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Structural Studies of the Heme Domain of Sulfite Oxidase: CNBr Fragments†

Dennis R. Winge,‡ William M. Southerland,§ and K. V. Rajagopalan*

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ABSTRACT: The cytochrome b_5 type heme domain of rat liver sulfite oxidase, prepared by limited tryptic digestion of the intact enzyme, is a 10 000 molecular weight peptide containing a single methionine residue. Cyanogen bromide treatment permitted cleavage of the heme domain into 2 peptides containing 49 and 38 residues, representing the NH_2- and COOH-terminal segments of the intact peptide, respectively. The circular dichroism spectra indicated the virtual absence of secondary structure in the isolated CNBr fragments. Addition of hemin to either fragment resulted in the enhancement of the CD spectrum, and also increased the antigenic activity of the NH_2-terminal fragment toward antibodies prepared against native sulfite oxidase. The CD spectrum of heme complex of the COOH-terminal fragment was indicative of the formation of extensive α-helical structure. Mixing the two CNBr fragments in the presence of heme resulted in about 40% reconstitution of heme in a b_5-type configuration. The heme-reconstituted complex appeared to attain the secondary folding structure of the intact domain as detected by circular dichroism and immunological properties. The COOH-terminal peptide is composed of a long sequence rich in hydrophobic residues followed by an acidic sequence. Results of these structural studies are discussed in terms of homology with other b_5-type heme proteins and the interaction of sulfite oxidase with its physiological electron acceptor, cytochrome *c*.

Sulfite oxidase catalyzes the terminal reaction in the oxidative degradation of sulfur-containing amino acids and uses cytochrome *c* as its physiological electron acceptor. The enzyme has a dimeric molecular weight of 120 000 and contains molybdenum and heme as prosthetic groups (Cohen et al., 1971; Kessler & Rajagopalan, 1972). Recently, the subunit of the enzyme was demonstrated in our laboratory to be composed of two domains, one containing molybdenum and the other, heme (Johnson & Rajagopalan, 1977). The heme domain was shown to be the NH_2-terminal portion of the native enzyme and the molybdenum domain the carboxyl end. The cleavage of the enzyme by trypsin into domains did not affect either the structural or functional integrity of the domains. Thus, the molybdenum domain retained the ability to oxidize sulfite with ferricyanide as acceptor and had identical EPR properties to those of native sulfite oxidase. Within the heme domain, the structure which confers the cytochrome b_5 type absorption spectrum is preserved.

Two other cytochrome b_5 type proteins have been studied extensively. Microsomal cytochrome b_5 is a component of a multienzyme system which catalyzes the desaturation of fatty acids. The protein is composed of two distinct parts—a polar segment which binds the heme and a hydrophobic sequence which anchors the protein to the membrane (Spatz & Strittmatter, 1971; Strittmatter et al., 1972). The primary structure has been determined for the polar core from a variety of sources and the three-dimensional structure has been resolved for the bovine protein (Ozols & Strittmatter, 1969; Ozols, 1970; Nobrega & Ozols, 1971; Ozols et al., 1976; Matthews et al., 1972). A second well-studied b_5-type heme protein is the flavocytochrome b_2 from baker's yeast which catalyzes the oxidation of lactate to pyruvate. Digestion of the protein by trypsin releases a hemopeptide of 11 400 molecular weight (Labeyrie et al., 1966). The hemopeptides of microsomal b_5 and yeast b_2 exhibit substantial homology, suggesting that these proteins may have been derived from a common ancestral gene (Guiard & Lederer, 1976).

Recently Guiard & Lederer (1977) reported that a 34 residue NH_2-terminal sequence of the hemopeptide of chicken liver sulfite oxidase displayed sequence similarities to corre-

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sponding regions of cytochrome b_5 and flavocytochrome b_2 implicating a common evolutionary background for the heme domains of all proteins containing the b_5-type structure. As part of an effort to investigate this relationship, we present in this report studies on the CNBr-digested heme domain from rat liver sulfite oxidase.

Sulfite oxidase is a natural cytochrome *c* reductase, since the latter is its physiological electron acceptor. Many studies have been carried out to investigate the interaction of cytochrome *c* with membrane-bound oxidoreductases. Sulfite oxidase is a soluble enzyme, and thus represents the ideal system for studying the interaction between cytochrome *c* and its specific reductase. Structural studies on the heme domain of sulfite oxidase should reveal features, such as asymmetry of charges, which may be important in the interaction with cytochrome *c*.

Materials and Methods

Sulfite oxidase was purified from fresh rat livers to yield an A_{413nm}/A_{280nm} ratio of greater than 0.9 as described previously (Johnson & Rajagopalan, 1976). The buffers used in the enzyme purification contained 0.1 mM phenylmethanesulfonyl fluoride ($PhCH_2SO_2F$).[1] The heme domain was prepared by proteolytic digestion of the native enzyme using Tos-$PheCH_2Cl$-trypsin, and subsequent separation of the cleavage products by gel filtration (Johnson & Rajagopalan, 1977). The animals used in the enzyme isolation were CD strain rats from Charles River and were fed commercial rat chow ad libitum.

Trypsin (Tos-$PheCH_2Cl$-treated) and carboxypeptidases A and B ($PhCH_2SO_2F$-treated) were obtained from Worthington, pyroglutamate aminopeptidase was from Boehringer Mannheim, hemin and cyanogen bromide were from Eastman, and ultrex hydrochloride and pyridine were from J.T. Baker. Reagents used in automated sequencing were from Beckman. Cellulose thin-layer chromatography plates were purchased from Brinkman, polyamide layer sheets from Pierce, dansyl chloride and dansyl amino acids from Sigma, and Sephadex gels from Pharmacia.

