COMPLEX FORMATION BETWEEN METHIONINE AND A HEME PEPTIDE FROM CYTOCHROME C*

BY HENRY A. HARBURY, JOHN R. CRONIN,† MICHAEL W. FANGER,‡ THOMAS P. HETTINGER,§ ALEXANDER J. MURPHY,** YASH P. MYER, AND SERGE N. VINOGRADOV

DEPARTMENT OF BIOCHEMISTRY, YALE UNIVERSITY

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It has generally been held that the only protein groups capable of binding to heme to form complexes exhibiting hemochrome spectra are the α-amino group, the ε-amino group of lysine, and the imidazole group of histidine.1,2 In the course of studies of cytochrome c and related heme peptide systems, we have come to question whether this view in fact is a valid one, and decided recently to examine whether in some instances the thioether group of methionine might not play a role. Our immediate interest concerned the possibility that mixed hemochromes might be formed, in which one of the available coordination positions about the heme iron is taken by the imidazole group of a histidine residue, and the other by a methionine side chain. Experiments were accordingly undertaken with a heme octapeptide (HSPT)3 in which one of the ligands at neutral pH is known to be contributed by the peptide’s single histidine residue.4–10 Measurements were also performed with HG and with iron protoporphyrin IX. We present here our initial findings, and comment briefly on their possible relevance to cytochrome c.

Materials and Methods.—N-Acetyl-ldl-methionine was a recrystallized commer-
However, ferromagnetic systems illustrated for corresponding changes of the case except for the acetylmethionine methyl ester (acetyl-DL-methionine).

30°; temperature, acetyl-DL-methionine methyl ester; (g) ferro HSPT + 0.05 M N-acetyl-DL-methionine methyl ester; (h) ferro HSPT + 0.05 M imidazole; (i) ferro HSPT + 0.05 M imidazole; (j) ferro HSPT + 2.5 M N-acetyl-DL-methionine; (k) ferro HSPT + 0.25 M N-acetyl-DL-methionine.

Spectral preparation, and its methyl ester prepared by esterification with methanol-HCl. β-Methylmercaptopyropionic acid was prepared by reaction of β-mercaptopyropionic acid with dimethylsulfate. Ethyl sulfide was a redistilled commercial product. HSPT was prepared as described previously. HG was a chromatographically purified sample of material prepared by reaction of the hydrobromide adduct of iron protoporphyrin IX with glutathione (Vinogradov, Tu, and Harbury, unpublished work). Absorption spectra were recorded with Cary model 11 and Bausch and Lomb Spectronic 505 spectrophotometers. Measurements on samples in the reduced state were conducted under helium in a closed assembly fitted with an absorption cell, glass, gold, and calomel electrodes, and microburettes for the delivery of ligand and other titrants. A Radiometer TTT1 titrator was used as a “potential-stat” to maintain full reduction with the addition of a minimum excess of reducing agent. Measurements of oxidation-reduction potentials were performed as described previously.

Results.—In Figure 1 are collected some representative spectra (pH 7) for HSPT and HG in the absence and presence of imidazole, N-acetylmethionine, and N-acetylmethionine methyl ester. The curves for the oxidized forms in the absence of added ligand are typical of the respective high-spin species (curves a, c). Except for the effects of some intermolecular coordination of the α-amino group in the case of ferro HSPT, and aggregation in the case of ferro HG, the curves for the reduced systems in the absence of added ligand are also of the high-spin type (b, d). However, upon the addition of N-acetylmethionine or its methyl ester to ferro HSPT or ferro HG, spectra characteristic of low-spin species are obtained (e, f). Corresponding changes can be observed with the systems in the oxidized state, but, as illustrated for N-acetylmethionine (j, k), these approach completion only at concentrations much higher than required by the reduced systems. This is in marked

