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Electron-transfer-mediated binding of optically active cobalt(III) complexes to horse heart cytochrome *c*

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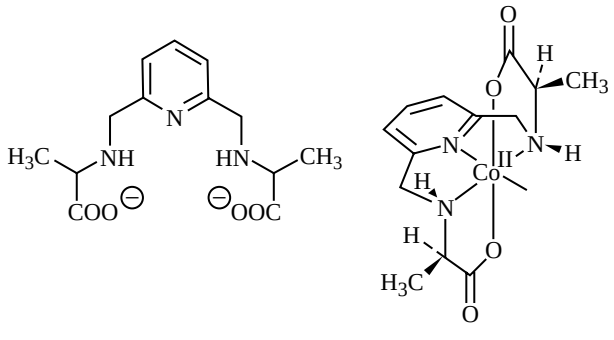
Optically active cobalt(II) complexes are used as reducing agents in the electron-transfer reaction involving horse heart cytochrome *c*. Analysis of the circular dichroism (CD) spectra of reaction products indicates that the corresponding cobalt(III) species of both enantiomers of [Co^{II}(alamp)] (H_2alamp = *N*,*N*′-[(pyridine-2,6-diyl)bis(methylene)]-bis[alanine]) are partly attached to the protein during electron transfer by coordination to an imidazole unit of one of the histidine residues. His-26 and His-33 are both solvent exposed, and the results suggest that one of these histidine residues acts as a bridge in the electron transfer to and from the haem iron of cytochrome *c*. The reaction is enantioselective: the ratio of the relative reactivity at 15 °C is 2.9 in favour of the *R*,*R*-enantiomer. A small induced CD activity in the haem chromophore reveals that some structural changes in the protein occur consecutively with the binding of the cobalt(III) complex.

Keywords: cytochrome *c*; cobalt complexes; electron transfer; stereoselectivity; circular dichroism

1. INTRODUCTION

In several previous communications, we have shown that chiral metal complexes can be used in electron-transfer reactions to identify chiral recognition on the protein surface, and to distinguish inner- and outer-sphere electron-transfer mechanisms. A prerequisite of the use of labile, redox-active metal centres is the formation of a single isomer of the complex showing predetermined chirality. In this way, the reduction of a spinach plastocyanin by the two enantiomers of [Fe(alamp)] (H_2alamp = *N*,*N*′-[(pyridine-2,6-diyl)bis(methylene)]-bis[alanine] (**I**)) allowed the first clear demonstration of a stereoselective electron transfer involving metalloproteins (Bernauer & Sauvain 1988), and the investigation of genetically modified plastocyanins showed that the stereoselectivity was caused by the side chain of the leucine-12 residue (Bernauer *et al.* 1998).



I Δ-[Co(*R*,*R*)-**I**]

By the electron-transfer-mediated coupling of cobalt complexes to the surface of the protein, inner- and outer-sphere mechanisms were distinguished. The analysis of the circular dichroism (CD) spectra of the complete redox cycle revealed that the inner-sphere electron transfer occurred through the coordinated imidazole unit of histidine-87 in plastocyanin, whereas in the same reaction with azurin the uncoordinated histidine-83, some 20 Å distant from the copper centre, was involved (Bernauer *et al.* 1999).

The use of chiral electron-transfer agents has also been applied to other metalloproteins, such as superoxide dismutase (Pladziewicz *et al.* 1994), plant ferredoxin (Bernauer *et al.* 1990) and cytochrome *c* (Sasaki *et al.* 1989, 1991; Ficke *et al.* 1991; Bernauer & Jauslin 1993). Nevertheless, cytochrome *c* chiral interactions using cobalt(II) complexes as reducing agents have not been studied until now. Considering the additional information obtained by the use of these compounds in the reduction of the blue copper proteins plastocyanin and azurin, we applied the same technique to cytochrome *c*, which contains the redox-active centre in a porphyrin system.