For heme removal, the b_5 heme domain was acidified to pH 1.5 with HCl and chromatographed on Sephadex G-25. The resulting preparation (typically 200 nmol) was degraded with a 300 molar excess of CNBr in 5 mL of 70% formic acid. Following incubation at 25 °C for 24 h the sample was diluted tenfold with deionized water and lyophilized twice to remove the volatile cyanogen bromide degradation products.

Peptides resulting from CNBr cleavage were routinely separated on Sephadex G-50 (2.5 × 82 cm) equilibrated and eluted with deaerated 50% acetic acid. Column eluates were monitored for ninhydrin reactivity in a modular technicon autoanalyzer system as described by Herman & Vanaman (1975).

Peptides were hydrolyzed in 6 N HCl containing 0.1% phenol and analyzed on a Beckman 120 C amino acid analyzer. Tryptophan was quantitated on the amino acid analyzer after acid hydrolysis in the presence of 4% thioglycolic acid as described by Matsubara & Sasaki (1969). Amino acids released by digestion with carboxypeptidase A and B according to the procedure of Ambler (1972) were identified by amino acid analysis. Carboxypeptidase A was initially washed free of contaminating amino acids, whereas carboxypeptidase B was used directly.

The purity of the peptides was assessed by ascending chromatography on thin layer cellulose plates (20 × 20 cm) and N-terminal amino acid analysis by dansylation. The solvent system used for TLC chromatography was pyridine/butanol/acetic acid/water (10:15:3:12 by volume). Peptides were identified by spraying the plates with 0.2% ninhydrin in 95% acetone. Dansylation was achieved in 0.2 M sodium bicarbonate according to the procedure of Gray (1967). The dansyl amino acids were identified by two-dimensional thin-layer chromatography on polyamide sheets using the following solvent systems: (solvent 1), 1.5% formic acid; (solvent 2) benzene–acetic acid (9:1 by volume); and (solvent 3) ethyl acetate–methanol–acetic acid (20:1:1 by volume).

Visible and ultraviolet absorption spectra were recorded on a Perkin-Elmer 575 spectrophotometer. Circular dichroism (CD) spectra were obtained with a Rousse! Jouan Dicrographie III. Ellipticities were calculated on a molar or mean residue basis. Atomic absorption spectroscopy was carried out on a Perkin-Elmer 107 spectrophotometer equipped with a heated graphite furnace (HGA-2000). Tryptophan fluorescence was recorded on an Aminco-Bowman fluorimeter.

A Model E Beckman analytical ultracentrifuge equipped with ultraviolet absorption optics was used for sedimentation equilibrium experiments. Acrylamide gel electrophoresis at pH 8.9 was carried out according to the procedure of Davis (1964). The electrophoretic procedure of Hedrick & Smith (1968) was used to determine the dependence of polypeptide mobility on acrylamide concentrations ranging from 6 to 12%. Antibodies to rat liver sulfite oxidase were elicited and isolated as described previously (Johnson & Rajagopalan, 1977).

Amino acid sequence analysis was performed by automatic Edman degradation on a Beckman 890 C sequencer using a 0.1 M Quadrol peptide program (011576) with a combined benzene and ethyl acetate wash. The phenylthiohydantoins were identified by gas chromatography using a Beckman gas chromatograph and by amino acid analysis following back hydrolysis under N_2 at 130 °C with 6 N HCl containing 1.0 mM β-mercaptoethanol and 0.1% phenol.

Results

Isolation and Characterization of CNBr Fragments. The 10 000 molecular weight heme domain (Tp-Hmf) generated by trypsin proteolysis of rat liver sulfite oxidase contains a single methionine and has been purified to homogeneity. Incubation of heme-free Tp-Hmf with CNBr may be expected to yield two polypeptide fragments with the NH_2-terminal peptide having a homoserine/homoserine lactone as the COOH-terminal residue. Figure 1 shows a typical elution profile of the cleavage products from a Sephadex G-50 column as monitored by a Technicon autoanalyzer. The three fractions were pooled separately and evaporated to dryness. Chromatography of the three samples (5 nmol) on cellulose thin-layer plates revealed two ninhydrin reactive peptides in peak I with R_f values of 0 and 0.7. Peak II showed only a ninhydrin positive spot at the origin, whereas peak III showed a single ninhydrin stained spot at R_f of 0.7. NH_2-terminal analysis by dansylation demonstrated a weak Glx spot in peak II and a leucine in peak III. Dansylation of peak I was inconclusive and extensive studies were precluded due to lack of material in this fraction. The three fractions (10–15 nmol) were digested with a mixture of carboxypeptidase A and B for 3 h at 37 °C in 0.2 M *N*-ethylmorpholine. The enzyme to substrate ratio was 1:25. At the end of the incubation period, the samples including blanks

[1] Abbreviations used: $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride; Tos-$PheCH_2Cl$, L-1-tosylamido-2-phenylethyl chloromethyl ketone; Tp-Hmf, the b_5 heme containing peptide of sulfite oxidase generated by trypsin; CNBr, cyanogen bromide; Glx, pyroglutamate, pyroglutamine.

