Pseudomonas Cytochrome *c*. II. Effect of Modification of the Methionine Residues*

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ABSTRACT: Reaction of *Pseudomonas* cytochrome *c* (551) with bromoacetate at pH 7 results in the carboxymethylation of methionine 22, and is unattended by change in the visible absorption spectrum. If the reaction is conducted in the presence of cyanide, then not only methionine 22 but also methionine 61 is modified, and the spectrum is altered markedly. The single histidine residue is unaffected by this treatment. Alkylation with iodoacetate at pH 3.0 and 5.4 yields results comparable to those obtained with bromoacetate in the presence and absence of cyanide, respectively. The possibility is considered that at neutral pH the heme iron is coordinated to histidine 16 and methionine 61.

Pseudomonas cytochrome *c* (*c*Ps)[1] contains only one histidine residue (Ambler, 1963a), and all of the amino groups of the protein can be trifluoroacetylated, or the ϵ-amino groups guanidinated and the α-amino group acetylated, without loss of the hemochrome character of the molecule (Vinogradov and Harbury, 1967). Thus, of the two structures that have been the most commonly considered for the cytochromes *c*—coordination of the heme iron to two histidine residues, or, alternatively, coordination to a histidine residue and a lysine residue—the first can be immediately ruled out in this instance, and the second is probably also inapplicable. We have, therefore, been examining other possibilities, one of which is that the thioether side chain of a methionine residue serves as ligand (Harbury *et al.*, 1965).

The latter type of structure would require that *S*-alkylation of the methionine residues lead to major alteration of the visible absorption spectra. In the studies described here, we have worked with preparations of *Pseudomonas* cytochrome *c* modified by treatment with bromoacetic acid and iodoacetic acid.

Materials and Methods

Materials. *Pseudomonas* cytochrome *c* and its trifluoroacetylated derivative were the same preparations used previously (Vinogradov and Harbury, 1967). Bromo- and iodoacetic acids were recrystallized from toluene and petroleum ether (bp 30–60°). TPCK-trypsin was prepared as described by Kostka and Carpenter (1964).

Alkylation with Bromoacetate. The conditions were the same as those used by Schejter and George (1965) for the carboxymethylation of horse heart cytochrome *c*. A 1% solution of the protein in 0.1 M phosphate buffer, pH 7.0, was treated with an equal volume of 0.32 M bromoacetic acid neutralized either with sodium hydroxide or sodium cyanide. The mixture was allowed to stand at 23–25° for 36 hr, and then desalted by passage through a column of Sephadex G-25.

Alkylation with Iodoacetate. A 1% solution of the protein was treated with 0.5 M iodoacetic acid at pH 3.0 or 5.4 for 24 hr at 23–25°, and then passed through Sephadex G-25.

Tryptic Peptides from Trifluoroacetylated Preparations. Hydrolysis with TPCK-trypsin was used to split trifluoroacetylated derivatives into a heme peptide of 47 amino acid residues and a nonheme peptide of 35 residues. Solutions of the trifluoroacetylated protein (10 mg/ml) plus TPCK-trypsin (0.2 mg/ml) in 0.05 M phosphate buffer pH 7.5, were kept for 10 hr at 23–25°, and then subjected to gel filtration through Sephadex G-50. A representative elution pattern is shown in Figure 1. Fractions corresponding to each

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[1] Abbreviations used in this work: *c*Ps, *Pseudomonas* cytochrome *c* (the preparation which has been referred to as *Pseudomonas* cytochrome *c*-551 (Ambler, 1963a,b; Horio *et al.*, 1960)); TFA-*c*Ps, trifluoroacetylated *c*Ps; *c*Ps(BrAc), *c*Ps treated with bromoacetate at pH 7; *c*Ps(BrAc,CN), *c*Ps treated with bromoacetate at pH 7 in the presence of cyanide; *c*Ps(IAc,5), *c*Ps treated with iodoacetate at pH 5.4; *c*Ps(IAc,3), *c*Ps treated with iodoacetate at pH 3.0; TFA-*c*Ps(BrAc), TFA-*c*Ps treated with bromoacetate at pH 7; TFA-*c*Ps(BrAc,CN), TFA-*c*Ps treated with bromoacetate at pH 7 in the presence of cyanide; H47T/TFA-*c*Ps, H47T/TFA-*c*Ps(BrAc), and H47T/TFA-*c*Ps-(BrAc,CN), heme peptides of 47 amino acid residues obtained upon tryptic hydrolysis of TFA-*c*Ps, TFA-*c*Ps(BrAc) and TFA-*c*Ps(BrAc,CN), respectively; N35T/TFA-*c*Ps, N35T/TFA-*c*Ps-(BrAc), and N35T/TFA-*c*Ps(BrAc,CN), nonheme peptides of 35 amino acid residues obtained upon tryptic hydrolysis of TFA-*c*Ps, TFA-*c*Ps(BrAc), and TFA-*c*Ps(BrAc,CN), respectively; TPCK-trypsin, trypsin treated with L-(1-tosylamido-2-phenyl)-ethyl chloromethyl ketone

