THE INTERACTION BETWEEN THE HEME c AND HEME d MOIETIES OF PSEUDOMONAS NITRITE REDUCTASE AS REVEALED BY MAGNETIC AND NATURAL CIRCULAR DICHROISM STUDIES

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SUMMARY

A possibility of a heme-heme interaction between the heme c and heme d moieties in Pseudomonas nitrite reductase was examined by using magnetic and natural circular dichroism. The MCD of the heme c moiety in the ferric enzyme was similar to that of mammalian ferricytochrome c in shape and intensity, whereas in the reduced state the MCD intensity was considerably smaller than that of ferrocytochrome c. When the heme d moiety was perturbed by the complex formation with CO, imidazole or cyanide as well as by pH changes, the depressed MCD was restored to the MCD level of mammalian ferrocytochrome c, accompanying conformational changes around the prosthetic groups. Thus, it was concluded that the heme-heme interaction exists only in the reduced enzyme and that this interaction is released under appropriate conditions.

Pseudomonas aeruginosa nitrite reductase contains heme c and heme d in a 1:1 ratio in a molecule of 70,000 daltons (1). This enzyme catalyzes the reduction of nitrite or molecular oxygen with ferrocytochrome c (551, P. aeruginosa) as an electron donor (2). In contrast to the oxygen reduction in which only heme d reacts with molecular oxygen forming an oxygenated intermediate (3), in the nitrite reduction the both hemes in Ps.NiR[a)] react simultaneously and directly with an electron acceptor NO^+ which is in equilibrium with nitrous ion in aqueous medium (4). This behavior strongly suggests that there exists

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[a)]Abbreviations: Ps.NiR, Pseudomonas aeruginosa nitrite reductase; CD, circular dichroism; MCD, magnetic circular dichroism.

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either a direct or indirect interaction through a polypeptide backbone between the two prosthetic heme moieties so as to affect their respective electronic states. In order to examine the nature of this interaction, the MCD, which is known to be sensitive to the heme electronic states, as well as the CD technique was employed in the present study.

MATERIALS AND METHODS

Ps.NiR was extracted from cells grown anaerobically in the presence of nitrate, according to the method of Horio et al. (5) and purified by chromatography on DEAE- and CM-cellulose. Finally the enzyme was crystallized from 10 mM Tris-HCl buffer (pH 7.5), and its concentration was determined spectrophotometrically taking a millimolar extinction coefficient of 30.2 at 549 nm for the reduced enzyme. The heme d moiety was depleted from the enzyme essentially according to the procedure of Yamanaka and Okunuki (6) and the protein moiety containing heme c and a small fraction of heme d was dissolved in 0.1 M dipotassium phosphate. The concentration was determined taking a millimolar extinction coefficient of 29.1 for pyridine ferrohemochrome prepared by dissolving the apoprotein in a mixture of 0.1 N NaOH and 10% (v/v) pyridine and reducing with sodium dithionite.

Absorption spectra were recorded on a Cary model 16 spectrophotometer equipped with accessories for automatic recording and a thermostatted cell holder. CD and MCD measurements were carried out on a JASCO MOE-1 spectropolarimeter using a cuvette of 10 mm light path. The magnetic field strength was 15 K gauss.

RESULTS AND DISCUSSION

Figure 1 illustrates the MCD spectra of Ps.NiR in the oxidized and reduced states. A small trough at 460 nm and an S-shaped spectrum with a zerocrossing at 634 nm of the oxidized enzyme originated from the heme d moiety, since these were lacking in the heme d-depleted enzyme. By reduction with sodium