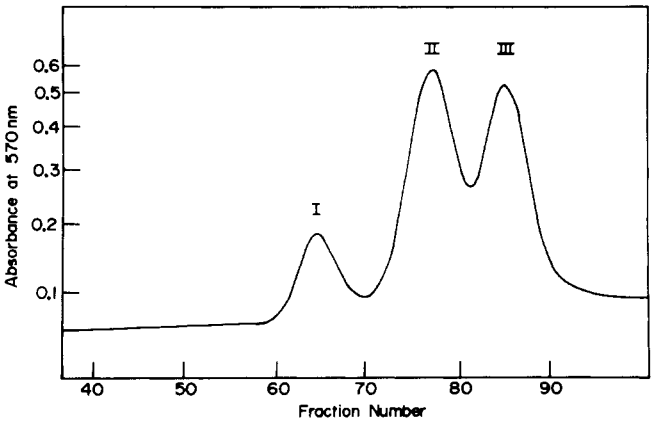

FIGURE 1: Gel filtration of CNBr digested heme domain of sulfite oxidase. The trace shown was obtained with the CNBr peptides from 200 nmol of dehemed Tp-Hmf. A portion of the column effluent (approximately 2%) was continuously removed and monitored by reaction with ninhydrin following alkaline hydrolysis. Separation was achieved on a Sephadex G-50 column (80 × 2.5 cm) equilibrated with 50% acetic acid. The column was operated at 25 °C with a flow rate of 30 mL/h. Fractions of 3 mL were collected.

TABLE I: Amino Acid Composition of CNBr Peptides of the Heme Domain from Sulfite Oxidase.[a]

Residue	Residues/molecule		
	Peptide II	Peptide III	Tp-Hmf
Lys	2.9 (3)	1.0 (1)	5
His	2.1 (2)	1.7 (2)	4
Arg	2.2 (2)	1.7 (2)	4
Asp	4.8 (5)	3.1 (3)	9
Thr	2.6 (3)	0.4 (0)	3
Ser	4.1 (4)	0.8 (1)	6
Glu	5.2 (5)	5.7 (6)	12
Pro	2.6 (3)	3.5 (4)	7
Gly	4.1 (4)	3.1 (3)	8
Ala	0.9 (1)	4.3 (4)	6
Val	5.5 (6)	1.9 (2)	8
Met[b]	(1)		1
Ile	0.9 (1)	1.0 (1)	2
Leu	4.3 (5)	5.4 (5)	10
Tyr	1.0 (1)	1.8 (2)	3
Phe	1.8 (2)	1.0 (1)	3
Trp[c]	0.6 (1)	0.9 (1)	2

[a] Values represent averages of 24-h hydrolysates from two different preparations; values in parentheses are assumed residues per mole. [b] Peptide II contains homoserine, so the single methionine is localized in this peptide. [c] Presence of tryptophan verified by fluorescence measurements.

were acidified and lyophilized. The released amino acids were analyzed and the contribution of amino acids from carboxypeptidase autodigestion was subtracted out. In peaks I and III the only significant release was arginine. In both fractions approximately 0.7 mol of Arg per mol of peptide was released in the 3-h digestion period. In peak II 0.41 and 0.46 mol of Lys and Leu respectively were released. Homoserine/homoserine lactone were also apparent, but quantitation was not possible. Tp-Hmf was found to have NH_2-terminal Glx and COOH-terminal Arg. Carboxypeptidase A,B released 0.7–0.9 residue of arginine from Tp-Hmf in a 90-min digestion period. These results demonstrate that peak II and peak III are the NH_2-terminal and COOH-terminal peptides of Tp-Hmf, respectively. These peptides will consequently be referred to as CNBr peptides II and III.

The CNBr fragments were characterized by amino acid

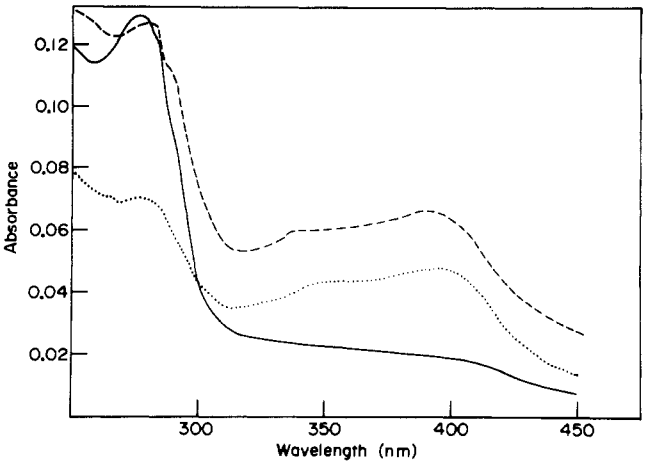

FIGURE 2: Absorption spectra of the CNBr peptides. Each peptide was dissolved in 0.2 M $NaHCO_3$. The dotted line represents peptide I at a concentration of 5 nmol/mL. The dashed and solid lines represent peptides II and III respectively at concentrations of 12 nmol/mL.

analysis. As can be seen in Table I the summation of amino acids in peptides II and III compares well with the composition of intact Tp-Hmf. Deviations from additivity are few and may be due to experimental error or conceivably to a small peptide hydrolyzed at an acid labile bond. Cases are known in which the 70% formic acid used in CNBr digestion is itself sufficient to hydrolyze peptide bonds adjacent to aspartyl residues (Kojima et al., 1976). Of the amino acids detected, peptide II contains about 49 residues and would have a molecular weight of about 5500. Peptide III has approximately 38 residues which would correspond to a molecular weight of about 4200. Thus, CNBr peptide II represents approximately the N-terminal 60% of Tp-Hmf.

The amino acid composition of peak I from Figure 1 revealed that it is a heterogeneous composite of CNBr cleaved and uncleaved Tp-Hmf molecules. The presence of 0.2 residue of methionine suggests that 20% of the molecules are uncleaved. The observations that peak A contains homoserine/homoserine lactone and that traces of fragment II and III are apparent on thin-layer chromatograms indicate that peptides II and III are eluting in peak I. Their coelution in a volume where undegraded Tp-Hmf fractionates suggests that a complex of peptides II and III exists. A complex may result or may be stabilized by the presence of heme. Absorption spectroscopy showed absorption near 400 nm suggesting that some heme was present in peak I (Figure 2). This was substantiated by iron analysis in which 0.5 mol of Fe/mol of peptide was found in peak I.