Horse heart cytochrome *c*, one of the most thoroughly studied metalloproteins (Pettigrew & Moore 1987, 1990 and references therein), contains three histidine residues at positions 18, 26 and 33. The imidazole group of His-18 ensures the link of the protein to the metal centre of the haem. As was seen with plastocyanin and azurin, all three histidines, including the one bound to the haem, are potential candidates for electron-transfer-mediated binding of the reagent. The reduction of the haem iron from the +III

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(ferricytochrome *c*) to the +II (ferrocytochrome *c*) oxidation state can easily be followed by UV–Vis measurements, since this reaction is revealed by the appearance of a characteristic absorption band at 550 nm.

2. EXPERIMENTAL DETAILS

2.1. Reagents

Horse heart ferricytochrome *c* 95% from Fluka was used without further purification. Ligands and the cobalt(III) complex were synthesized as described previously (Bernauer & Pousaz 1984; Bernauer *et al.* 1988). All other reagents were of analytical grade.

2.2. Solutions

Solutions were prepared using dd H_2O, and those used in the reduction processes were freed from dissolved O_2 by gently bubbling Ar through the solution for at least 30 min.

2.2.1. Buffer. A stock solution at pH 7.5 and an ionic force $I=0.5$ M was prepared from 6 M HCl and solid tris(hydroxymethyl)aminoethane.

2.2.2. Cobalt(II) complexes. Both enantiomers of the optically active cobalt(II) complex were prepared *in situ* at a concentration of 0.021 M from $CoSO_4$, a 1.5-fold excess of the corresponding ligand and the buffer solution, as a way to obtain a final ionic force $I=0.1$ M.

2.2.3. Ferricytochrome c. A 3.6×10^{-4} M solution in a 0.1 M buffer was prepared with freshly unfrozen solid protein.

2.3. Measurements

The reductions were carried out in parallel with 0.021 M **Δ**-[Co^{II}((*R*,*R*)-**I**)], **Λ**-[Co^{II}((*S*,*S*)-**I**)] and 0.05 M ascorbic acid (two samples). Under an Ar stream, ferricytochrome *c* (0.5 ml) was mixed with the reducing agent (0.5 ml). To one of the samples containing ascorbic acid, 0.02 M **Δ**-[Co^{III}((*R*,*R*)-**I**)]ClO_4 (0.5 ml) was added. For the reaction at 15 °C, the sealed test tubes were left for 10 h. The samples were introduced separately into four dialysis bags (molecular porous membrane tube Spectra/Por 1, molecular weight cut-off 6–8 kDa) and gently stirred in 2×10^{-3} M buffered ascorbic acid (1 l) for 16 h. After 8 h, the ascorbic acid solution was replaced by a fresh one. The contents of the dialysis bags were diluted to 2 ml, and their UV–Vis spectra (Varian Cary 1E) and CD spectra (Jasco J-710) were recorded. The actual ferrocytochrome *c* concentration, used in the calculation of bound cobalt(III), was deduced from the absorption at 550 nm.

3. RESULTS

Figure 1 shows the CD spectra of cytochrome *c* reduced either by the two enantiomers of [Co^{II}(**I**)] or by ascorbic acid. The spectra of the former were recorded after a reaction time allowing the reduction to be more than

