# Carboxymethylation of Horse Heart Ferricytochrome *c* and Cyanferricytochrome *c**

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ABSTRACT: One of the two methionyl residues of horse heart ferricytochrome *c* is carboxymethylated by reaction with bromoacetate at neutral pH. The properties of the modified protein are virtually identical with those of native ferricytochrome *c*. Replacement of one of the two protein–heme coordinate covalent bonds by cyanide to form cyanferricytochrome *c*, followed by reaction with bromoacetate, results in the carboxymethylation of both methionyl residues. Carboxymethylation of the second methionyl residue selectively alters those properties of the protein associated with the heme moiety, particularly the absorption band at 695 mμ and the extent of the solvent perturbation of the Soret absorbance. Similar selective changes are observed when the properties of ferricytochrome *c* and cyanferricytochrome *c* are compared. The correspondence of these changes supports the view that cyanide replaces a methionyl ligand in ferricytochrome *c*.

Substitution of one of the two protein ligands of the heme iron of ferricytochrome *c* by cyanide causes a formerly unreactive methionyl residue (residue 80 in horse heart cytochrome *c*) to be carboxymethylated by bromoacetate at neutral pH (Harbury *et al.*, 1965; Harbury, 1966a; Fanger *et al.*, 1967). The changes in the properties of ferricytochrome *c* following carboxymethylation of this residue, the invariance of this residue in the amino acid sequences of 15 different cytochrome *c*'s (Margoliash and Schejter, 1966) and the similarity of the visible absorption spectrum of a heme peptide in the presence of *N*-acetyl-DL-methionine methyl ester with that of native cytochrome *c* (Harbury *et al.*, 1965) strongly suggest that the sulfur atom of methionyl 80 is coordinated to the heme iron in ferricytochrome *c*. However, it may be argued that the reactivity of methionyl 80 in cyanferricytochrome *c* is the result of a conformational change accompanying the formation of the cyanide derivative. In this report the properties of horse heart ferricytochrome *c*, cyanferricytochrome *c*, and their carboxymethyl derivatives are compared. The differences observed were confined to properties associated with the heme moiety, supporting the view that methionine 80 is a ligand for the heme iron of horse heart ferricytochrome *c*.

## Materials and Methods

*Materials.* Horse heart cytochrome *c*, type VI, was obtained from the Sigma Chemical Co. The protein was converted into the oxidized form by treatment with excess ferricyanide, exhaustively dialyzed against water, and lyophilized.

*Alkylation.* Ferricytochrome *c* was carboxymethylated at room temperature (23–26°) with 0.2 M bromoacetate in 0.1 M phosphate buffer adjusted to pH 7.0, using a protein concentration of 10 mg/ml. Carboxymethylation of ferrocytochrome *c* or cyancytochrome *c* was performed in 0.01 M ascorbate or 0.1 M NaCN, respectively. For measurement of the properties of carboxymethylated cytochrome *c*, the protein was treated with bromoacetate for 3 days and then dialyzed against 0.2 M KCl–0.01 M phosphate (pH 7.0).

For measurement of the kinetics of carboxymethylation of the methionyl residues, aliquots were removed from the reaction mixture at specified intervals, diluted about 25-fold with water, and applied to a 1 × 2 cm column of Amberlite IRC-50 adjusted to neutral pH. The protein, which binds to the column, was washed free of the reaction solvent with water, eluted with 1 N $NH_4OH$, and lyophilized.

*Amino Acid Analyses.* Lyophilized protein was subjected to performic acid oxidation as described by Hirs (1956) prior to acid hydrolysis in 6 N HCl for 24 hr at 110°. All amino acid analyses were performed on a Spinco 120C amino acid analyzer. Methionine sulfone was resolved from aspartic acid in the analytical column effluents by reducing the flow rate of the pH 3.28 buffer to 50 ml/hr and that of the ninhydrin solution to 25 ml/hr. The methionine sulfone content was calculated from the ratio of sulfone and aspartic acid, assuming that the ninhydrin color value is the same for both amino acids and that the moles of aspartic acid represent eight aspartate residues per molecule of cytochrome *c*.

*Other Measurements.* Solvent perturbation difference spectra, absorption spectra, and reduced viscosities were measured as described previously (Stellwagen and Van Rooyan, 1967). Extinction coefficients for carboxymethylated ferricytochrome *c* were calculated from the $\Delta A_{243\ m\mu}$ observed between pH 7.0 and 13.3, assuming

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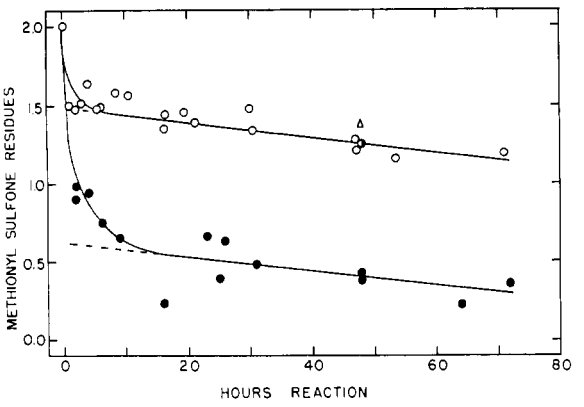


FIGURE 1: Kinetics of the carboxymethylation of the methionyl residues of cytochrome *c*. The reaction conditions are described in Materials and Methods. (○) Ferricytochrome *c*, (◑) ferricytochrome *c* carboxymethylated with 0.2 M iodoacetamide instead of bromoacetate, (△) ferrocytochrome *c*, and (●) cyanferricytochrome *c*.