The NH_2-terminal residue of peptide III observed by dansylation was verified by Edman degradation. Nineteen cycles of degradation were completed on 120 nmol of CNBr peptide III with the results shown in Table II. As can be seen, the sequence contains predominantly hydrophobic residues. The sequence of the first half of peptide III has a polarity index calculated according to Fisher (1964) of 26%, whereas the second half of the molecule has an index of 60%. Thus, it appears that there is a cluster of charged residues in the COOH-terminal region of Tp-Hmf. Sequence analyses on the CNBr peptide II and intact Tp-Hmf were attempted, but the apparent presence of pyroglutamine (pyro-Glx) as the amino terminus precluded degradation. Treatment of peptide II with pyroglutamate aminopeptidase did not successfully remove the terminal Glx.

Heme Reconstituted CNBr Fragments. Intact Tp-Hmf molecules bind heme reversibly analogous to microsomal b_5

TABLE II: Sequencer Analysis of 120 Nanomoles of CNBr Peptide III.

Cycle	Amino acid identified[a]	Yield[b] (nmol) GC	AAA
1	Leu	70	45
2	Ala	65	47
3	Ala	57	39
4	Gly	23	33
5	Gly	11	21
6	Pro	9	
7	Leu	22	16
8	Glu		16
9	Pro	6	
10	Phe	15	14
11	Trp	8	
12	Ala	11	12
13	Leu	9	9
14	Tyr	7	6
15	Ala	11	8
16	Val	10	9
17	His		2
18	Asx		5
19	Glu		5

[a] Each of the first sixteen residues was identified by both gas chromatography (GC) and amino acid analysis (AAA) following back hydrolysis. [b] Blanks indicates cases where quantitation was precluded or too difficult to accurately measure.

(Strittmatter & Velick, 1956). Apo Tp-Hmf molecules can be reconstituted 70% by the addition of free heme, and the resulting heme-containing molecules exhibit native-like oxidized and reduced absorption spectra.[2] It was of interest to determine whether the two CNBr peptides retained the ability to bind heme. As can be seen in Figure 2, peptide II and to a lesser extent peptide III exhibit visible light absorption in the region around 400 nm. Iron analysis revealed less than 0.15 mol of Fe/mol of peptide for both CNBr fragments II and III. Thus, some residual heme (0.15 mol of heme/mol of peptide) remained associated with each peptide during gel filtration in 50% acetic acid. For heme reconstitution studies, a stock solution of hemin was prepared in 0.1 N sodium hydroxide and dilutions were made in 0.1 M potassium phosphate, pH 7.0. Aliquots of buffered hemin were added to known concentrations of CNBr peptides II and III, and binding was determined by visible spectroscopy. Additions were continued until the presence of free heme shifted the absorption band of the solution from 413 nm toward the peak of free heme, 390 nm. The A_{413nm} should increase linearly until a point is reached at which heme concentration exceeds the binding capacity. Subsequently, the rise in absorption at 413 nm should show a break as free heme begins to accumulate. The intersection of the two linear segments is a measure of the heme binding capacity. Figure 3 shows the A_{413nm} increase as a function of added heme for both peptides II and III. Results from two different preparations of CNBr fragments showed that very little binding occurred in each peptide. The heme titrated fragments were reduced with sodium dithionite and in each case a spectrum similar to native Tp-Hmf was generated (Figure 4). Assuming that the extinction coefficient at 556 nm is the same for reduced hemopeptides II and III as for reduced Tp-Hmf, the CNBr peptides were calculated to bind only approximately 0.1–0.15 mol of additional heme. The two fragments retain the ability to bind heme, but the efficiency is low.

An equimolar mixture of peptides II and III was similarly

[2] Johnson, J. L., & Rajagopalan, K. V., unpublished observations.

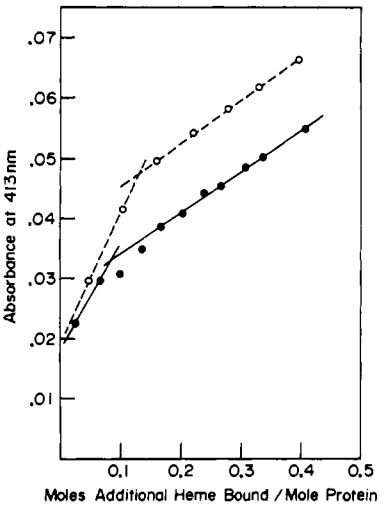

FIGURE 3: Heme binding by the individual CNBr peptides. Each peptide was titrated with heme as described in the text. Titrations were conducted at 25 °C in 0.1 M potassium phosphate, pH 7.0. The solid line represents the titration of peptide II at a concentration of 4.5 nmol/mL. The dashed line represents the titration of peptide III at a concentration of 9 nmol/mL.

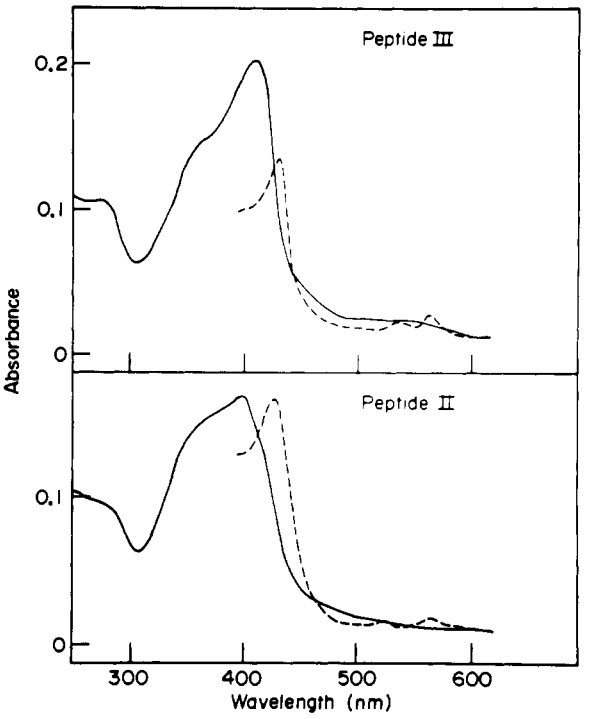

FIGURE 4: Absorption spectra of heme-reconstituted peptides II and III. The spectra were recorded at 25 °C in 0.1 M potassium phosphate, pH 7.0. The solid lines represent the oxidized heme peptides, whereas the dashed lines represent spectra of sodium dithionite reduced samples. The spectra were obtained from 4.5 nmol of peptide and heme.