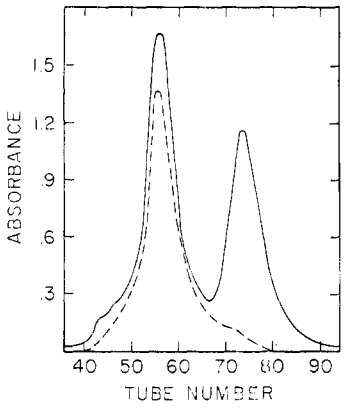


FIGURE 1: Gel filtration pattern of tryptic hydrolysate of TFA-*c*Ps. Sephadex G-50 (1.4 × 80 cm column), eluted with 0.05 M phosphate buffer, pH 7.5, 1.1-ml fractions. ———, absorbance at 280 mμ; -----, absorbance at 430 mμ.

of the two bands were combined, concentrated, and passed through the column a second time. The heme peptide, corresponding to the faster moving component, was not purified further. The slower moving fraction, containing the nonheme peptide, still included some material with an absorption band in the Soret region. To remove this material, the solution was passed through a column of TEAE-cellulose equilibrated with 0.05 M phosphate buffer, pH 7.5. The impurity remained on the column.

Tryptic Peptides from Nontrifluoroacetylated Preparations. A 1% solution of the protein in 0.1 M ammonium acetate buffer of pH 8.5 was treated with TPCK-trypsin (0.2 mg/ml) for 10 hr at 37°. The resulting peptides were separated by paper electrophoresis in pyridine–acetate buffer of pH 6.4 (100 ml of pyridine, 4 ml of acetic acid, 900 ml of water; 30 v/cm; Whatman No. 3MM paper).

Removal of Trifluoroacetyl Groups. Samples were kept for 30 hr at room temperature in 0.1 M potassium carbonate buffer of pH 10.7 (Fanger and Harbury, 1965).

Cyanogen Bromide Cleavage. Treatment with cyanogen bromide was carried out as described by Gross and Witkop (1962).

Analyses. Samples were hydrolyzed with constant-boiling hydrochloric acid in evacuated ampoules at 110°, either for 16 (dinitrophenyl derivatives) or 24 hr (all others). In certain instances, the hydrolysis was preceded by oxidation with performic acid (Gundlach *et al.*, 1959). Quantitative amino acid analyses were obtained with a Beckman-Spinco Model 120B automatic amino acid analyzer (Spackman *et al.*, 1958).

Amino-terminal residues were identified by the fluorodinitrobenzene method, essentially as described by Fraenkel-Conrat *et al.* (1955). Methionine-containing peptides in paper electrophoresis patterns were identified by reaction with iodoplatinic acid (Easley, 1965).

Spectrophotometry. Absorption spectra were recorded with a Bausch and Lomb Spectronic 505 spectrophotometer. Samples were oxidized with potassium ferricyanide and reduced with sodium dithionite.

Results

Extent of Carboxymethylation. Table I gives the results of amino acid analysis of the carboxymethylated preparations obtained under the various conditions employed. Reaction with bromoacetate at pH 7, in the absence of cyanide, resulted in a reduction of the methionine content from 1.8 to 1.1 residues/mole. When, on the other hand, the reaction was carried out in the presence of cyanide, a value of 0.3 residue/mole was obtained. Since, upon acid hydrolysis, approximately 20% of the *S*-carboxymethylmethionine present would be expected to be reconverted to methionine (Gundlach *et al.* 1959), it appears that in this case both methionine residues of the protein were fully alkylated.

Carboxymethylation of TFA-*c*Ps yielded very similar results. When the reaction was carried out in the absence of cyanide, the methionine analysis dropped to one residue per mole, and, when the reaction was performed in the presence of cyanide, both methionine residues were modified.

Results similar to those obtained in the presence of cyanide could be obtained also by carrying out the reaction at low pH. To keep the conditions comparable to those used by Ando *et al.* (1965) and by Tsai and Williams (1965) in studies of beef and horse cytochrome *c* at low pH, iodo- rather than bromoacetic acid was employed. At pH 5.4, one residue of methionine was recovered unchanged. On the other hand, at pH 3.0, both methionine residues reacted. Residues other than methionine were, as judged from the amino acid analyses, essentially unaffected by any of the alkylation procedures described.

