Thiol-coordinated Heme Octapeptides of Cytochrome *c*; Model Compounds of Cytochrome P-450*

ABU JAFAR Md. SADEQUE, TORU SHIMIZU and MASAHIRO HATANO

Chemical Research Institute of Non-aqueous Solutions, Tohoku University, Katahira, Sendai 980, Japan

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Abstract

Thiol (alkylmercaptan) bindings to cytochrome *c* (cyt. *c*) and a heme octapeptide (H8PT) of cyt. *c* were studied in terms of optical absorption and magnetic circular dichroism (MCD) spectra. It was found that there is an intermediate state in the thiol binding to the H8PT and cyt. *c*. Association constants (K_a), nearly 10^3 M^{-1}, of alkylmercaptans to cyt. *c* (for making the intermediate complex) were similar to those (10^2–10^3 M^{-1}) of the H8PT (for making the intermediate complex). Addition of a large excess of the alkylmercaptans to the intermediate thiol–cyt. *c* complex freely reduced cyt. *c*. MCD spectrum of the thiol-coordinated complex of the H8PT was very similar to that of ferric low-spin cytochrome P-450 (P-450), suggesting that the thiol-coordinated H8PT has a heme structure similar to that of ferric low-spin P-450 even though the heme of the H8PT is covalently bound to the peptide in contrast to P-450. Furthermore, it was found that alkylmercaptans reduce the heme iron of cyt. *c* and modify the heme plane of the H8PT much more easily than those of metMb, indicating a difference in the reactivity of the heme iron and the heme plane against thiol between the H8PT (or cyt. *c*) and metMb (or P-450).

Introduction

Heme of cytochrome *c* (cyt. *c*) is covalently bound to the peptide and has internal His and Met as its axial ligands, forming a low-spin complex [1–3]. By adding cyanide anion or a nitrogenous ligand such as imidazole to cyt. *c*, Met is substituted for the exogenous ligand, again forming a low-spin complex with an exogenous axial ligand of cyanide or imidazole *trans* to the internal axial ligand, His [1–3]. Heme iron of cytochrome P-450 (P-450) is bound to the peptide only through coordination with thiol of Cys as an internal axial ligand [4]. Although an axial ligand *trans* to Cys of P-450 is vacant or water, the spin state of P-450 is low-spin [4, 5]. The electronic character of sulfur of Met is very different from that of Cys. Cyt. *c* is an electron-transfer heme protein, while P-450 utilizes oxygen to hydroxylize organic compounds. Thus, the structure of the heme environment of cyt. *c* is different from that of P-450.

Here we used cyt. *c* and a heme octapeptide of cyt. *c* (H8PT) as model compounds of P-450. Heme in the H8PT prepared from cyt. *c* by trypsin digestion is covalently linked to octapeptide and has only one axial imidazole (of His) ligand, the other axial site *trans* to imidazole being vacant. By using these compounds, we attempted to obtain cyt. *c* derivatives whose spectra would be very similar to those of P-450. Hitherto, interactions of cyt. *c* with exogenous axial ligands have been limited to nitrogenous ligands. Thus, interactions of thiol compounds with cyt. *c* and the H8PT are studied spectrometrically for the first time in this paper. It was found in this study that there is an intermediate state in the thiol binding to the H8PT and cyt. *c* and that a thiol-coordinated H8PT is very similar to ferric low-spin P-450 in terms of MCD spectra. We spectrometrically determined the association constants (K_a) of the thiol compounds to the H8PT and cyt. *c*. It was also found that thiol compounds reduce the heme iron of cyt. *c* and modify the heme plane of the H8PT much more easily than metMb [6].

Experimental

Horse heart cyt. *c* was purchased from Sigma. To oxidize the ferrous form of cyt. *c* mixed in the purchased batch, we used potassium ferricyanide. The H8PT was prepared from cyt. *c* by the method described previously [7]. Other reagents were of the highest guaranteed grade and were used without further purification. pH values were kept at 7.2 in 100 mM potassium phosphate buffer for cyt. *c* or in 50% ethylene glycol–100 mM potassium phosphate

*Abbreviations used are: cyt. *c*, cytochrome *c*; P-450, cytochrome P-450; H8PT, heme octapeptide prepared from cytochrome *c* by trypsin digestion; MCD, magnetic circular dichroism; K_a, association constant.