![Figure 1](https://example.com/fig1.png)
contrast to the situation with imidazole (g, h, i), which is bound approximately as well to ferri as to ferro H8PT. It is assumed that in H8PT and those of its complexes dealt with here, the predominant species at pH 7 is in each instance one in which the single histidine residue of the peptide remains coordinated to the heme iron. HG, on the other hand, would be expected to behave more nearly like a simple iron porphyrin, and, indeed, spectra analogous to those obtained upon the addition of N-acetylmethionine to HG can be observed also upon its addition to free iron protoporphyrin IX. We have used HG rather than iron protoporphyrin IX in most of the comparisons thus far undertaken, principally because the former is less subject to aggregation, and, in terms of its porphyrin side chains and general composition, more closely resembles H8PT.

As a solution of the imidazole complex of ferro H8PT is brought to values of pH below 7, both the added imidazole and the side chain of the histidine residue begin to be protonated, and, at pH 4, no evidence of a hemochrome spectrum remains. With ferro H8PT in the presence of 0.25 M N-acetylmethionine methyl ester, on the other hand, a very different change is seen. Although a family of curves is obtained which exhibits approximately the same midpoint of change (pH 5.4) as that observed upon dissociation of the imidazole complex, this change does not, as in the latter case, lead to the formation of a spectrum typical of high-spin species in aggregated form, but, rather, to the type of double-banded spectrum seen throughout this range with solutions of HG in the presence of acetylmethionine methyl ester (Fig. 2). The simplest interpretation would seem to be that, upon the conversion of the histidine residue to the imidazolium form, an imidazole-heme-thioether complex is being converted to a thioether-heme-thioether complex. Adjustment of the solution to still lower values of pH introduces progressively greater difficulties in maintaining full reduction with dithionite, but, with the use of methyl viologen as reducing agent (Fig. 2, insert), a double-banded curve can be recorded to values of pH as low as 2, the limit of the range covered in this study.

Figure 3a shows the family of curves obtained with a solution of $2 \times 10^{-4} M$ ferro H8PT in the presence of increasing concentrations of acetylmethionine methyl ester. With a midpoint of change near 0.006 $M$, the spectrum changes from one characteristic of a sample predominantly in the high-spin form, to one exhibiting sharp, hemochrome-type bands. Comparable changes in spectra have been obtained with N-acetylmethionine in nonesterified form, $\beta$-methylmercaptopropionic acid, 2,2'-thiodiglycol, and ethyl sulfide. In the case of the last-named compound,
limitations of solubility restricted the extent to which conversion of H8PT to the complex could be attained, but, since this is structurally the simplest thioether examined, we illustrate in Figure 3b its effect over the range of concentrations accessible. It is clear that the induction of a two-banded spectrum can occur with a ligand in which the thioether group is the only functional group present.

As indicated earlier, much higher concentrations of acetylhistidine or its methyl ester are required to shift the spectrum of ferri H8PT in a manner indicative of a high-spin to low-spin transition than are needed in the case of ferro H8PT. This is borne out also by the results of oxidation-reduction potential measurements. Titrations were conducted with concurrent determination of potentials and spectra (Fig. 4a), at concentrations of acetylhistidine up to 2.0 M. In Figure 4b are summarized the results obtained at pH 7. The potentials at the higher ligand concentrations are some 0.15 v less negative than the corresponding value for H8PT in the absence of added ligand or for H8PT in the form of its imidazole complex.

Discussion.—Cytochrome c is generally considered to typify a heme protein in which positions 5 and 6 about the iron are both occupied by nitrogenous side-chain groups of the molecule. According to one view, these groups are the imidazole side chains of two histidine residues. According to another, they are an imidazole group of histidine and an ε-amino group of lysine. The latter hypothesis is, however, difficult to reconcile with the finding that all of the lysine residues of horse and tuna heart cytochromes c can be guanidinated without loss of activity in the succinate oxidase system, and without appreciable effect on the absorption spectra and oxidation-reduction potentials at neutral and acid pH. The hemochrom spectrum of horse heart cytochrome c at neutral pH is, furthermore, retained also upon trifluoroacetylation of all of the amino groups. Thus, in a choice between a central coordination complex involving two histidine residues, and a complex involving a histidine residue and a lysine residue, the former alternative would appear to be the more likely one.