Location of Modified Residues. It is known from the work of Ambler (1963b) that the two methionine residues of *Pseudomonas* cytochrome *c* can readily be distinguished by tryptic hydrolysis of the protein and separation of the resulting peptides by electrophoresis at pH 6.4. The peptide containing methionine 22 is cationic at this pH, whereas that containing methionine 61 is anionic. Since thioethers give a positive test with iodoplatinic acid (Easley, 1965), whereas sulfonium compounds do not, a simple procedure was available for the identification of the methionine residues carboxymethylated under the various conditions. Figure 2 gives a comparison of the patterns yielded by tryptic hydrolysates of *c*Ps, *c*Ps(BrAc), and *c*Ps(BrAc,CN). It is clear that, of the two methionine-containing peptides, the cationic one could not be detected with iodoplatinic acid in the hydrolysates of either *c*Ps(BrAc) or *c*Ps(BrAc,CN). The anionic methionine-containing peptide, on the other hand, failed to give a positive test only in the case of *c*Ps(BrAc,CN). Similar experiments with *c*Ps(IAc,5) and *c*Ps(IAc,3) yielded essentially identical results, with the pattern for *c*Ps(IAc,5)

TABLE I: Amino Acid Composition of *Pseudomonas* Cytochrome *c* and Carboxymethylated Derivatives of *Pseudomonas* Cytochrome *c*.

	Residues/Mole of Protein[a]							
Amino Acid	*c*Ps	*c*Ps(BrAc)	*c*Ps(BrAc, CN)	TFA-*c*Ps(BrAc)	TFA-*c*Ps(BrAc, CN)	*c*Ps(IAc,5)	*c*Ps(IAc,3)	*c*Ps (lit.)[b]
Lysine	7.9	7.6	7.6	8.0	7.9	7.9	8.0	8
Histidine	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1
Arginine	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1
Aspartic acid	8.0	8.5	8.4	8.2	8.2	8.0	8.2	8
Threonine	2.0	2.3	2.3	2.3	2.2	2.1	2.3	2
Serine	2.8	3.3	3.2	3.9	3.4	3.1	3.4	3
Glutamic acid	10.1	9.9	9.8	9.6	9.6	9.7	9.5	10
Proline	5.4	6.5	6.1	6.1	6.2	6.0	6.1	6
Glycine	6.9	7.4	7.4	7.3	7.3	7.1	7.4	7
Alanine	13.1	13.0	13.3	12.9	12.6	12.5	12.9	13
Half-cystine[c]	1.3	1.2	1.3	1.2	1.0	1.1	1.0	2
Valine	6.4	7.0	6.9	6.7	6.5	6.6	6.6	7
Methionine[d]	1.8	1.1	0.3	1.1	0.4	1.1	0.3	2
Isoleucine	2.6	2.6	2.7	2.8	2.7	2.7	3.0	3
Leucine	3.9	4.1	4.1	4.2	4.1	4.0	4.2	4
Tyrosine	0.8	0.9	0.9	1.0	0.8	1.1	0.8	1
Phenylalanine	1.6	2.0	2.0	2.0	1.9	2.1	2.1	2

[a] No corrections were made for loss on hydrolysis. [b] Ambler (1963a,b). [c] Thioether bridges not cleaved prior to hydrolysis. [d] Includes methionine sulfoxides.

corresponding to that of *c*Ps(BrAc), and the pattern for *c*Ps(IAc,3) corresponding to the one for *c*Ps(BrAc,CN). These results suggest that, at pH 5–7 and in the absence of cyanide, only methionine 22 is readily modified, whereas in the presence of cyanide or at pH 3, both methionine 22 and methionine 61 are alkylated.

Further support for this view was obtained by a study of the products of cyanogen bromide cleavage of *Pseudomonas* cytochrome *c* and its derivatives. Reaction of *c*Ps with cyanogen bromide, followed by dinitrophenylation and acid hydrolysis, yielded approximately equal amounts of DNP-glutamic acid and DNP-valine. The DNP-glutamic acid arises from the amino-terminal glutamic acid residue of the protein, and DNP-valine results from cleavage of the bond which follows methionine 22 (Ambler, 1963b). Fission of the peptide bond after methionine 61, followed by dinitrophenylation and acid hydrolysis, would be expected to produce, in addition, DNP-proline. The fact that none was found is probably attributable to its destruction during acid hydrolysis (Fraenkel-Conrat *et al.*, 1955). Upon application of the cyanogen bromide procedure to *c*Ps(BrAc) and *c*Ps(BrAc,CN), the yield of DNP-valine was found in each case to be only 10% of that obtained with the nonalkylated preparation, which would indicate that methionine 22 was at least 90% modified. The latter figure is a minimum one, since decomposition of the carboxymethylmethionine under the conditions of cyanogen bromide treatment would lead to high yields of DNP-valine.