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buffer for the H8PT. To avoid aggregation of the H8PT in aqueous solutions, we added ethylene glycol [7–9].

Optical absorption spectra were obtained on a JASCO UNIDEC-510 digital-recording spectrophotometer using cuvettes with a 10 mm optical path at 20 °C. MCD spectra were obtained at 20 °C on a JASCO J-500C spectropolarimeter equipped with a JASCO electromagnet which produces longitudinal magnetic field up to 11.4 kG at the sample. Cells of 10 mm optical path were used for MCD measurements. The MCD magnitude was expressed by the molar ellipticity per Gauss ($[\theta]_M$ = deg cm^2 dmol^{-1} G^{-1}) on the basis of the molar concentration of the heme unit. Other general experimental conditions of MCD were: time constant 4 s; band width, 2 nm; scanning speed, 10 nm; sensitivity, 1 m°/cm.

Results

Optical Absorption Spectra

To obtain K_a values of methylmercaptan, ethylmercaptan and n-propylmercaptan to cyt. *c*, an optical titration study was done for cyt. *c* by adding these mercaptans. As observed in Fig. 1 (solid line), the Soret absorption band of cyt. *c* (5 μM) first decreased by adding mercaptan up to nearly 1 mM and formed clear isosbestic points at 350 nm and 475 nm. The absorption spectrum of this thiol-bound cyt. *c* had a Soret absorption maximum at 408 nm and visible absorption bands at 529 nm and 560 nm (lower part in Fig. 2). Further addition of mercaptan to the cyt. *c* solution resulted in deviation of the Soret spectral change (dotted line in Fig. 1), giving rise to the Soret absorption at 412 nm (broken line in Fig. 1). This spectral deviation from the isosbestic

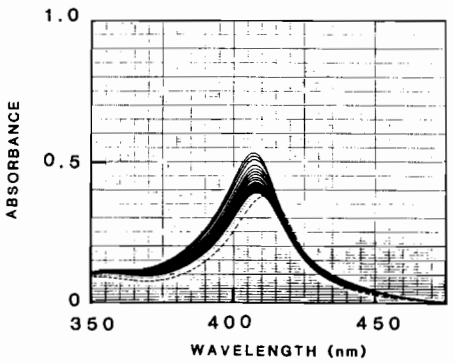

Fig. 1. Soret absorption spectral change of cyt. *c* (5 μM) caused by adding n-propylmercaptan. The solid lines indicate spectral changes to form the intermediate thiol-bound form by adding up to 1.08 mM n-propylmercaptan. The dotted and broken lines indicate deviations from the first spectral change by adding excess (more than 1.33 mM) n-propylmercaptan.

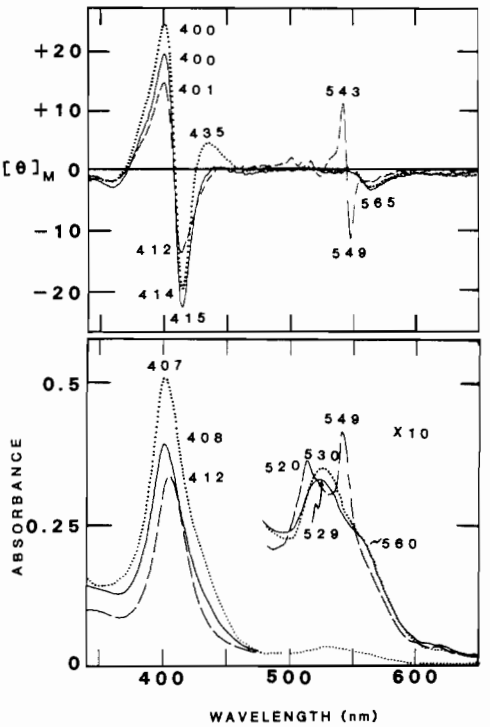

Fig. 2. MCD (upper) and absorption (lower) spectra of cyt. *c* (5 μM) (·····), the intermediate thiol-bound form of cyt. *c* (5 μM) in the presence of 1.08 mM n-propylmercaptan (——) and the reduced form of cyt. *c* (5 mM) in the presence of 1.33 mM n-propylmercaptan (- - - -).

points was concomitant with the appearance of new narrow absorption bands at 520 nm and 549 nm (lower part in Fig. 2), which will be ascribed to a ferrous low-spin type of cyt. *c*. Addition of a reducing reagent, sodium dithionite, to the cyt. *c* solution in the presence of excess mercaptan did not change the absorption spectral pattern at all, suggesting again that cyt. *c* was already reduced by the excess mercaptan. Additions of methylmercaptan, ethylmercaptan and n-propylmercaptan to the cyt. *c* solution resulted in essentially the same spectral changes. K_a values of these mercaptans to cyt. *c* are summarized in Table 1. We could not obtain exact K_a values for the second binding of mercaptans to the already thiol-bound cyt. *c*, which led to the reduction of cyt. *c*.