titrated with hemin. As can be seen in Figure 5, the initial rate in the A_{413nm} increase plateaued after an increase in binding of 0.36 mol of heme per mol of polypeptide. The spectra of the oxidized and dithionite-reduced heme reconstituted complex of II + III are shown in Figure 6. The reduced spectrum is similar to that of dithionite-reduced Tp-Hmf and sulfite-reduced sulfite oxidase. Calculations based on the extinction coefficients at 556 nm and 413 nm for native sulfite oxidase suggest that 0.43 and 0.5 mol of heme, respectively, are bound per mol of complex. These quantities represent the total heme content which includes the small amount bound to the isolated

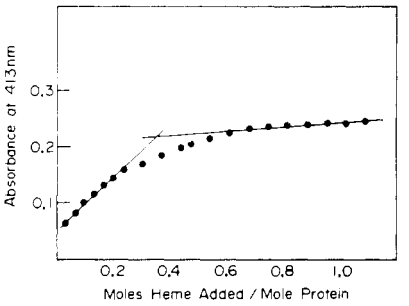

FIGURE 5: Heme reconstitution of a mixture of CNBr peptides II and III. Titrations were carried out as described in text and Figure 3. The concentration of each peptide was 4.5 nmol/mL in 0.1 M potassium phosphate, pH 7.0.

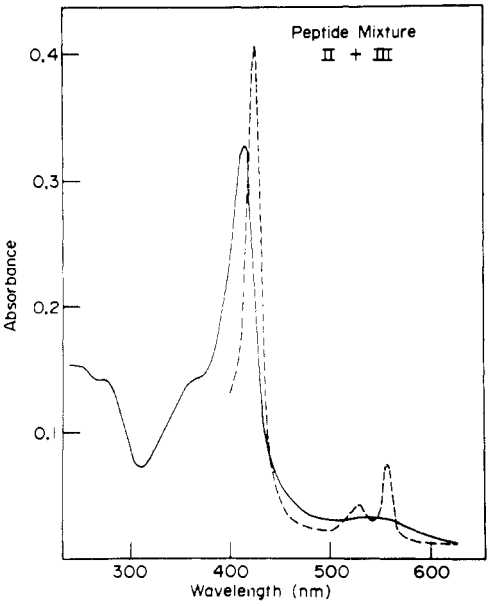

FIGURE 6: Absorption spectra of the oxidized and reduced heme-reconstituted peptide mixture. Spectra were obtained for an equal molar mixture of heme and peptides II + III. The concentration was 4.5 nmol/mL in 0.1 M potassium phosphate, pH 7.0. The solid line is the oxidized samples, and the dashed line is the sodium dithionite reduced spectrum.

peptides. Free heme would contribute more to the absorption at 413 nm than to that at 556 nm, so the results based on the 556-nm data may be more accurate. Attempts to achieve a more stoichiometric binding of heme by either slow dilution of peptides II + III and heme in 6 M guanidine hydrochloride or slow neutralization of the mixture at pH 4.5 were unsuccessful. The 0.4 mol of heme bound to the peptide mixture remained bound after either dialysis or gel filtration.

Circular Dichroism. The fragments derived from CNBr digestion were analyzed for their ability to absorb circularly polarized light. The intact heme domain has a significant amount of α-helical secondary structure as will be described in a subsequent report.[3] Removal of the heme markedly reduces the helical structure (Figure 7). Circular dichroic studies of the CNBr peptides showed that in the absence of heme neither fragment had any appreciable periodic structural characteristics, but in the presence of heme α helix was reconstituted in peptide III with only slight changes apparent in peptide II. Addition of heme to a mixture of peptides II + III resulted in slightly higher molar ellipticities compared to the

[3] Southerland, W. M., Winge, D. R., & Rajagopalan, K. V., unpublished observations.

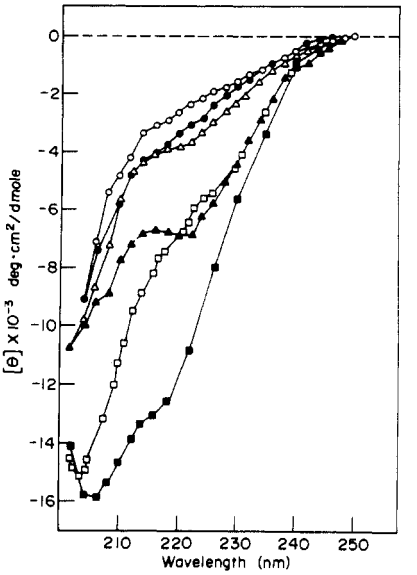

FIGURE 7: Circular dichroism of CNBr peptides in the presence and absence of heme. Spectra shown are mean residue ellipticities. Open symbol lines indicate samples in the absence of heme, and closed symbol lines are samples in the presence of heme. The spectra represent the following: (●) peptide II at a concentration of 15.8 nmol/mL; (▲) peptide III at a concentration of 15.8 nmol/mL; and (■) native heme domain from sulfite oxidase at a concentration of 9 nmol/mL. Spectra were recorded at 0.4 M potassium phosphate, pH 7.0.

