results suggest the following.

(a) The route for ET to Cu(II) in Cr–Az is not via the Cr(III) label by a Cr(II)–Cr(III) electron exchange reaction. If so, at least partial loss of the original Cr(III) label would occur. Also, the Cr(II)–Cr(III) exchange rate constant[27] at pH 4.5 is very small ($\approx 2\ M^{-1}\ s^{-1}$). Besides the link to the protein Cr(III) is coordinated only to solvent, and an increase in rate of Cr(II) to Cr(III) ET to 300 $M^{-1}\ s^{-1}$ (the rate observed here) because of this change in coordination seems unlikely. Further, the activation enthalpy for the reduction of Cr–Az by Cr(II) ions is ≈ 0 kJ/mol, while that of Cr(II)–Cr(III) electron exchange is much larger, $\Delta H^{\ddagger} \approx 60$ kJ/mol.[28]

(b) The actual protein ET site for the reduction of Cr–Az probably lacks an effective ligand since the second Cr(III) ion does not bind to the protein.

(c) The original Cr(III) coordination site is located close to the ET site since the kinetic parameters of the reduction of Cr–Az at pH 4.5–5.0 are different from the parameters for the corresponding reaction with Az in the same pH range.

The hydrophobic patch surrounding His117 has been suggested as a potential site for ET for both artificial and physiological redox partners.[12,29] The activation parameters, however, do not support reduction of Cr–Az via this site. A value of $\Delta H^{\ddagger} \approx 0$ kJ/mol would suggest some sort of compensation effect from the formation of a precursor complex. It is established from the X-ray structure that water molecules are bound to the protein.[24] We suggest that Cr(II) reduces Cr–Az via such water bridges. Judged from the solvent accessibility and the distance to the copper center the water molecules hydrogen bonded to N-ϵ of Gln8 or Gln14, or N-δ of Asn16 are likely candidates as bridges to the protein. ET via one or more of these water molecules could prevent a direct coordination of Cr(III) to protein residues. In a recent theoretical study of ET self-exchange in azurin we have demonstrated the importance of bridging water molecules.[30]

Stellacyanin. It is known from previous studies that when Cr(III)–St[Cu(II)] is reduced with Cr(II) the original Cr(III) label remains bound to the protein while the Cr(III) ion produced in the reduction reaction does not bind to stellacyanin.[9] As indicated in Table I the kinetics of the reduction reaction of native oxidized St[Cu(II)] with Cr(II) differ dramatically from that of Cr(III)-St[Cu(II)]. The biphasic nature of the latter reduction reaction can be rationalized in terms of eq 3 assuming the existence

$$\begin{aligned} &\text{Cr(III)–St[Cu(II)] + Cr(II)} \xrightarrow{k_1} \\ &\qquad \text{Cr(III)–St[Cu(I)] + Cr(III)} \\ &\text{Cr(III)–St'[Cu(II)] + Cr(II)} \xrightarrow{k_2} \\ &\qquad \text{Cr(III)–St'[Cu(I)] + Cr(III)} \end{aligned} \quad (3)$$

of two protein conformers (Cr–St and Cr–St') induced by the chromium modification which do not interconvert readily compared with the rate of the reaction under study.

Upon Cr(III) binding to St, the rate constants decrease 3 orders of magnitude and also the activation parameters of the reaction change. Thus, it is obvious that the Cr(III) coordination site is located at a redox active site on stellacyanin, preventing a second reduction process from taking place here. From earlier binding studies it was found that reduction of St by Cr(II) leads to Cr(III) coordination, probably to the carboxylate of Asp49.[10] The three-dimensional structure of St is not known. ENDOR studies on St demonstrate that the copper ion is relatively exposed to the solvent.[31] Computer models based on comparison with the known structures of poplar plastocyanin and cucumber blue protein have been presented,[32,33] and both models predict an O-δ(Asp49) to Cu distance of approximately 9 Å. Even in the absence of solid structural details, an ET pathway based on the amino acid sequence may be estimated. His46 in St is homologous with His37 in the plastocyanins and His39 in cucumber blue protein. The imidazoles of the latter two proteins coordinate Cu(II).[34,35] Thus, His46 is most likely a Cu ligand in stellacyanin. The short Asp49 to His46 peptide patch may therefore provide an effective through-bond pathway (15 covalent bonds). Using the decay factors for covalent bonds we calculate an electronic coupling factor EC = 4.7×10^{-4}. This is 10 times larger than that of the pathway using backbone hydrogen bonding in Az. At 298 K the rate of reduction of Cu(II) in Az is 2000 times slower than the corresponding reduction of St[Cu(II)]. This difference in rate may therefore in part be rationalized by a shorter through-bond ET pathway in St.

The larger effect of chromium binding on the rate of reduction of St/Cr–St compared with that of Az/Az–Cr may be due to the large carbohydrate content in the former protein, making the possibilities of a close approach to the Cu center more limited in Cr–St. The site from which the Cr(II) ion donates the electron to the blue copper center in Cr–St is unknown. A Cr(II)–Cr(III) electron exchange mechanism can be excluded as for Cr–Az (see above). A direct reduction of the Cu(II) center in Cr–St is not likely since the decrease in ET rate is due to the larger negative activation entropy in the modified protein and not an increase in activation enthalpy. We suggest that a mechanism similar to that proposed for Cr–Az, involving hydrogen-bonded water molecules, may also apply to Cr–St.

Concluding Remarks

We have demonstrated that Cr(III) coordinates to the blue copper proteins, Az and St, at sites where it perturbs the reduction kinetics by Cr(II). We therefore conclude that in reduction of the proteins with Cr(II) the resulting Cr(III) does indeed bind to the surface of the protein at or near a redox-active center. In Az the redox-active site is concentrated around Glu91 while in St Asp49 seems to be the point of origin for ET. Calculations show that in both proteins these centers provide effective ET pathways to Cu(II). These centers may very well also be involved in the biological function of the proteins.[12,13]

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