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Stereo- and site selection by enantiomers in electron-transfer reactions involving native and recombinant metalloproteins

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Abstract: Electron transfer kinetics between the blue copper proteins spinach plastocyanin or wt-azurin and optically active Fe^{II} or Co^{II} complexes have been measured as a function of temperature and pH. From the observed stereoselectivity, the analysis of the reaction products, and the influence of site directed mutagenesis it is concluded, that low molecular weight electron transfer reagents can react at different sites of a narrow area of the protein surface and that considerable selection of the reactive sites can occur between enantiomeric reagents.

Introduction

Since their discovery in the late fifties blue copper proteins have been thoroughly studied with respect to their physical, structural and chemical properties. The physiological function of all these proteins, the best known of which are stellacyanin, azurin and plastocyanin, is electron transfer. For this reason they are also known as cupredoxines. Despite many investigations involving natural and artificial redox partners, with as objective the identification of pathways and points of attack, the exact nature of the groups at the surface of the protein responsible for the electron exchange with the surrounding is in general not known. It is an open question whether there is a single specific point of attack or that the electrons can enter and leave proteins over extended areas (1).

In the case of plastocyanin two such electron-transfer active areas have been identified: a hydrophobic one around His-87 and a polar one near Tyr-85, the latter being formed by the protein segments including the negatively charged residues of the amino acids 42-45, 59 and 61 (2). In plastocyanin, His-87, one of the two histidines forming the copper binding site, represents the only solvent exposed imidazole unit and has therefore been designated as a possible electron-transfer site (3). Azurin contains two extra histidines at positions 35 and 83, which are not involved in copper binding but considered, together with Glu-85, as possible electron transfer sites. On the other hand, azurin has no negatively charged area comparable to the one found in plastocyanin.

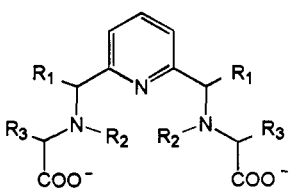
Stereoselectivity and site selection in electron transfer

In order to obtain more detailed information about the interactions occurring between chemical reagents and a protein surface, we began a series of measurements to collect evidence on kinetic stereoselectivity in electron-transfer reactions between chiral, redox active metal complexes and metalloproteins. More or less pronounced stereoselectivity was found with plastocyanin (4,5), plant ferredoxin (6) and cytochrome c (7). Similar observations made by other groups (8-13) indicate that stereoselectivity can be considered as a general behavior in electron transfer reactions involving metalloproteins. However, even if stereoselectivity is an interesting phenomenon as such, the known results show that stereoselectivity measurements alone do not provide clear information about the exact binding site on the protein surface. Therefore, we

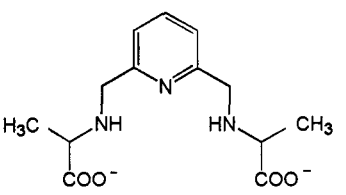
extended our measurements, involving metalloproteins and low molecular weight optically active reagents, to the following fields:

- Determination of the stereoselectivity as a function of temperature (activation parameters)
- Electron-transfer mediated binding of chiral reagents to the protein surface
- Chiral recognition in the electron transfer involving mutant recombinant proteins
- Catalysis and stereoselectivity of electron transfer in the presence of small monodentate ligands

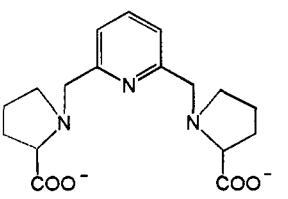
In order to use inert as well as labile metal complexes as chiral one-electron transfer agents, one needs to use ligands forming metal complexes with definite, predetermined chirality. Furthermore, the ligands should permit various substitution patterns and control various properties of the complexes formed, such as overall charge, hydrogen bonding ability, chelate ring conformation, etc. These prerequisites are largely fulfilled by ligands of type (I) (14), of which the derivatives alamp (L^1) and promp (L^2) will be used in the present work. L^1 and L^2 form complexes with predetermined chirality, Δ-[M(*R*,*R*)-L] and Λ-[M(*S*,*S*)-L].