An optical titration study was also done for the H8PT by adding mercaptans in the same way as for cyt. *c* (Fig. 3). The Soret absorption magnitude of the H8PT was reduced by adding mercaptan together with forming two isosbestic points at 409 nm (arrow 1 in Fig. 3) and 470 nm. A saturation of this first spectral change was obtained by adding up to 4.91 mM methylmercaptan or up to about 0.50 mM ethylmercaptan or n-propylmercaptan. K_a values of this binding are described in Table 1. It was noted

TABLE I. Association Constants (K_a) (M^{-1}) of Mercaptan Compounds to Cyt. *c* and the H8PT Estimated from the Soret Absorption Spectral Change[a]

Thiol compounds		Cyt. *c*	H8PT
Methylmercaptan	K_a^1	1.3×10^3	1.1×10^2
	K_a^2	$<1.1 \times 10^3$	$<2.9 \times 10^2$
Ethylmercaptan	K_a^1	1.0×10^3	1.4×10^3
	K_a^2	$<7.1 \times 10^2$	$<1.3 \times 10^3$
n-Propylmercaptan	K_a^1	9.5×10^2	1.0×10^3
	K_a^2	$<7.5 \times 10^2$	$<1.3 \times 10^3$

[a] K_a^1 designates the first association constant for forming the intermediate complex while K_a^2 designates the second association constant for reducing cyt. *c* or forming the thiol–H8PT complex.

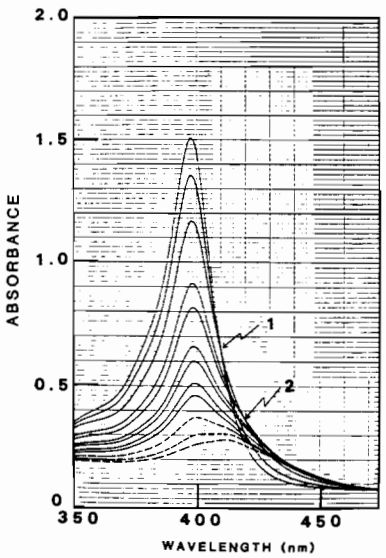

Fig. 3. Soret absorption spectral change of the H8PT (10 μM) caused by adding ethylmercaptan. The arrow 1 indicates the first isosbestic point to form the intermediate thiol-bound form complex by adding up to 0.50 mM ethylmercaptan. The arrow 2 indicates the second isosbestic point to form the second thiol-coordinated complex by adding up to 0.99 mM ethylmercaptan. The broken lines are indicative of modification of the heme ring of the H8PT by adding excess (more than 1.96 mM) ethylmercaptan.

that K_a of methylmercaptan is less than others by one order. These thiol-bound H8PT complexes had the Soret absorption peak at 398 nm and visible absorption peaks at 495 nm, 530 nm and 569 nm (lower part in Fig. 4). Further addition of mercaptan to this thiol–H8PT solution resulted in a deviation of the isosbestic points of the spectral change of the H8PT (arrow 2 in Fig. 3). Although the second spectral change did not form a clear isosbestic point, the apparent K_a for the second binding of mercaptan seemed to be less than 10^3 M^{-1}. The second thiol-coordinated H8PT had a Soret absorption peak