Still additional evidence on the same point was provided by analysis of the heme peptide and nonheme peptide obtainable from trifluoroacetylated preparations upon tryptic cleavage of the bond following the single arginine residue (Tables II and III). The heme peptide, 47 residues long, contains methionine 22, and the nonheme peptide, 35 residues in length, includes methionine

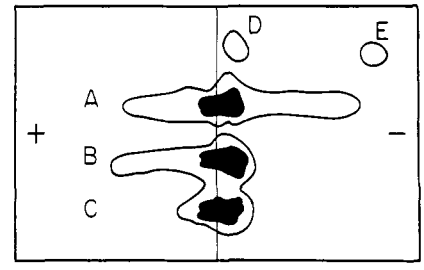


FIGURE 2: Electrophoresis patterns of tryptic hydrolysates of *c*Ps, *c*Ps(BrAc), and *c*Ps(BrAc,CN). Pyridine–acetate buffer, pH 6.4. Darkened areas indicate positions of heme peptides. Areas bleached in iodoplatinic acid test enclosed by solid lines (A–C). Methionine and histidine detected with ninhydrin. (A) *c*Ps; (B) *c*Ps(BrAc); (C) *c*Ps(BrAc,CN); (D) methionine; and (E) histidine.

TABLE II: Amino Acid Composition of Tryptic Peptides of Trifluoroacetylated *Pseudomonas* Cytochrome *c*.

Amino Acid	Residues/Mole of Peptide[a] H47T/TFA-*c*Ps	N35T/TFA-*c*Ps
Lysine	5.0 (5)	3.0 (3)
Histidine	0.9 (1)	0.1 (0)
Arginine	0.9 (1)	0.2 (0)
Aspartic acid	4.3 (4)	4.0 (4)
Threonine	1.3 (1)	1.0 (1)
Serine	1.1 (0)	3.2 (3)
Glutamic acid	5.9 (6)	3.8 (4)
Proline	2.2 (2)	3.8 (4)
Glycine	4.3 (4)	3.3 (3)
Alanine	9.3 (10)	3.3 (3)
Half-cystine[b]	1.1 (2)	0 (0)
Valine	3.8 (4)	2.6 (3)
Methionine[c]	0.9 (1)	1.0 (1)
Isoleucine	1.1 (1)	1.7 (2)
Leucine	2.1 (2)	1.8 (2)
Tyrosine	0.9 (1)	0.1 (0)
Phenylalanine	2.0 (2)	0.1 (0)

[a] No corrections were made for loss on hydrolysis. Literature values (Ambler, 1963b) in parentheses. [b] Thioether bridges not cleaved prior to hydrolysis. [c] Includes methionine sulfoxides.

TABLE III: Methionine and Methionine Sulfone Analysis of Tryptic Peptides Treated with Performic Acid.

Peptide	Residues/Mole of Peptide[a] Methionine[b]	Methionine Sulfone
H47T/TFA-*c*Ps	0	0.9
H47T/TFA-*c*Ps(BrAc)	0.1	0.1
H47T/TFA-*c*Ps(BrAc,CN)	0.2	0.1
N35T/TFA-*c*Ps	0	0.9
N35T/TFA-*c*Ps(BrAc)	0.1	0.8
N35T/TFA-*c*Ps(BrAc,CN)	0.2	0.1

[a] No corrections were made for loss on hydrolysis. [b] Includes methionine sulfoxides.

61. Table III gives the results of experiments in which the samples to be analyzed were oxidized with performic acid to convert noncarboxymethylated methionine to the sulfone (Gundlach *et al.*, 1959). The peptides isolated from the nonalkylated protein, H47T/TFA-*c*Ps and N35T/TFA-*c*Ps, each yielded approximately one residue of methionine sulfone per mole of peptide, as did N35T/TFA-*c*Ps(BrAc). H47T/TFA-*c*Ps-(BrAc), on the other hand, yielded essentially no methionine sulfone.

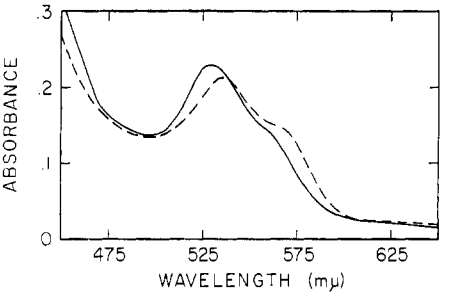


FIGURE 3: Spectra of *c*Ps(BrAc), oxidized form. Concentration ≅ 2 × 10^{-5} M; room temperature; 1-cm cuvet. ———, pH 4.3 and 7.3; -----, 0.9 M NaOH.

Absorption Spectra. As shown in Figures 3 and 4, the visible absorption spectra of *c*Ps(BrAc) closely approximate those of the noncarboxymethylated protein, and remain of the hemochrome type to the lower limit of the pH range studied (pH 4.3). In contrast, *c*Ps(BrAc,CN) exhibits hemochrome spectra only at alkaline pH (Figures 5 and 6). At pH 4.3, curves typical of high-spin iron porphyrin systems are obtained with this derivative, and the system is predominantly in the high-spin state even at neutral pH.

A similar change is seen in the case of preparations alkylated at pH 3. Figure 7 shows the spectra recorded at pH 4.4 for *c*Ps(IAc,3) and *c*Ps(IAc,5). The curves for *c*Ps(IAc,5) correspond to those for *c*Ps(BrAc). The spectra for *c*Ps(IAc,3), on the other hand, are similar to those obtained with *c*Ps(BrAc,CN).