(I)



L^1
(alamp)



L^2
(promp)

First measurements of electron transfer kinetics with Fe^{II} and Co^{II} complexes of L^1 and L^2 showed weak to moderate stereoselectivities with all the reagents used, indicating sufficiently close approach of the reagent to the protein surface to allow energetic differentiation of the diastereoisomeric transition states. Temperature dependence of the reaction rates revealed that the observed selectivity is the result of combined effects of activation enthalpy and activation entropy, the reagent favored by a lower activation enthalpy being always disadvantaged by a more negative activation entropy. The consequence of this behavior is the inversion of the stereoselectivity at a given temperature. The differences in the activation parameters observed for two enantiomeric reagents are generally rather small (≤ 10%) but some cases exist were these differences are significantly larger suggesting different electron-transfer mechanisms and/or electron-transfer pathways. Table 1 gives some typical examples for which the observed activation enthalpy for one reagent can be up to 40% higher than for its enantiomer.

TABLE 1: Differences of the Activation Parameters Between Enantiomers in Some Electron-Transfer Reactions Involving Metalloproteins.

Protein	Reagent	$\Delta\Delta H^{\#}_{\Delta-\Lambda}$ kJ mol^{-1}	$\Delta\Delta S^{\#}_{\Delta-\Lambda}$ J K^{-1} mol^{-1}	Ref
Plastocyanin	[Co^{II}(alamp)]	16.1	55	15
wt-Azurin	[Fe^{II}(promp)]	- 5.6	- 18	16
"	[Co^{II}(promp)]	- 7.6	- 24	16
Plant Ferredoxin	[Co^{III}(alamp)(py)]$^{+}$	- 11.8	- 38	6

As previously communicated (17), the system plastocyanin/[Co(L)] offers a unique possibility to verify the hypothesis which designates His-87 as a possible electron transfer site. As in the case of Cr^{2+}, which has also been used to identify reactive sites of metalloproteins (18,19), Co^{II} complexes become inert upon oxidation and should therefore, in an inner-sphere reaction, remain attached to the protein together with the optically active ligand, as illustrated in Fig. 1. In contrast to the reaction with the positively charged Cr^{2+} during which Cr^{3+} is bound to the negatively charged amino acid sequence 42 to 45 (18), the neutral

Co^{II} complexes react preferentially at the hydrophobic site. At the same time the copper(II) center is reduced to colorless copper(I) and the reaction product is easily identified by circular dichroism (CD) spectroscopy.

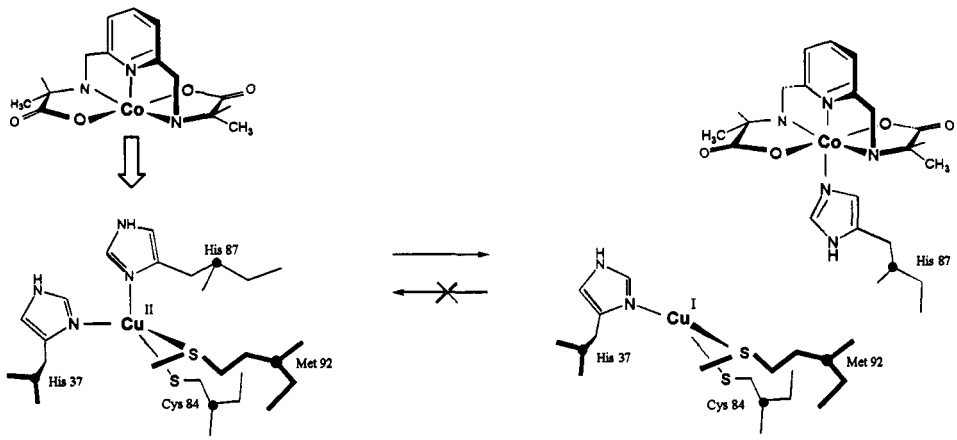


Fig. 1. Inner-sphere electron transfer between PcCu(II) and $[Co(L^1)]$ (Λ-(*S,S*)-isomer represented).