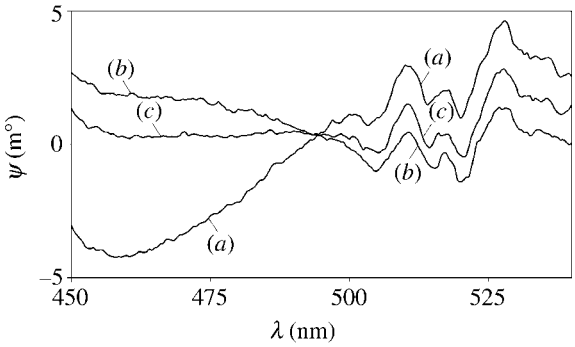

Figure 1. CD spectra of horse heart cytochrome *c* after reduction by (*a*) **Δ**-[Co^{II}((*R*,*R*)-**I**)H_2O], (*b*) **Λ**-[Co^{II}((*S*,*S*)-**I**)H_2O] and (*c*) ascorbic acid. Reaction conditions: pH = 7.5 ($I=0.1$ mol l^{-1}, Tris/HCl), $\Theta=15$ °C; reaction time: 10 h; [cytochrome *c*] $=8.2\times10^{-5}$ mol l^{-1}; optical path length $l=1.00$ cm.

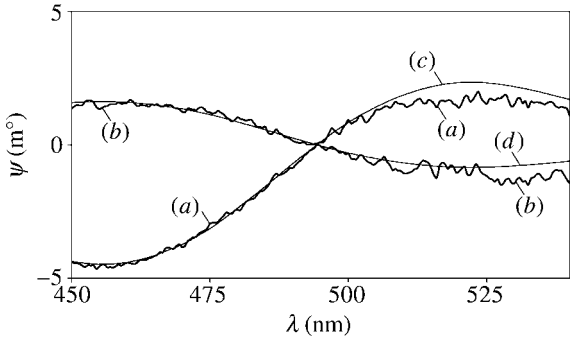

Figure 2. Difference CD spectra of the curves in figure 1: (*a*) 1*a*–1*c*; (*b*) 1*b*–1*c*. The spectra of (*c*) **Δ**-[Co^{III}((*R*,*R*)-**I**)(imidazole)]$^+$ at 43% and (*d*) **Λ**-[Co^{III}((*S*,*S*)-**I**)(imidazole)]$^+$ at 15% of the cytochrome *c* concentration are superimposed (thin lines).

95% complete and after the elimination of excess [Co^{II}(**I**)] by dialysis against large volumes of the same buffer solution as was used for the reaction. To prevent reoxidation of the ferrocytochrome *c* during the dialysis process, ascorbic acid was added to the solution.

Figure 2 shows the spectra of cytochrome *c* reduced by the two enantiomers of [Co^{II}(**I**)], from which the spectrum of cytochrome *c*, reduced by ascorbic acid, was deduced. Assuming independent chromophores, these spectra should represent the contribution of any protein-bound Co^{III} species. In order to identify the chemical nature of the bound species, the spectra of the corresponding enantiomers of [Co^{III}(**I**)(imidazole)]$^+$ are superimposed in figure 2 over the calculated spectra of the bound species.

The concentrations of the solutions of these spectra correspond to 43% and 15% for the *R*,*R*- and the *S*,*S*-enantiomer, respectively, compared with the concentration of cytochrome *c*. The good agreement of the curves of each enantiomer in the spectral region between 450 and 500 nm, where cytochrome *c* produces only weak CD activity, indicates, on the one hand, that the cobalt species have been bound to the protein by an imidazole group, and that there are no significant modifications of the enantiomeric cobalt(III) units by vicinal effects owing to the neighbourhood of the protein. On the other hand, the agreement of the

calculated and measured spectra is less in the region between 500 and 560 nm, where cytochrome *c* has significant CD activity, indicating some structural modifications in the protein owing to the presence of the Co^{III} complex. Very similar results were obtained for runs at temperatures between 15 and 35 °C with stereoselectivities of 2.9 ± 1.0 in favour of the *R*,*R*-enantiomer. The possibility of coordinating to one of the different amino groups of the lysine side chains has been checked by measuring the CD spectrum of $[Co^{III}((R,R)\text{-}\mathbf{I})(\text{butylamine})]^{+}$. As the spectrum of the latter strongly differs from the corresponding compound with imidazole, this possibility can be disregarded. Finally, the possibility of binding the cobalt(III) complex to the protein in a reaction subsequent to electron transfer has been tested by reducing cytochrome *c* using ascorbic acid under identical reaction conditions in the presence of a large excess of $[Co^{III}(\text{alamp})H_2O]^{+}$, ascorbic acid being inactive against the cobalt(III) complex. Some binding to the protein has been observed only at temperatures above 45 °C; at lower temperatures the CD spectra of the reduced cytochrome *c* were identical in the presence and absence of the cobalt(III) complex.