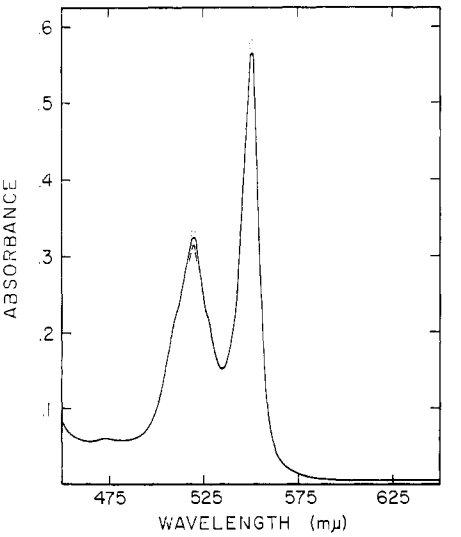


FIGURE 4: Spectra of *c*Ps(BrAc), reduced form. Concentration ≅ 2 × 10^{-5} M; room temperature; 1-cm cuvet. . . . . , pH 4.3; ———, pH 7.3; -----, 0.9 M NaOH.

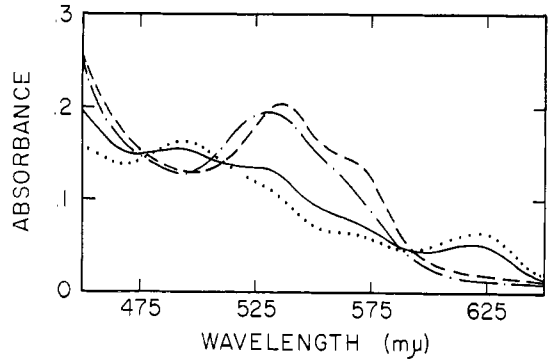


FIGURE 5: Spectra of *c*Ps(BrAc,CN), oxidized form. Concentration ≅ 2 × 10^{-5} M; room temperature; 1-cm cuvet. ....., pH 4.3; ———, pH 7.3; -·-·-, pH 10.9; -----, 0.9 M NaOH.

TFA-*c*Ps(BrAc), like *c*Ps(BrAc), yields typical hemochrome curves (Figure 8). In contrast, the spectra obtained at pH 7.5 for TFA-*c*Ps(BrAc,CN) (Figure 9) closely resemble those of model heme peptide systems in which the heme is coordinated to only one strong-field ligand (Harbury and Loach, 1960; Y. P. Myer and H. A. Harbury, to be published). There is no indication in this instance of incipient hemochrome formation similar to that observed with *c*Ps(BrAc,CN) at neutral pH. Furthermore, the well-developed hemochrome spectrum exhibited by reduced *c*Ps(BrAc,CN) at higher pH is not observed with reduced TFA-*c*Ps-

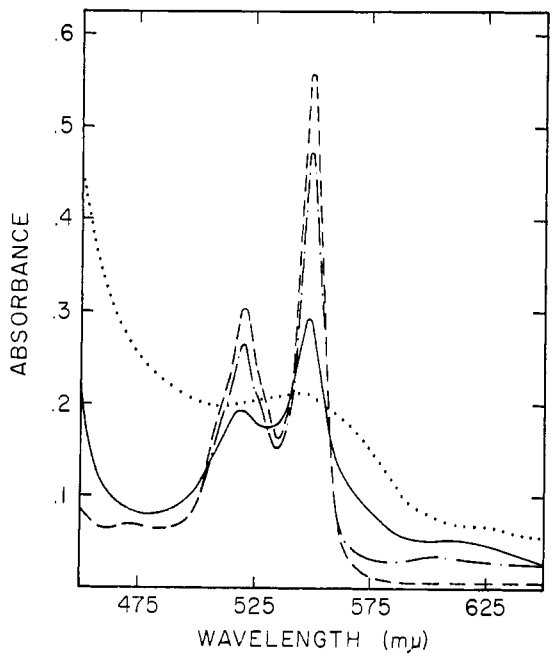


FIGURE 6: Spectra of *c*Ps(BrAc,CN), reduced form. Concentration ≅ 2 × 10^{-5} M; room temperature; 1-cm cuvet. ....., pH 4.3; ———, pH 7.3; -·-·-, pH 10.9; -----, 0.9 M NaOH.