Quantitative determinations of the reaction products have been done with both enantiomers of $[Co(L^1)]$ and $[Co(L^2)]$, and in some selected cases as a function of pH and temperature. CD spectra were monitored during the reaction and the final spectra were determined taking into account the contribution of the unreacted Co^{II} species. After elimination of all the species not bound to the protein by extensive dialysis, the CD spectra were determined once again. Striking differences were observed between $[Co(L^1)]$ and $[Co(L^2)]$, as well as between the enantiomers of $[Co(L^1)]$. Whereas the aqua-Co^{III} complex is exclusively formed as the oxidation product with both enantiomers of $[Co(L^2)]$ and no metal complex remains attached to the protein, Λ-$[Co^{II}(S,S)$-$(L^1)]$ is quantitatively bound to the protein at pH > 6. With Δ-$[Co(R,R)$-(L^1) on the other hand, both, the inner- and the outer-sphere reactions take place simultaneously and after dialysis only a fraction of the formed Co^{III} complex remains bound to the protein. In a control experiment with the mixed ligand complex $[Co^{III}(alamp)(imidazole)]^+$ no hydrolysis was observed during one week at 40 °C at pH 5-7 (20).

TABLE 2: Amount (%) of Co^{III} Complexes Covalently Bound to Plastocyanin After Electron Transfer at Different Temperatures and pH (error limit ± 5%).

pH	T (°C)	Δ-[Co(alamp)]	Λ-[Co(alamp)]	Δ-[Co(promp)]	Λ-[Co(promp)]
5.5	20	13	80	---	---
6.0	20	21	95	---	---
7.0	10	35	---	----	---
	20	40	100	0	0
	30	48	---	---	---
	50	62	---	---	---
8.0	20	50	100	---	---

The results given in Table 2 show that in the case of Δ-[Co(alamp)] the amount of the protein-bound complex varies greatly with pH and with temperature, suggesting that the reaction at His-87 takes place by a bridging, deprotonated imidazole unit (Fig. 2), and that the activation enthalpy for the inner-sphere reaction is considerably higher than for the outer-sphere reaction which yields the aqua-complex.

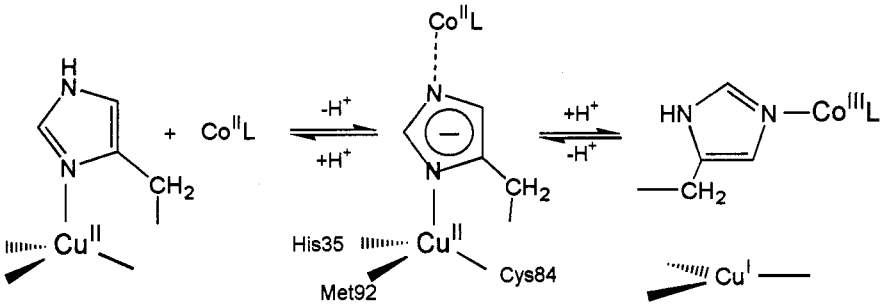


Fig. 2: Possible transition state for the inner-sphere electron transfer between Co^{II} complexes and plastocyanin.

From the temperature dependence of the overall reaction and the amount of the bound reaction product individual activation parameters can be calculated for both reaction pathways used by Δ-[Co(*R*,*R*)-(L^1)] giving $\Delta H^{\#}$ = 67.5 and 45.9 kJ mol^{-1} and $\Delta S^{\#}$ = -24 and -95 J K^{-1} mol^{-1} for the inner- and the outer-sphere reaction, respectively. Compared to the values obtained for Λ-[Co^{II}(*S*,*S*)-(L^1)] ($\Delta H^{\#}$ = 73.7 kJ mol^{-1}, $\Delta S^{\#}$ = + 5 J K^{-1} mol^{-1}), the inner-sphere reaction shows a stereoselectivity of $k_{\Lambda(IS)}/k_{\Delta(IS)} \cong 2.5$ at 20 °C. Interestingly, at this temperature the overall reaction rate is exactly the same (crossing Eyring plots) for both enantiomers. This is only possible if the corresponding outer-sphere reaction shows opposite stereoselectivity. **Therefore, the system PcCu(II)/[Co^{II}(alamp)] represents, to our knowledge, the first known case in which two enantiomers of the same electron-transfer reagent react with significant preference not only at two different reactive sites but also according to two different reaction mechanisms**. The existence of different reactive sites also explains the large difference in the activation parameters observed in this case.