Recently, these modification experiments have been extended to cytochrome c
Fig. 4.—(a) Spectra recorded during a reductive titration of HSPT in the presence of 2.0 M N-acetyl-DL-methionine. HSPT $\cong 4 \times 10^{-5} M$; pH 7.02; buffer, 0.05 M phosphate $+$ 0.05 M acetate; reducing agent, methyl viologen; temperature, 30°; 1-cm absorption cell. Percentage of reduction: (A) 100; (B) 80; (C) 59; (D) 36; (E) 16; (F) 0. (b) Oxidation-reduction potentials of half-reduced HSPT in the presence of different concentrations of N-acetyl-DL-methionine. HSPT $4 \times 10^{-5} M$; pH 7.0; buffer, 0.05 M phosphate $+$ 0.05 M acetate; reducing agent, methyl viologen; temperature, 30°. Insert: comparison of potentials of half-reduced systems at pH 7.

from *Pseudomonas fluorescens* (Vinogradov, Hettinger, Fanger, and Harbury, unpublished work). This protein differs greatly from those of horse and tuna heart. It has a molecular weight only some two thirds that of horse or tuna cytochrome c, is acidic rather than basic, has a free $\alpha$-amino group, and possesses an amino acid sequence bearing little resemblance to those determined for “mammalian-type” cytochromes c. As in the “mammalian-type” cytochromes c, there is a histidine residue in the position immediately following the second of the thioether bridges between the cysteine residues and the prosthetic group, but, notably, this is the only histidine residue present. The occurrence of an intramolecular di-histidine complex is thus, in this instance, excluded. Nevertheless, we find that, as in the case of the “mammalian-type” cytochromes c studied, the reduced form displays a typical hemochrome spectrum, and the oxidized form a typical hemichrome spectrum, down to very low values of pH. Furthermore, all of the lysine residues can be guanidinated or trifluoroacetylated without appreciable change in the visible spectra observed at neutral pH, and the guanidinated derivative remains a hemochrome to approximately the same low values of pH as does the unmodified protein. Not only a histidine-histidine complex, but also a histidine-lysine complex, would appear to be excluded for this molecule.

Guanidination, under the conditions used, did not lead to elimination of the $\alpha$-amino group, but the product obtained upon subsequent treatment with acetic anhydride was found to remain a hemochrome at pH 7, in keeping with the data for trifluoroacetylated preparations. It thus appears as though the $\alpha$-amino group
too, is not coordinated to the iron at neutral pH. Should further experiments confirm that the preparations having no unmodified amino groups are monomeric at the concentrations used for the spectral measurements, and that no binding of arginine side chains takes place at neutral and acid pH, then it clearly will become necessary to consider possibilities not involving the coordination of nitrogenous side-chain groups other than the imidazole group of the one histidine residue present.

The finding that methionine and other thioethers can bind to H8PT to yield complexes having spectra typical of heme systems in the low-spin state, and the observation that such complex formation is maintained in acid solution and is favored when the metalloporphyrin is in the reduced form, suggests that one of the possibilities to be checked, for Pseudomonas cytochrome c, and perhaps other cytochromes c as well, would be the occurrence of binding of a methionine residue to the prosthetic group. Clearly, however, such a possibility is not the only one that could be considered. Perhaps, for example, the induction of a low-spin state does not require, in the case of the intact protein, the coordination of two groups of the type necessary in the case of model systems in aqueous solution. Indeed, some of the spectral characteristics of the thioether-H8PT complexes could be argued to rule against the occurrence of methionine-heme binding in cytochrome c; e.g., the relative intensities of the α- and β-bands of the reduced systems studied correspond less well to those of Pseudomonas, tuna, and horse cytochromes c than do those of the imidazole-H8PT complex at neutral pH. There is, however, very little known about the manner in which these parameters vary with conditions, and simple comparisons of this sort could easily be misleading.