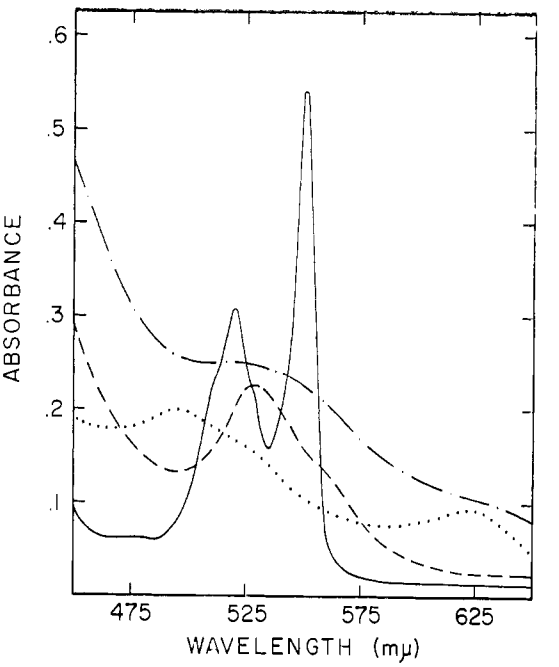


FIGURE 7: Spectra of *c*Ps(IAc,5) and *c*Ps(IAc,3). Concentration ≅ 2 × 10^{-5} M; pH 4.4; room temperature; 1-cm cuvet. -----, *c*Ps(IAc,5), oxidized form; ———, *c*Ps(IAc,5), reduced form; ....., *c*Ps(IAc,3), oxidized form; -·-·-, *c*Ps(IAc,3), reduced form.

(BrAc,CN) in alkaline solution. The curve for the latter system has the same form at pH 9.7 as that shown for pH 7.5. The low-spin character obtained in the case of *c*Ps(BrAc,CN) at high pH appears thus to be dependent upon the presence of unblocked amino groups.

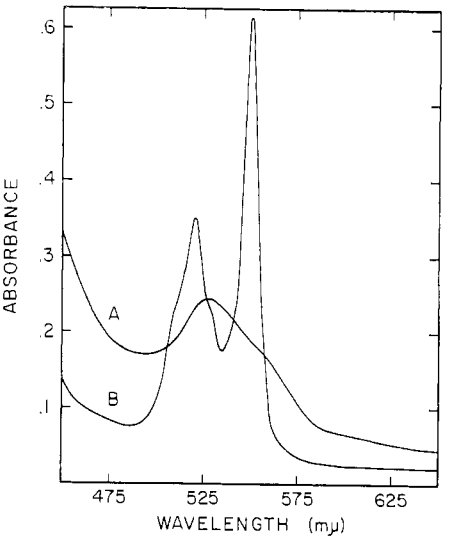


FIGURE 8: Spectra of TFA-*c*Ps(BrAc). Concentration ≅ 2.5 × 10^{-5} M; pH 7.5; room temperature; 1-cm cuvet. (A) Oxidized form; (B) reduced form.

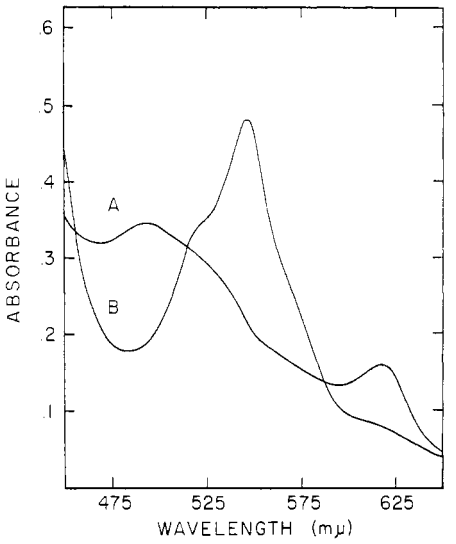


FIGURE 9: Spectra of TFA-*c*Ps(BrAc,CN). Concentration $\cong 5 \times 10^{-5}$ M; pH 7.5; room temperature; 1-cm cuvet. (A) Oxidized form; (B) reduced form.

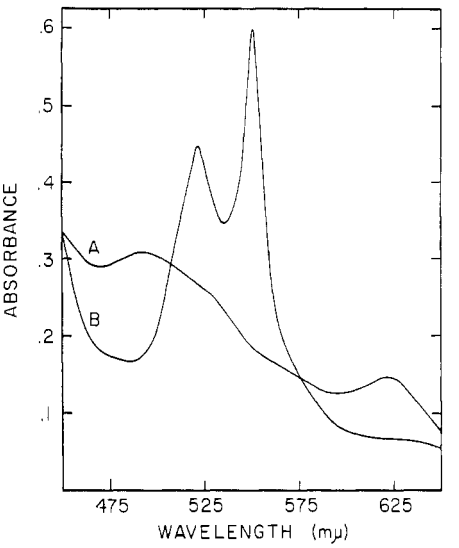


FIGURE 10: Spectra of H47T/TFA-*c*Ps. Concentration $\cong 3 \times 10^{-5}$ M; pH 7.5; room temperature; 1-cm cuvet. (A) Oxidized form; (B) reduced form.