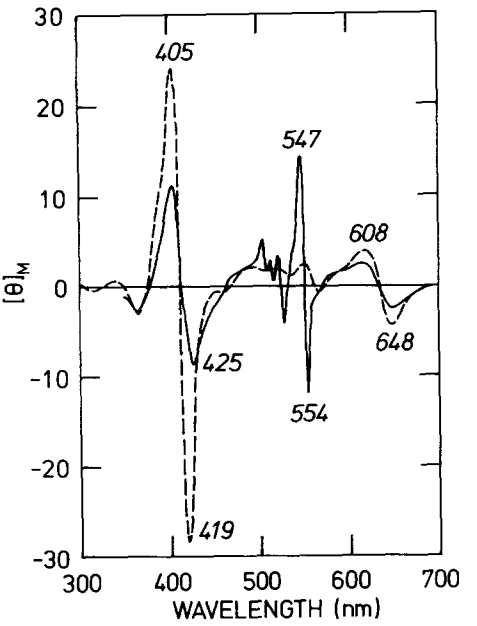

Fig. 1. MCD spectra of Pseudomonas nitrite reductase. Ps.NiR(4.47 μM) was dissolved in 0.1 M Tris-HCl, pH 7.93. -----, oxidized form; ———, reduced with sodium dithionite. $[\theta]_M$ is molar ellipticity per unit magnetic field strength.

dithionite, the S-shaped spectrum was intensified and the 460-nm trough became obscure. The shape of the MCD in the wavelength regions from 380 to 440 nm and from 480 to 560 nm was similar to that of mammalian cytochrome c (7) in the both redox states, respectively, thus being ascribed to the heme c moiety. However, the MCD intensity of the reduced enzyme was lower than that of ferrocytochrome c by a factor of 1/2 to 1/3 depending on wavelength, although with the oxidized enzyme the MCD was as large as that of ferricytochrome c.

When cyanide or imidazole was added to oxidized Ps.NiR, the peak-to-trough MCD intensity of the heme c Soret band increased maximally 30% without accompanying a change in shape. Since the Soret absorption band increased in intensity 10% at the same time, most of the increment in MCD would be accounted for

by the increased absorbance. A change of the MCD profiles in the visible region was less discernible. Upon addition of these ligands, the CD profiles did not change either except that a small trough appeared around 470 to 480 nm, a region for the heme d moiety. Therefore, there was no sign for an interaction between the two heme groups in oxidized Ps.NiR.

Carbon monoxide (8) and imidazole selectively attacked the heme d moiety in reduced Ps.NiR, altering its absorption bands as summarized in Table I. In the presence of either of these ligands, however, the MCD of its heme c moiety increased in intensity two-fold in the Soret region and three-fold in the visible region (Table I). By addition of cyanide the absorption bands of the both prosthetic groups were altered, and the MCD intensity of heme c was nearly doubled in the both spectral regions. The absorbance decrease of the heme c bands upon the cyanide complex formation may partly account for this relatively small extent of increase observed especially in the visible region. When reduced Ps.NiR was brought to pH 12.5, the heme c MCD was also intensified as in the case of CO or imidazole complex formation. On the contrary, although the MCD of the heme d-depleted enzyme was intensified in the visible region, it remained in the Soret region at the same intensity as for the native enzyme. A decreased absorption of the Soret peak would explain the latter result. Thus, only in the reduced enzyme, the heme c MCD increased in intensity to the magnitude comparable to that of mammalian ferrocytochrome c as the heme d moiety was perturbed by the complex formation, pH changes or even by its removal. This result indicates that the electronic states of heme c are strongly affected by those of heme d. The occurrence of such an interaction, however, does not necessarily imply that the two hemes are juxtaposed close to each other so as to yield the direct interaction between the transition moments of each heme.

Table I summarizes the Soret CD profiles for Ps.NiR and its derivatives in the reduced state. The native enzyme showed negative and positive extrema at 430 and 405 nm, respectively, with a shoulder at around 395 nm. Upon addition of CO, imidazole or cyanide, two negative extrema appeared, one lying at

Table I. Absorption, MCD and CD indices of Pseudomonas nitrite reductase in the reduced state.

Sample solutions were prepared as described in the legend to Fig. 1 except that necessary additions were made. The final pH values and ligand concentrations were as follows. Intact enzyme, pH 7.93; alkali, pH 12.5; cyanide, 50 mM, pH 8.12; imidazole, 50 mM, pH 7.67; CO, saturated, pH 7.93 and apoenzyme, pH 8.43.