The question then arises, whether the observation of such differences is a general indication of a selection of different reactive sites by enantiomeric reagents. This question can not yet be answered. Nevertheless, for one other case indicated in Table 1, preliminary measurements showed that for the reaction between wt-azurin and Δ-[Co(*R*,*R*)-(L^2)] or Λ-[Co^{II}(*S*,*S*)-(L^2)] at 30 °C, no detectable complex is fixed after electron transfer with the former, whereas 16 ± 2 % of the reagent remains bound to the protein with the latter (16). In this case again, differentiated activation parameters were determined giving $\Delta H^{\#}$ = 37 and 63 kJ mol^{-1} and $\Delta S^{\#}$ = -129 and - 57 J K^{-1} mol^{-1} for the outer- and the inner-sphere reaction respectively. Interestingly, the activation parameters for the outer-sphere reactions are similar for both enantiomers. The observed stereoselectivity is therefore mostly due to the inner-sphere reaction. Since the CD spectrum of the reoxidized solution, after deduction of the contribution due to the bound Co^{III} species, is superimposable to the initial spectrum of azurin, contrary to what is observed with plastocyanin, we tentatively suggest that the inner-sphere reaction between wt-azurine and Λ-[Co^{II}(*S*,*S*)-(L^2)] takes place at His-83 and not at His-117.

Mutant plastocyanines

For the inner-sphere electron transfer involving plastocyanin, the reactive site can be exactly located. On the other hand, for the outer-sphere reaction we only know from competitive experiments with highly charged, redox inactive cations that the site of attack is not located on the negatively charged area of the protein. Furthermore the question arises, whether or not the outer-sphere electron transfer with [Co^{II}(L^1)] and [Co^{II}(L^2)] occurs on the same site. This problem has been tackled using site directed mutagenesis. Mutations of plastocyanin were performed at the positions Leu-12 and Glu-68 and the following recombinant proteins were prepared: Leu12Val, Leu12Ala, Leu12Gly, Leu12Phe, Glu68Gly, and Leu12Ala Glu68Gly. With all these mutants, electron-transfer kinetics with both enantiomers of [Co^{II}(L^1)]

and $[Co^{II}(L^2)]$ have been measured as a function of temperature at pH 7. The results can be summarized as follows:

- With both enantiomers of $[Co^{II}(L^2)]$ the reaction rates and the stereoselectivities, as well as the activation parameters are, within error limits, almost the same for all mutants and correspond to the values obtained for the native protein. No complex remains bound to the protein after electron transfer.
- With $[Co^{II}(L^1)]$ significant differences appear. A clearly biphasic behavior is observed in the reaction with the mutants Leu12Ala, Glu68Gly, and Glu68Gly Leu12Ala and initial rates vary over a factor of about 20, all mutants reacting faster than the native protein except Leu12Phe. With the latter, the reaction is two times slower than with the native protein and both enantiomers react in exactly the same manner over the whole temperature range.
- The amount of Δ-$[Co^{III}(L^1)]$ bound to the protein gradually increases by shortening the side chain of the amino acid on position 12: wt 50%, Leu12Val 64%, Leu12Ala 75% and Leu12gly 100% (26 °C). At the same time the difference of the activation enthalpies $\Delta\Delta H^{\#}_{\Delta\text{-}\Lambda}$ becomes smaller.

All these results are consistent with the hypothesis that the outer-sphere reactions with $[Co^{II}(L^2)]$ and Δ-$[Co^{II}(L^1)]$ take place at different sites: near Leu-12 for $[Co^{II}(L^1)]$, but at a site sufficiently distant to leave mutations at this position exerting no influence on the electron-transfer rate for $[Co^{II}(L^2)]$. The special behavior of the mutant Leu12Phe might be due to a conformational change, the latter becoming rate limiting. Interestingly, the reaction with $[Co^{II}(L^2)]$ is two times faster, indicating that the reactive site used by this complex must be much less accessible for $[Co^{II}(L^1)]$.