As would be expected, H47T/TFA-*c*Ps(BrAc) and H47T/TFA-*c*Ps(BrAc,CN) also yield curves of the type shown in Figure 9; like TFA-*c*Ps(BrAc,CN), these systems contain no free amino groups and no free methionine side chain. H47T/TFA-*c*Ps, on the other hand, has a nonalkylated methionine residue in position 22, and, in the reduced form, yields a curve that is predominantly of the low-spin type (Figure 10). Apparently, in the reduced peptide, the single thioether side chain coordinates to the iron in the position opposite the histidine residue. In the oxidized form, the spectrum is predominantly of the high-spin type, in keeping with the fact that thioether binding is far less favored in oxidized than in reduced heme complexes (Harbury *et al.*, 1965).

Discussion

The carboxymethylation of cytochrome *c* at pH 7 has been studied previously by Schejter and George (1965). In an important experiment, these authors demonstrated that alkylation of the cyanide complex of horse heart ferricytochrome *c*, followed by removal of the cyanide, leads to marked changes in the visible absorption spectra, whereas no change is obtained upon reaction in the absence of cyanide. When it was subsequently found that these results reflect an effect of cyanide on the susceptibility to carboxymethylation of methionine 80 (Harbury *et al.*, 1965; Harbury, 1966), the possibility suggested itself that this methionine residue might be one of the groups involved in coordination to the heme iron. The studies outlined here were undertaken to see whether results consistent with a possible ligand role for methionine could be obtained in experiments with *Pseudomonas* cytochrome *c*, a molecule likely to have only one nitrogenous side-chain group bound to the iron (Harbury *et al.*, 1965; Vinogradov and Harbury, 1967), and for which the ligand in coordination position 6 remained to be identified.

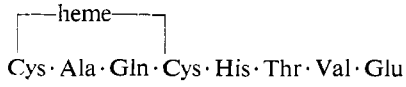
The data are easily summarized. Reaction with bromoacetate at pH 7, in the absence of cyanide, led to the modification of only one of the two methionine residues of the molecule, methionine 22, and was without effect on the visible absorption spectra. On the other hand, when the reaction was carried out in the presence of cyanide, not only methionine 22 but also methionine 61 was modified, and the spectra were altered greatly. Whereas the unmodified molecule remains a hemochrome to very low pH, the derivative with a modified methionine 61 side chain exhibited spectra that were predominantly of the high-spin type at neutral pH, and essentially fully so at pH 4. Spectra similar to those of the parent protein were obtained only at alkaline pH, and when the carboxymethylation was carried out with a preparation in which the amino groups were trifluoroacetylated, hemochrome character failed to develop even at pH 10. Since amino acid analysis gave no indication that the alkylation procedure led to change in the content of residues other than methionine, the simplest inference is that the change in spectra obtained upon reaction in the presence of cyanide results specifically from the modification of methionine 61.

It is of course possible that modifications occurred which were not detected by the analyses performed. However, it seems likely that such alterations, if any did occur, were of minor consequence. In a control experiment in which the protein was treated with bromoacetate and then, after replacement of the bromoacetate with acetate, exposed to the action of cyanide, the spectrum remained unchanged. Further-

more, upon learning of the studies of Tsai and Williams (1965) and Ando *et al.* (1965), in which alkylated derivatives of horse and beef cytochrome *c* were prepared by reaction with iodoacetate at pH 3 and 5–6, we repeated the carboxymethylation of *Pseudomonas* cytochrome *c* under comparable conditions, and obtained results fully compatible with those gained with bromoacetate at pH 7. Reaction with iodoacetate at pH 5.4 led to the modification of methionine 22, but not of methionine 61, and was unattended by a change in spectrum. Reaction at pH 3, on the other hand, led to the modification of both of the methionine residues, and resulted in the changes in spectra obtained with the cyanide procedure.

The relationship between methionine 61 and the hemochrome character of the molecule could be either a direct or an indirect one. It might be that the conversion of the uncharged thioether side chain to the charged sulfonium salt results simply in a conformation change which affects the heme–protein interaction secondarily. An effect of this type would not necessarily require even that the methionine be proximal to the heme group. On the other hand, it also could be argued that the loss of the low-spin spectrum reflects a much more direct disruption of the central coordination complex; *i.e.*, that methionine 61 is one of the groups bound to the iron. Such a structure would help to account for the fact that the effect under study is not limited to *Pseudomonas* cytochrome *c*, but has been obtained also with "mammalian-type" systems. The amino acid sequences of the latter (*cf.* review by Margoliash and Schejter, 1966) differ markedly from the sequence of the *Pseudomonas* protein (Ambler, 1963b), and especially so in the vicinity of the relevant methionine residues. Yet, in each instance, it apparently is the modification of the one particular residue—methionine 61 in *Pseudomonas* cytochrome *c*, and methionine 80 in cytochrome *c* from horse, tuna, cow, and moth (Ando *et al.*, 1965, 1966a; Tsai *et al.*, 1965; Harbury *et al.*, 1965; Harbury, 1966)—that results in alteration of the characteristic hemochrome structure. Furthermore, this modification is greatly facilitated by formation of the cyanide complex, or by adjustment of the pH to the range in which ferricytochrome *c* undergoes transition to a high-spin structure (Theorell and Åkeson, 1941). This obviously would have an especially simple basis if the methionine were coordinated to the metal, and the function of the extrinsic ligand or low pH were to sever or weaken this linkage.