Absorption spectra: $\lambda_{nm}(\varepsilon_{mM})$

Addition	intact	alkali	cyanide	imidazole	CO	apoenzyme
	417(181)	416(166)	412(161)	417(171)	416(174)	417(136)
	460(47.7)	460(40.3)	443(64.9)	460(49.2)		
	522(28.0)	521(20.6)	522(19.9)	522(23.2)	522(28.6)	523(18.4)
	549(30.2)	549(23.3)	548(19.7)	549(26.0)	550(32.9)	551(19.9)
	553(29.1)	553(25.0)	553(19.5)	554(25.5)	554(32.4)	554(19.9)
	656(17.4)	629(23.3)	630(24.6)	631(22.1)	655(14.8)	

MCD spectra: $\lambda_{nm}([\theta]_M)$

Addition	intact	lakali	cyanide	imidazole	CO	apoenzyme
	405(+11.3)	420(+21.5)	417(+23.3)	417(+24)	418(+23)	420(+11)
	413(0)	425(0)	425(0)	426(0)	425(0)	429(0)
	425(-9)	432(-9.5)	430(-9.6)	430(-10.7)	430(-10)	435(-7.4)
	547(+14.6)	551(+45)	547(+24)	551(+47)	549(+52)	557(+38)
	551(0)	555(0)	552(0)	555(0)	550(0)	562(0)
	554(-12.2)	557(-44)	555(-22)	558(-46)	555(-50)	565(-49)

CD spectra: $\lambda_{nm}([\theta] \times 10^{-4})$

Addition	intact	cyanide	imidazole	CO
	430(-5.5)	445(-26.8)	447(-16.8)	435(-6.3)
	412(0)	420(0)	422(0)	423(-2.3)
	405(+2.4)	413(+2.6)	415(+4.0)	
	392(0)	407(0)	410(0)	403(-12.7)

435-447 nm and the other at 403-395 nm. The former trough can be attributable to heme d and the latter to heme c. In each case, since the anisotropy factor $[\theta]_\lambda/\varepsilon_\lambda$ changed depending on wavelength, a possibility of a sole change in the orientation of the Soret transition moments in the plane of each heme was rendered less likely to explain the observed CD changes (see ref. 9). On the contrary, it is probable that conformational changes were induced around the heme c moiety, affecting its rotational strength even when only heme d was ligated with CO or imidazole. Changes occurred at or around heme d might have been transmitted to neiborhood of heme c through a polypeptide skelton. The induced Cotton effects became significant not only in the α- and β-bands of the heme c moiety but also in the spectral region for the heme d from 580 to 700 nm.

Thus, the present investigations revealed that the heme c and heme d moieties in a molecule of Ps.NiR interact each other only when the hemes are in the reduced state, although their closeness remained as a matter of further studies. This kind of interaction is in accord with the finding of Shimada and Orii that the reactions of the both ferrous hemes with NO^+ synchronized during the enzymic reduction of nitrite (4).

REFERENCES

1. Nagata, Y., Yamanaka, T., and Okunuki, K. (1970) Biochim. Biophys. Acta, 221, 668-671.
2. Yamanaka, T. (1964) Nature, 204, 253-255.
3. Shimada, H., and Orii, Y. (1976) J. Biochem, 80, 135-140.
4. Shimada, H., and Orii, Y. (1975) FEBS Lett, 54, 237-240.
5. Horio, T., Higashi, T., Yamanaka, T., Matsubara, H., and Okunuki, K. (1961) J. Biol. Chem, 236, 944-951.
6. Yamanaka, T., and Okunuki, K. (1963) Biochim. Biophys. Acta, 67, 407-416.
7. Vickery, L., Nozawa, T., Sauer, K. (1976) J. Am. Chem. Soc, 98, 351-357.
8. Yamanaka, T., and Okunuki, K. (1963) Biochim. Biophys. Acta, 67, 394-406.
9. Nicola, N. A., Minasian, E., Appleby, C. A., and Leach, S. J. (1975) Biochemistry, 14, 5141-5149.