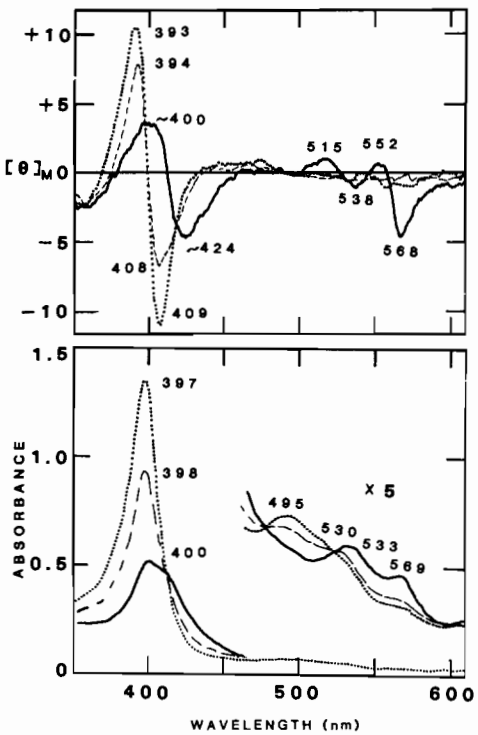

Fig. 4. MCD (upper) and absorption (lower) spectra of H8PT (10 μM) (·····), the intermediate thiol-bound form of H8PT (10 μM) in the presence of 4.91 mM methylmercaptan (- - - -) and the second thiol-coordinated form of H8PT (10 μM) in the presence of 6.55 mM methylmercaptan (——).

around 400 nm and visible absorption peaks around 533 nm and 569 nm (Fig. 4). Addition of more mercaptan (more than 1.96 mM ethyl- or n-propyl-mercaptan or more than 6.6 mM methylmercaptan) to the second thiol-coordinated H8PT remarkably reduced the absorption spectra of H8PT (broken lines in Fig. 3), suggesting that excess mercaptan modified the heme ring of the H8PT. The spectral changes observed were essentially the same for methylmercaptan, ethylmercaptan and n-propyl-mercaptan additions to the H8PT solution. No time-dependent change was observed during absorption spectral titration.

MCD Spectra

It is reported [6, 10, 11] that thiol coordination to metMb provides an MCD spectral pattern characteristic of the thiol coordination. To study the coordination structure of the thiol-coordinated cyt. *c* and H8PT, we obtained MCD spectra of those complexes whose absorption spectra were already shown in the preceding section. The dotted line in Fig. 2 (upper part) shows the MCD spectrum of cyt. *c* in the absence of mercaptan, which is of typical ferric low-spin heme iron [12]. The solid line and broken line in Fig. 2 (upper part) show MCD spectra of cyt. *c* in the presence of 1.08 mM and 1.33 mM n-propyl-

mercaptan, respectively. MCD pattern of cyt. *c* in the presence of 1.08 mM mercaptan was not very different from that of cyt. *c* in the absence of mercaptan. The MCD pattern of cyt. *c* in the presence of 1.33 mM is of typical ferrous low-spin heme iron [12], suggesting that cyt. *c* was reduced by n-propylmercaptan in accordance with the suggestion from the absorption spectral peaks at 520 nm and 549 nm (lower part in Fig. 2).

The MCD spectrum of H8PT (10 μM) in the absence of mercaptan is shown in Fig. 4 (·····). This MCD is of typical ferric high-spin form [12]. The MCD spectrum of the H8PT in the presence of 4.91 mM methylmercaptan is shown in upper part in Fig. 4 (- - - -). This MCD spectrum seems to be still of the ferric high-spin complex. The solid line in Fig. 4 shows MCD spectrum of the second thiol-coordinated H8PT complex. This MCD spectrum is very different from that of the H8PT itself in that the Soret MCD magnitude of the thiol-coordinated H8PT is smaller than that of the H8PT and the α-transition of the second thiol-coordinated H8PT is much larger than those of the H8PT. The MCD pattern of the second thiol-coordinated H8PT is quite similar to that of ferric low-spin P-450 [10]. It has been reported [6, 10, 13, 14] that the magnitude ratio of the Soret- *vs.* α-MCD bands of the ferric low-spin complexes sensitively reflects the axial coordination structure (Table II). The magnitude ratio of the Soret- *vs.* α-MCD bands for the second thiol-coordinated H8PT was 1.6, which is in the range (1.5–2.7) of low-spin P-450, but in contrast to that (7.8–15.1) of non-thiol coordinated ferric low-spin heme complexes (Table II).