One of the more promising approaches for obtaining further information would seem to be through the examination of cytochrome c derivatives containing modified methionine residues. The results of two recent studies of the reaction of horse heart cytochrome c with iodoacetic acid have been interpreted as indicating that carboxymethylation of the methionine residues, and, more specifically, carboxymethylation of methionine residue 80, leads to loss of the electron-transfer activity of the molecule. Other experiments (Hettinger and Harbury, unpublished data) suggest that the changes in spectra obtained upon treatment of horse heart cytochrome c with bromoacetic acid in the presence of cyanide reflect an effect of cyanide on the susceptibility to carboxymethylation of methionine-80. Such an effect could, of course, arise in any of a number of ways—all that need be invoked is a suitable conformation change attendant upon the cyanide binding—but, viewed in the most direct way possible, it could be accounted for very simply were the thioether group of methionine-80 bound to the prosthetic group at neutral pH, and were this binding disrupted upon the coordination of cyanide. It will be of interest to extend these experiments to some of the cytochromes c of simpler composition.

Summary.—N-Acetyl-DL-methionine, N-acetyl-DL-methionine methyl ester, diethyl sulfide, and several other thioethers have been shown to form complexes with a heme octapeptide from horse heart cytochrome c, with a glutathione adduct of iron protoporphyrin IX, and, in a few instances tested, with iron protoporphyrin IX itself. Binding in the peptide systems occurs to the lower limit of the pH-range covered (pH 2), is stronger in the reduced than in the oxidized state, and results in the appearance of hemochrome- and hemichrome-type spectra. The oxidation-reduction potential of the heme octapeptide in the presence of N-acetyl-
dl-methionine is less negative than that of the imidazole-heme octapeptide system at pH 7. The possibility is considered that methionine-heme binding might occur among the cytochromes c.

Note added in proof: Reaction of trifluoroacetylated *Pseudomonas* cytochrome c with bromoacetic acid in the presence of cyanide (same conditions as for horse heart cytochrome c25) results in a product with spectra in the visible region that resemble closely those of H8PT at pH 7 (Fig. 1, curves a and b). If the cyanide is omitted, there is no change in the visible spectra. Amino acid analysis of the product obtained in the presence of cyanide shows that carboxymethylation of methionine has taken place, but that the histidine residue remains unmodified. Tryptic cleavage at the only arginine residue20 (position 47) has been used to separate the two methionine residues of the protein (positions 22 and 61). Initial comparisons of the peptides obtained from preparations treated with bromoacetic acid in the presence and in the absence of cyanide point to a cyanide-dependent carboxymethylation of residue 61.

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3 Abbreviations: H8PT, the heme octapeptide obtained by tryptic hydrolysis of a heme undecapeptide prepared by peptic hydrolysis of horse heart cytochrome c; HG, "heme glutathione," 2,4,α,α'-bis(5-γ-glutamyl-L-cysteinyl glycine)-iron mesoporphyrin IX.
9 Ibid., p. 3646.
14 The term ligand is used in this paper in its most general sense. Although it seems a reasonable assumption that the observed complex formation with thioethers involves Fe—S bonding, this remains to be established.
17 Margoliash, E., in *Brookhaven Symposia in Biology*, No. 15, BNL 738 (C-34) (1962), p. 266.
18 Hettinger, T. P., and H. A. Harbury, these PROCEEDINGS, 52, 1469 (1964).
22 It appears to be a commonly held view that the groups functioning as ligands in the different cytochromes c will turn out in all instances to be the same, but there is little evidence either in support or refutation of this premise.
26 Of the histidine residues of horse heart cytochrome c, only histidine-33 was found to be carboxymethylated under the conditions used. This residue reacts also in the absence of cyanide.