4. DISCUSSION

Possible reactive sites for electron transfer with cytochrome *c* have been investigated with physiological partners (e.g. cytochrome c1, cytochrome oxidase and cytochrome peroxidase), as well as with low molecular weight metal complexes. For the binding of the latter in the precursor complex, NMR and kinetic measurements, as a function of ionic strength and chemical modifications of the surface residues of the protein, have allowed researchers to determine three generally reactive areas (Brautigan *et al.* 1978; König *et al.* 1980; Webb *et al.* 1980; Ahmed & Millett 1981; Butler *et al.* 1981, 1983; Boswell *et al.* 1982; Eley *et al.* 1982; Koppenol & Margoliash 1982; Williams *et al.* 1982; Moore *et al.* 1984; Armstrong *et al.* 1986; Drake *et al.* 1989). These areas, located on both sides of the haem, are found to react preferentially either with anionic or cationic species. Interestingly, the solvent-exposed histidine residues, despite the use of His-33 for the studies of intramolecular electron transfer by covalently bound ruthenium moieties (Isied *et al.* 1982; Winkler *et al.* 1982; Yocom *et al.* 1982; Nocera *et al.* 1984; Sun *et al.* 1996), have not been considered as possible electron-transfer sites in intermolecular reactions. The present study shows that this inner-sphere pathway can effectively compete with the outer-sphere reactions occurring close to the solvent-exposed haem edge, provided the imidazole side chains can act as bridging ligand to the electron-transfer active reagent.

The difference between the two enantiomers in the amount of electron-transfer-mediated binding indicates an important chiral recognition between the protein and $[Co^{II}(\mathbf{I})]$. As this stereoselectivity is of a kinetic nature, it is *a priori* not possible to know whether the effect is caused by a higher reaction rate of the *S*,*S*-enantiomer in the outer-sphere or of the *R*,*R*-enantiomer in the inner-sphere part of the reaction. Nevertheless, the observed modification of the CD spectra of the protein indicates steric interactions between the latter and the chiral framework of the ligand (**I**). Furthermore, in a similar case involving the electron transfer between the blue copper protein azurin and the complex $[Co^{II}(\text{promp})]$ ($H_2\text{promp} = N,N'$-[(pyridine-2,6-diyl)bis(methylene)]-bis[proline]), for which the individual stereoselectivity of the inner- and outer-sphere reactions could be determined, we have shown that the observed stereoselectivity of the overall reaction is almost entirely due to the inner-sphere pathway (Bernauer *et al.* 1999). We therefore tentatively suggest that, in the present case, the stereoselectivity is a consequence of the chiral interaction of $[Co^{II}(\mathbf{I})]$ and the protein in the precursor complex, probably formed with His-33. This hypothesis is also supported by the observation that the reaction between cytochrome *c* and [Fe(alamp)] shows only weak stereoselectivity (Bernauer & Jauslin 1993).

Initially, the technique of electron-transfer-mediated binding of optically active cobalt(II) complexes to identify electron-transfer active sites in metalloproteins was applied to the blue copper proteins plastocyanin and azurin. In these cases, the protein part was colourless after the reaction, and the determination of the bound cobalt(III) species by circular dichroism was straightforward. In the present case, the reaction product has two chromophores. In this case an exact determination is possible only if there is a wavelength region where the protein has no CD activity, or if the two chromophores are independent in a way that allows the additivity rule to be applied. As the results show, the latter is not true in the system discussed in this communication.

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