TABLE II. Magnitude Ratios of the Soret- *vs.* α-MCD Bands

Ferric low-spin complexes	Ratios	References
H8PT-mercaptan	1.6	this work
P-450$_4$	1.5	14
P-450$_1$	1.8–1.9	14
P-450$_{scc}$	2.2	14
P-450$_{cam}$	2.7	13
P-450$_{scc}$-aminoglutethimide	4.8	14
P-450$_{cam}$-metyrapone	5.6	13
P-450$_{cam}$-pyridine	7.6	13
Cytochrome b_5	7.8	12, 14
metMb-imidazole	8.7	12, 14
metMb-CN	12.6	12, 14
Cytochrome *c*	13.9	12, 14

Discussion

It seems likely from absorption and MCD spectral findings that there is an intermediate state in the thiol binding to the H8PT and cyt. *c* before thiol is perfectly bound to the heme iron of the H8PT and cyt. *c*. It is known that the Soret MCD magnitudes of the thiol-bound low-spin heme iron are smaller than those of nitrogen-bound low-spin heme iron [6, 10, 11]. The Soret MCD magnitude of the intermediate complex of cyt. *c* was decidedly smaller than that of cyt. *c*, but did not show any peculiar MCD pattern characteristic of the thiol ligation [6, 10, 13, 14]. The Soret MCD magnitude of the intermediate H8PT complex was decidedly smaller than that of the H8PT itself, but the spin-state of the intermediate H8PT complex is still in the high-spin state. It is very difficult to determine the coordination structure of the intermediate H8PT and cyt. *c* complexes. There are two possibilities about the structure of the intermediate complexes:

(1) Thiol is partially bound to the heme iron only from one side for the intermediate H8PT or cyt. *c* complex. On the other hand, the thiol-coordinated H8PT complex has thiol axial ligands on both sides of the heme iron. Presence of excess thiol in the cyt. *c* solution reduces the heme iron concomitant with the thiol ligation to both the sides of the heme iron.

(2) Thiol partially saturates the heme ring, giving the smaller Soret MCD magnitude of the intermediate complex compared with that of the original complex.

We would favor the second possibility, because:

(1) The Soret CD bands (not shown) of the intermediate H8PT and cyt. *c* complexes, which are ascribed to interactions of the heme plane with neighbor aromatic amino acids [15], were almost the same as those of the original complex. If an axial ligand is exchanged for cyt. *c*, CD bands would be significantly changed [16].

(2) If thiol is bound to the heme iron of the H8PT (to a vacant site *trans* to His), the spin state would be changed from the high-spin to low-spin state. The intermediate H8PT complex is still in the high-spin state.

(3) If thiol is bound to the heme iron of cyt. *c* from the axial position *trans* to His, the intermediate cyt. *c* complex must have an MCD pattern characteristic of the thiol ligation [6, 10, 13, 14]. The MCD pattern of the intermediate cyt. *c* complex is still almost the same as that of cyt. *c*.

(4) It is reported [17] that the pyrrole β–β double bond of the heme in metMb and metHb is saturated by sulfur to form an episulfide bridge. This may be a partial reason why Soret MCD magnitudes of the thiol complexes of cyt. *c* and the H8PT are smaller than those of P-450.

It is interesting to note that addition of excess methylmercaptan, ethylmercaptan or n-propylmercaptan to metMb did not easily reduce the heme iron of metMb, and the thiol-coordinated metMb complexes were stable for more than 2 h at room

temperature [6]. This difference in the stability against thiol compounds between cyt. *c* (or H8PT) and metMb may be related to the fashion of the heme binding to the peptide in that the heme in cyt. *c* is covalently bound to the peptide, while that in metMb is ionically bound to the peptide. In addition, peptides surrounding the heme periphery may be related to the reduction of the heme iron by excess mercaptan.

Ferric low-spin P-450 has thiol on only one site of the heme iron, and the binding of a nitrogenous ligand *trans* to the internal thiol (–N–Fe–S– structure) gives rise to an MCD pattern different from the original ferric low-spin P-450 (Table II) [13, 14]. Thus, it is interesting to note that the MCD pattern of the thiol-coordinated H8PT (–N–Fe–S– structure) is quite similar to that of ferric low-spin P-450 (–O–Fe–S– structure).

In conclusion, we have shown here that: (1) there is an intermediate thiol-bound complex of the H8PT and cyt. *c*; (2) the ferric low-spin H8PT complex having a thiol ligand on one axial position *trans* to internal His gave an MCD spectrum quite similar to that of ferric low-spin P-450; (3) thiol compounds reduce the heme iron of cyt. *c* and modify the porphyrin ring of the H8PT more easily than those of metMb.

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