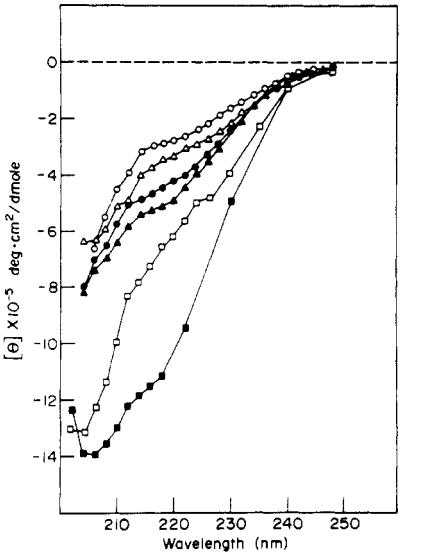

FIGURE 8: Molar ellipticities of CNBr peptides in presence and absence of heme. Circular dichroism spectra presented in Figure 7 were recalculated to yield molar ellipticities. Open symbol lines indicate sample in the absence of heme, and closed symbol lines are samples in the presence of heme. The spectra represent the following: (●) calculated summation of spectra of peptide II and III; (▲) heme-reconstituted peptide II, III complex; and (■) intact Tp-Hmf.

summation of spectra of the individual heme-containing fragments (Figure 8). Since heme binding is more efficient in the peptide mixture than in the isolated peptides, a greater appearance of secondary structure in the complex is not surprising. The ellipticities seen in the heme-reconstituted peptide mixture are approximately 40% of those calculated for the intact cytochrome b_5 domain. This agrees well with the heme-binding data which reveals that only 40% of the molecules bind heme. As can be seen in Figure 8, the summation of ellipticities of the individual peptides in the absence of heme

TABLE III: Protection by CNBr Fragments of the Antibody Inhibition of Sulfite Oxidase.[a]

Sample	Concn (nM)	Protection (%)
Native Tp-Hmf	0.036	100
II + III	0.036	8
	0.36	91
II + III + heme	0.036	43
	0.09	100
	0.36	100
II	0.36	21
II + heme	0.36	84
III	0.36	11
III + heme	0.36	43

[a] Sulfite oxidase was titrated with antibody such that 90% of the enzymatic activity was inhibited. The incubation time required for the mentioned inhibition was 15 min. Samples tested for protection against this inhibition were initially preincubated with the antibody for 15 min prior to the addition of sulfite oxidase. Incubations were carried out in 0.05 M potassium phosphate, pH 7.0, and assays were conducted in 0.1 M Tris-HCl, pH 8.5, containing 0.1 mM EDTA.

does not approximate the ellipticities observed for the apo-Tp-Hmf domain. This discrepancy is intriguing, but cannot be rigorously explained by the present knowledge of circular dichroism. Both the peptide mixture II + III and apo-Tp-Hmf molecules have similar dichroic spectra when dissolved in 6 M guanidine hydrochloride. Qualitatively, comparison of spectral line shapes indicates that heme regenerates native-like secondary structure in the CNBr peptide mixture with the majority of structure reconstitution occurring in peptide III. Considering that less than 20% of the peptide III molecules bind heme in a cytochrome b_5 configuration, the heme-containing molecules may be predominantly folded in α helices.

Antibody Reactivity. Antibodies to rat liver sulfite oxidase are known to inhibit the enzyme catalyzed reduction of cytochrome *c* by sulfite (Johnson & Rajagopalan, 1976). Preincubation of the antibody with Tp-Hmf eliminates the inhibition, suggesting that the heme domain contains at least one critical antigenic determinant.[2] The CNBr peptides were tested to determine whether they possessed any reactivity toward the antibody population. The peptides were preincubated with partially purified antibody for 15 min at 25 °C. A slight excess of sulfite oxidase with respect to the antibody was added and the incubation continued for an additional 15 min. The enzymatic activity was then assayed and the results are shown in Table III. When assayed at the same concentration as intact cytochrome b_5, the heme-reconstituted peptide mixture was the only sample which gave partial relief of the antibody inhibition. Complete protection was apparent when the heme-peptide II + III complex was present at 2.5-fold higher concentration. The individual peptides gave significant protection only in the presence of heme and at high peptide concentrations. No protection of antibody inhibition was observed with heme alone. Peptide II possessed a greater reactivity toward the antibody than did peptide III suggesting that fragment II possesses a substantial part of the antigenicity. The structure of peptide III presumably has an additional determinant or contains a portion of the antigenic site which resides mainly in peptide II.

Formation of Complex by CNBr Peptides II + III. The protection against antibody inhibition observed in the heme-reconstituted peptide mixture was markedly greater than the additivity of results from individual fragments suggesting that peptides II and III form a complex. In the absence of heme the somewhat greater protection observed in the peptide mixture when compared with the summation of individual fragment data may be attributed to a small degree of heme–peptide complex formation arising from intrinsic heme. Alternatively, the possibility of a peptide complex in the absence of heme cannot be completely ruled out. The existence of a complex of peptides II and III is suggested by the antibody data, circular dichroism, heme reconstitution, and the coelution of the fragments in peak I of the gel filtration in Figure 1. A complex should exhibit a molecular weight of 10 000 which is the weight of the native cytochrome b_5 fragment. To test this prediction, the heme-reconstituted mixture was subjected to equilibrium ultracentrifugation. One species of 10 000 molecular weight was apparent in addition to species of higher molecular weight. The optical density in the cell was monitored at 413 nm, so the weights represent only heme-containing species. The identity of the aggregated species is unresolved.