A very surprising, completely reproducible behavior is observed with the two mutants containing the alanine residue at position 12. Absorption changes as a function of time of the recombinant protein Leu12Ala with both enantiomers of $[Co^{II}(L^1)]$ and $[Co^{II}(L^2)]$ are shown in Figures 3a and 3b, respectively. Whereas the whole curves of the reactions with Δ- and Λ-$[Co^{II}(L^2)]$ can be fitted by pseudo-first order kinetics, the above mentioned biphasic behavior is clearly observed with $[Co^{II}(L^1)]$, suggesting the existence of two different forms of the protein. Towards Λ-$[Co^{II}(L^1)]$, both forms are redox active and react with comparable rates. On the other hand, with Δ-$[Co(L^1)]$ the reaction rate is similar to that observed with its enantiomer up to about 60% of the reaction but then suddenly slows down. Apparently only one form of the protein is redox actif towards the Δ-$[Co(L^1)]$ enantiomer and the conversion of the inactive into the active form becomes the rate determining step for the second phase of the reaction. These results suggest again, as mentioned above for the Leu12Phe mutant, that the site used by $[Co^{II}(L^2)]$ is not, or considerably less, accessible for $[Co^{II}(L^1)]$.

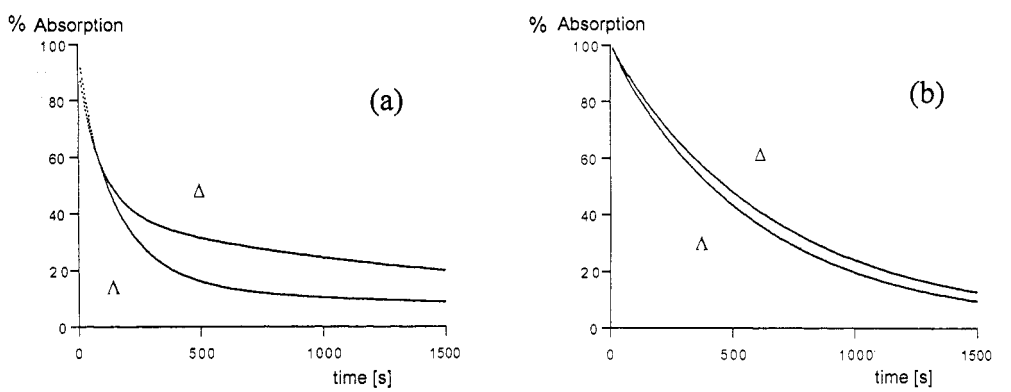


Fig 3. Absorption change of Leu12Ala mutant plastocyanin during the electron-transfer reaction with the enantiomers of $[Co^{II}(L^1)]$ (a) and $[Co^{II}(L^2)]$ (b). $[Co^{2+}] = 1{\cdot}10^{-3}$ M, [alamp] = [promp] = $1.2{\cdot}10^{-3}$ M, [PcCu] $\approx 1{\cdot}10^{-5}$ M, pH = 7,0 (phosphate); μ = 0,12 (NaCl); T = 25°C. (λ = 597 nm)

It is an interesting question to know where such an important difference in the general behavior of $[Co(L^1)]$ and $[Co(L^2)]$ comes from. We believe that, besides the difference in the redox potentials (21), this is mostly due to the ability to form hydrogen bonds by the coordinated N-H groups of $[Co^{II}(L^1)]$, a possibility which is absent in $[Co^{II}(L^2)]$. The predominant influence of hydrogen bonding in the formation

of transition states has been shown for the selection of pathways in the metal ion exchange of $[Cu^{II}(L^1)]$ and $[Cu^{II}(L^2)]$ with multidentate ligands (22).