That the thioether group of methionine can form complexes with hemochrome-type spectra is known from studies of model systems (Harbury *et al.*, 1965). In experiments with a heme octapeptide, complex



formation with *N*-acetylmethionine methyl ester was found to be greatly favored when the metalloporphyrin is in the reduced form, and to be maintained to low values of pH.[2] These are, of course, attractive features in connection with two of the characteristic properties of the parent *Pseudomonas* and "mammalian-type" protein molecules: retention of hemochrome character to low pH, and a high oxidation–reduction potential. Moreover, the results of the present studies suggest that comparable complex formation can be effected in the case of H47T/TFA-*c*Ps, a model in which the methionine content is restricted to that of the peptide chain. At pH 7, this system exhibits a spectrum that is essentially of the high-spin type when the heme is in the oxidized state, but predominantly low spin in the case of the reduced form. In contrast, H47T/TFA-*c*Ps(BrAc) and H47T/TFA-*c*Ps(BrAc,CN) exhibit spectra of the high-spin type in both oxidation states. The implication is that, in ferro- but not in ferri-H47T/TFA-*c*Ps, the formation of a histidine–heme–methionine complex is a sufficiently favorable process to pull into position the one available thioether group.

It could thus well be that methionine does serve as a ligand in the protein systems studied; *i.e.*, that the iron is bound, in the case of *Pseudomonas* cytochrome *c*, to histidine 16 and methionine 61, and, in the "mammalian-type" cytochromes *c*, to histidine 18 and methionine 80. Such binding to residues near opposite ends of the single peptide chain could contribute to the compactness and unusual stability of these molecules, and, since the far end of the chain would be more firmly linked in the reduced than in the oxidized system, might help to account for the fact that reduction results in a lessened susceptibility to enzymic hydrolysis (Nozaki *et al.*, 1957, 1958; Yamanaka *et al.*, 1959), and a greater resistance to change in structure upon increase in temperature (Butt and Keilin, 1962) or exposure to guanidine hydrochloride or urea (Urry, 1965; Harbury *et al.*, 1967). Similarly, a structure of this type would account very simply for the observation that the alkylation of methionine 80 of beef or moth cytochrome *c* leads to a complete loss of electron-transfer activity (Ando *et al.*, 1965, 1966a; Tsai *et al.*, 1965), and, particularly relevant, that the modification of this residue at pH 5–6 (beef cytochrome *c*) proceeds much more slowly with the reduced than with the oxidized system (Matsubara *et al.*, 1965; Ando *et al.*, 1966b).

However, it is clear that the evidence in support of a heme–methionine linkage is by no means conclusive. The changes in properties obtained upon modification of methionine 61 or 80 demonstrate only that once these residues have been modified, the original structure of the central coordination complex, whatever it may have been, cannot reestablish itself. Indeed, there is at least one observation that could be taken to weigh against methionine coordination in the protein systems. This relates to the absorbance ratios of the α and β bands of the model heme octapeptide complexes (reduced form, pH 7). In the case of the complex of the

[2] Experiments at low pH were conducted only with the system in the reduced form.

peptide with *N*-acetylmethionine methyl ester, this $\alpha:\beta$ ratio agrees less well with that for the proteins than does the ratio for the complex with imidazole (Harbury *et al.*, 1965). The significance of this difference is, however, difficult to assess at present. Information is needed on the effects of factors not operative in the simple model systems studied, but that potentially are of importance in the case of the protein systems (*e.g.*, steric constraint of ligand groups; the presence of a hydrophobic environment).

If methionine is not a ligand in *Pseudomonas* cytochrome *c*, it may become necessary to reexamine the long-held view that the heme of the cytochromes *c* is joined to two strong-field groups, both contributed by side chains of the protein, and to explore what have seemed thus far to be less likely possibilities; for example, structures in which the imidazole group is the only strong-field ligand bound to the heme, or in which a peptide nitrogen is coordinated. On the other hand, if the results obtained do reflect a ligand role for methionine, there remains the question of how general a role this is. It seems reasonable to assume that in all cytochromes *c* the iron is bound to the histidine residue corresponding to number 18 of the horse heart protein. However, a second coordinated side chain would have to vary in sequence position relative to this histidine (*cf.* amino acid sequences summarized by Margoliash and Schejter (1966) and Heller and Smith (1965); Ambler, 1963b), and the possibility exists that there could be differences also in the kind of group that is involved. Comparative studies of some other cytochromes *c*, differing markedly in amino acid composition and sequence, should permit a more adequate judgment on this point.

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