The peptides in the presence and absence of heme were analyzed by polyacrylamide gel electrophoresis to determine whether a complex could be visualized. Gels containing the heme-reconstituted complex revealed no differences from the summation of Coomassie blue stained bands of the individual peptides. Electrophoresis did show that peptide II resolved into two bands, whereas peptide III moved as a single species with a greater mobility than either of the two peptide II bands. Each peptide appeared free of contamination from the other. The two bands of peptide II could arise from different aggregation states or net charge. Gel electrophoresis according to the procedure of Hedrick & Smith (1968) suggested that the multiple bands were due to different charged species. The intact cytochrome b_5 fragment also shows on gel electrophoresis two heme–protein bands which appear to have a different charge to mass ratio.

Discussion

Cyanogen bromide cleaves the cytochrome b_5 domain of rat liver sulfite oxidase into two fragments. Each peptide possesses the capability of binding heme in a b_5-type configuration, but the binding efficiency is low. It appears that a portion of the heme binding site must reside on each of the two fragments. The partial site may allow some low affinity binding, but the equilibrium constant may favor dissociation. Each peptide contains structural regions which confer properties similar to those found in the native sulfite oxidase heme domain. The appearance of native-like properties in the CNBr peptides depends on the presence of heme. Heme–peptide II possesses an antigenic determinant. Some antigenicity was observed in heme–peptide III, but it cannot be concluded whether it represents a second determinant or a residual part of the determinant located on peptide II. In studies with proteolytic fragments of serum albumin, Atassi et al. (1976) found that peptides with an intact antigenic determinant would not appreciably inhibit the interaction of antibody and albumin except at a large molar excess (50–100-fold relative to albumin).

The addition of heme to peptide III results in the appearance of significant α-helical structure—a situation analogous to the heme reconstitution of apo-Tp-Hmf. Heme binding to peptide II does not elicit any marked changes in its circular dichroism. The pronounced changes seen in antigenic and circular dichroism properties occur with the peptides binding less than 0.2 mol of heme in a cytochrome b_5 configuration. It is conceivable that some changes result from non-b_5-type heme binding. Sulfite oxidase can be digested into two functional

domains by proteolysis, and now it is apparent that the heme domain can be fractionated into substructural regions with the retention of some physical and biological properties.

In the presence of heme, peptides II and III form a complex which resembles native Tp-Hmf. Both the peptide complex and native Tp-Hmf exhibit a molecular weight of 10 000, although the complex has a tendency to aggregate. Circular dichroism ellipticities and antigenicity are quantitatively lower in the complex than in the intact heme domain. The most likely explanation is the inability to exceed 50% heme reconstitution. The propensity of the peptide mixture to aggregate may reduce the extent of reconstitution. Alternatively, the lack of complete additivity in amino acid compositions of the peptide mixture and native Tp-Hmf may indicate that an acid-labile peptide has been lost which is important in high-affinity heme binding.

Crystallographic studies have indicated that the heme in microsomal cytochrome b_5 is axially liganded by two histidine residues, His-43 and -67 (Ozols & Strittmatter, 1969; Mathews et al., 1972). If the sulfite oxidase heme domain is evolutionarily related to microsomal b_5, sequence homology would be expected. Since CNBr cleaves the heme domain approximately midway in the sequence, one His ligand should reside in peptide II and the other in peptide III. If each peptide contained a single His ligand and a partial binding crevass, some heme binding may occur, but the affinity would be low. Cytochrome b_5 type spectrum requires two axial nitrogenous ligands. Both heme peptides II and III show b_5-type binding, so presumably there is some flexibility in the molecules to allow a second nitrogenous ligand to occupy the second axial position.

The histidine seen at cycle 17 in the partial sequence of CNBr peptide III may correspond to His-67 of microsomal cytochrome b_5. The position of the peptide III histidine would approximately correspond to residue 70 in the total sequence. Comparing the sequence of peptide III with those of microsomal b_5 and yeast b_2, there does not appear to be any homology with either of the well characterized heme proteins. In the same region yeast b_2 and microsomal b_5 exhibit striking sequence homology (Ozols & Strittmatter, 1969; Guiard & Lederer, 1976). There are 8 identities in the 19 residue sequence, whereas peptide III has only 2 identities comparing with either microsomal b_5 or yeast b_2. Guiard & Lederer (1977) reported that the heme core of chicken liver sulfite oxidase is similar in sequence to both the yeast b_2 and microsomal b_5. Their limited sequence was from the amino-terminal end of the molecule. In this region yeast b_2 and microsomal b_5 show 17 identities in about 40 residues, whereas the heme core of sulfite oxidase has 8–9 identities in 31 residues with either yeast b_2 or microsomal b_5 (Guiard & Lederer, 1977). The heme domain of sulfite oxidase appears to be more distantly related to either the yeast b_2 and microsomal b_5 than are the b_2 and b_5 to each other. If the heme domain of sulfite oxidase is evolutionarily related to b_2 or b_5, there appears to be a greater similarity in the amino terminal region than in the carboxyl terminal portion.

Electrophoresis of the heme domain of sulfite oxidase on 7.5% acrylamide gels revealed that the molecule migrated with the dye front. The electrophoretic mobility of peptide III is similar to that of Tp-Hmf, whereas that of peptide II is markedly different. The extreme electronegativity of peptide III and Tp-Hmf may be due to a clustering of negatively charged groups or a particular secondary structure. Within peptide III there is a clustering of amino acids in the COOH-terminal portion of the peptide, whereas the distribution of charges in peptide II is unknown. This clustering of negative charges in Tp-Hmf may explain its electrophoretic mobility, but it also may be significant as an interaction site for cytochrome *c*. Sulfite oxidase is a natural cytochrome *c* reductase, and the reduction occurs through the heme domain of the enzyme (Johnson & Rajagopalan, 1977). The region around the heme crevice in cytochrome *c* is known to contain a cluster of lysines which are believed to be involved in the interaction with physiological oxidoreductases (Salemme, 1977). Presumably, the association which is stabilized by complementary charge interaction brings the prosthetic groups into proper orientation for electron transfer. The complementary charged region in the oxidoreductases must be negative, and it is conceivable that the negatively charged sequence in peptide III represents the site for the electrostatic interaction of sulfite oxidase and cytochrome *c*. A similar mechanism has been advanced for the interaction of cytochrome *c* and microsomal b_5 (Salemme, 1976).