Catalysis of electron transfer by small ligands

An important electron transfer rate enhancement between azurin or plastocyanin and the different Co^{II} complexes is observed in the presence of small monodentate ligands such as imidazole or *N*-substituted imidazoles. The values given for plastocyanin in Table 3 reveal an acceleration of the reactions with both, $[Co^{II}(L^1)]$ and $[Co^{II}(L^2)]$, by a factor of about 400-500 and show at the same time that substitution at the noncoordinated nitrogen atom of the imidazole has apparently no significant influence on the rate enhancement.

TABLE 3: Second Order Rate Constants, Stereoselectivity and Activation Parameters of the Electron Transfer Between Plastocyanin and Mixed-Ligand Complexes $[Co^{II}(L)(L')]$; (L' = H_2O, imidazole, 1-methylimidazole and 1-(*n*-butyl)imidazole; T = 25 °C; pH = 7.0; I = 0.12 (H_2O) , 0.5 (imidazoles)).

	alamp				promp			
L'	k^a $M^{-1}s^{-1}$	k_Δ/k_Λ	$\Delta H^{\#\,a}$ kJ mol^{-1}	$\Delta S^{\#\,a}$ J mol^{-1}K^{-1}	k^a $M^{-1}s^{-1}$	k_Δ/k_Λ	$\Delta H^{\#\,a}$ kJ mol^{-1}	$\Delta S^{\#\,a}$ J mol^{-1}K^{-1}
H_2O (Δ)	1.37[b]	0.73	67.5[c] 46.0[d]	- 24[c] - 95[d]	1.15	0.85	39.4	- 111
H_2O (Λ)	1.84		73.7	+ 5	1.36		39.7	- 107
imidazole	725	1.27	20.0	- 124	623	0.76	31.6	-88
1-Meim	607	1.27	25.8	- 105	557	0.63	33.8	- 81
1-n-Buim	863	1.20	21.7	- 118	553	0.87	33.8	- 79

[a] Mean values of rate constants, $\Delta H^{\#}$ and $\Delta S^{\#}$ of the Δ- and Λ-complexes with the three imidazole derivatives are given, [b] Global rate constant for inner- and outer-sphere reactions, [c] inner sphere, [d] outer sphere

Again, the complexes with the two pentadentate ligands exhibit a different behavior. Even if the acceleration is almost the same for both ligands, it is seen from the activation parameters that for alamp this is due to a dramatic decrease in the activation enthalpy which largely overcompensates the effect of the more negative activation entropy. With promp on the other hand, the mean decrease in the free activation energy of 15 kJ mol^{-1} is due, by nearly two thirds, to the less negative activation entropy.
The increase of electron-transfer rate induced by imidazoles seems too high to be explained by the more negative redox potentials of the mixed ligand complexes according to Marcus' theory (23). The existence of a more efficient electron-transfer pathway due to the presence of the imidazole moiety is more likely. The possibility of stacking interactions has been mentioned for the oxidation of PcCu(I) with complexes containing aromatic ligands such as pyridine, bipyridine or phenanthroline (24). On the other hand, the observation that N^1-substituted imidazoles react almost in the same way as imidazole itself contradicts this possibility.

Conclusions

From the above described results we conclude that the electron transfer between neutral, low molecular weight metal complexes at the hydrophobic area of plastocyanin can take place by an inner- or an outer-sphere mechanism and occurs at least at four different points on the protein surface. Similar results are obtained for the reduction of wt-azurin. The selection of the most favorable reactive site and of the reaction mechanism is the result of a subtle equilibrium between different energetic factors stabilizing the transition state such as the nature of the ligand, its ability to form hydrogen bridges, the presence of small, electron conducting ligands and the **chirality** of the reactive complex.

Furthermore, the results show that one has to be very careful in the interpretation of activation parameters. Several pathways, involving different reaction mechanisms, may be followed in parallel reactions. Thus, the observed global activation parameters reflect the temperature dependence of the formation of various precursor complexes, of the rates of several rate determining steps as well as of the relative importance of each of these steps involved in the global reaction. A sound comparison of global activation parameters is therefore not possible in such a case.

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