Reconstitution of native-like properties by mixing constituent peptides is not unique to sulfite oxidase. Mixture of the two peptides generated by digestion of horse heart cytochrome *c* with cyanogen bromide results in a 1:1 complex with properties resembling the native protein (Corradin & Harbury, 1971). Recently, it was demonstrated that the two CNBr peptides of cytochrome *c* can reform the severed polypeptide linkage resulting in a fully active protein with only homoserine replacing the methionine (Barstow et al., 1977).

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Interaction of Protocatechuate-3,4-dioxygenase with Fluoro-Substituted Hydroxybenzoic Acids and Related Compounds†

Sheldon W. May,*,‡ Robert S. Phillips, and Charlie D. Oldham

ABSTRACT: The substrate analogues 3-fluoro-4-hydroxybenzoic acid (3-FHB) and 4-fluoro-3-hydroxybenzoic acid (4-FHB) were synthesized and examined as active site probes for protocatechuate-3,4-dioxygenase (PCD). Upon incubation of 3-FHB with PCD, a complex is formed which causes alterations in both the visible and CD spectra of the enzyme, and these changes can be reversed by dialysis or by displacement with other ligands. Upon prolonged incubation of 3-FHB with PCD, neither oxidation of the fluoro compound nor irreversible inactivation of the enzyme occurs. Steady-state kinetic measurements established that 3-FHB acts as a simple competitive inhibitor of PCD. Inhibition constants were determined in the pH range 6.0 to 9.4, with maximal potency at pH 7.0 where 3-FHB is the most potent PCD inhibitor known ($K_I = 0.3\ \mu M$). 3-FHB is an ideal active site titrant for PCD, and titration data indicate that 8.0 mol of ligand bind per mol of enzyme. Mathematical analysis of the titration data gave dissociation constants in excellent agreement with the kinetically determined K_I values at several different pHs. In contrast, the isomeric 4-FHB was found to be a very weak PCD inhibitor with a K_I approximately 1000-fold greater than that of 3-FHB. The contrasting behavior of the isomeric fluorohydroxybenzoates was found to be mirrored in the behavior of the corresponding defluoro ligands, 3- and 4-hydroxybenzoic acid (3-HB and 4-HB, respectively), but the presence of the fluorine substituent in 3-FHB increases the ligand's affinity for the enzyme approximately 500-fold. Analysis of the pH dependencies of inhibition constants indicated preferential binding of the protonated forms of 3-FHB and 4-HB. That the efficient binding of 3-FHB at the active site cannot be attributed solely to simple chelation of the iron atom is supported by spectral examination of Fe(III) complexation by 3-FHB and related compounds. Displacement studies were carried out using stopped-flow techniques in order to examine the kinetics of dissociation of the complexes of PCD with 3-FHB and other inhibitors. The utility of fluoro substituted ligands as active site probes for analysis of binding interactions and for evaluation of catalytic mechanisms for dioxygenases such as PCD is discussed.

Protocatechuate-3,4-dioxygenase (PCD)[1] catalyzes the cleavage of the aromatic ring of protocatechuic acid with the concomitant insertion of oxygen to give β-carboxy-*cis,cis*-muconic acid (Stanier & Ingraham, 1954). It has been identified and isolated from a number of microbial sources, but the enzyme from *Pseudomonas aeruginosa* is particularly suitable for study, due to the ease with which it can be isolated and crystallized, and also to its unusually high stability (Fujisawa & Hayaishi, 1968). The strict specificity of the enzyme is remarkable, with protocatechuic acid and its homologues, catechol, 3,4-dihydroxyphenylacetic acid, and 3,4-dihydroxyphenylpropionic acid, being among the few known substrates. Other substrate analogues such as 4-hydroxybenzoic acid, protocatechualdehyde, and 4-nitrocatechol are all nonmetabolizable competitive inhibitors of varying potency (Fujisawa et al., 1972a,b; Tyson, 1975; Zaborsky et al., 1975).

In order to facilitate investigations of the mechanisms and binding parameters operative at the active sites of oxygenases, we have been designing various fluorine-substituted substrate analogues (May & Phillips, 1977). The fluorine atom, if properly positioned, would be expected to present minimal steric interference with normal binding at the active site (see examples in Barnett, 1972), and an examination of the catalytic consequences of the replacement of hydroxyl groups or hydrogens by fluorine provides valuable mechanistic information regarding the enzymatic oxygen insertion process. Also, a fluorine substituent perturbs the pK_as of adjacent ionizable groups such as amines or phenols and thus can be of considerable value in studies on the pH dependencies of inhibitor binding and catalytic activity. Furthermore, fluoro analogues are exceedingly useful in NMR studies (Dwek, 1972) on the

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[1] Abbreviations used: PCD, protocatechuate-3,4-dioxygenase; 4-NC, 4-nitrocatechol; 3-FHB, 3-fluoro-4-hydroxybenzoic acid; 4-FHB, 4-fluoro-3-hydroxybenzoic acid; 4-HB, 4-hydroxybenzoic acid; 3-HB, 3-hydroxybenzoic acid.

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