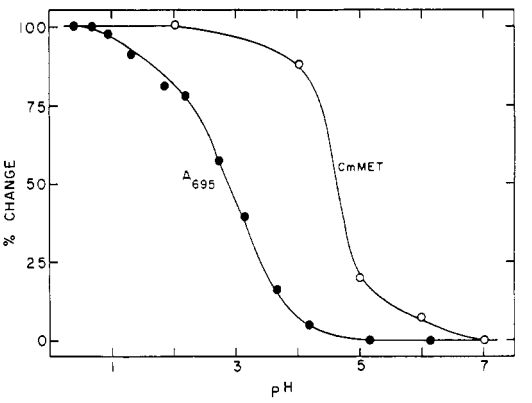


FIGURE 2: Reactivity of the methionyl residues of ferricytochrome *c* as a function of pH. (○) Decrease in methionine sulfone content following reaction of ferricytochrome *c* (10 mg/ml) with 0.2 M bromoacetate for 48 hr in 0.1 M phosphate or acetate adjusted to the indicated pH values. The percentage decrease in methionine sulfone content is related to 1.26 methionyl sulfone residues observed after reaction at pH 7.0 designated as 0% and no methionyl sulfone residues detected at pH 2.0, designated as 100% change. (●) Decrease in the absorbance of ferricytochrome *c* in 0.2 M KCl at 695 mμ as a function of pH.

that the modified protein contains four tyrosyl residues and that $\Delta\epsilon^{M}_{243}$ for tyrosine is $1.10 \times 10^4$ (Stellwagen, 1964).

## Results

*Extent of Carboxymethylation.* Only one of the three histidyl residues of horse heart ferricytochrome *c*, ferrocytochrome *c*, or cyanferricytochrome *c* is carboxymethylated by reaction with 0.2 M bromoacetate at room temperature and pH 7 for 3 days (Stellwagen, 1966; Harbury, 1966b). Because of the marked reversion of carboxymethylmethionyl residues to methionine during acid hydrolysis, an indirect procedure must be employed to measure the extent of modification of the methionyl residues concomitant with the carboxymethylation of the single histidyl residue. The procedure described by Neumann *et al.* (1962) was employed in which methionyl but not CmMET[1] residues are oxidized by performic acid to acid-stable methionyl sulfone residues prior to acid hydrolysis. Reduction in the methionine sulfone content of cytochrome *c* following treatment with bromoacetate can then be attributed to formation of CmMET residues.

The kinetics of the carboxymethylation of the methionyl residues of ferricytochrome *c* and cyanferricytochrome *c* are compared in Figure 1. Both forms of the protein exhibit a rapid initial reaction followed by a slow reaction continuing for a period of days. Extrapolation of the slow reaction observed for both forms to zero reaction time indicates that an additional methionyl residue is carboxymethylated in cyanferricytochrome *c*.

As shown in Figure 1, the extent of carboxymethylation of the methionyl residues of ferri- and ferrocytochrome *c* by bromoacetate at pH 7 is virtually identical after 48 hr. Carboxymethylation of ferricytochrome *c* with iodoacetamide rather than bromoacetate results in the same degree of modification of the methionyl residues of ferricytochrome *c* after reaction for 48 hr, as shown in Figure 1. The effect of pH on the extent of carboxymethylation of the methionyl residues of ferricytochrome *c* by bromoacetate and on the absorbance at 695 mμ are compared in Figure 2.

*Absorption Spectra.* As shown in Table I and Figure 3, carboxymethylation of one methionyl and one histidyl residue of ferricytochrome *c* produces no marked changes in the absorption spectra of the protein. Carboxymethylation of a second methionyl residue, however, reduces the Soret extinction and causes the disappearance of the 695-mμ absorption band in the oxidized form, $CmMET_2$-ferricytochrome *c*. In the reduced form, $CmMET_2$-ferrocytochrome *c*, the extinctions at 416, 520, and 550 mμ are all lower than the corresponding values observed for native and CmMET-ferrocytochrome *c* (Table I and Figure 3).

Acidification of ferricytochrome *c* causes an absorption band to appear at 620 mμ characteristic of a high-

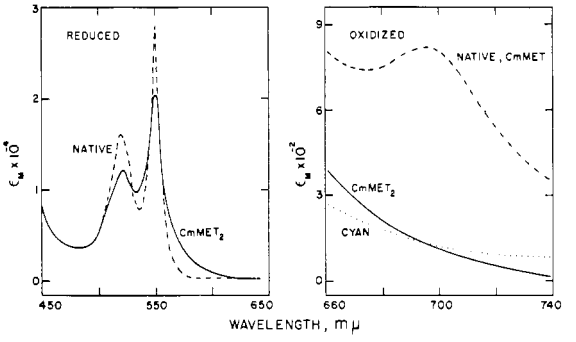


FIGURE 3: Absorption spectra of cytochrome *c*. (– – –) Native or CmMET-cytochrome *c*, (——) $CmMET_2$-cytochrome *c*, and (· · ·) cyanferricytochrome *c*. The solvent was 0.2 M KCl–0.01 M phosphate buffer (pH 7.0). The reduced form of the protein, ferrocytochrome *c*, was obtained by addition of sodium dithionite to the oxidized form, ferricytochrome *c*.

[1] Abbreviation used that is not listed in *Biochemistry 5*, 1445 (1966), is: CmMET, carboxymethylmethionyl.

TABLE I: Comparison of the Properties of Ferricytochrome *c*, Cyanferricytochrome *c*, and Carboxymethylated Ferricytochrome *c*.

| Measurement | Ferricytochrome *c* | | | |
|---|---|---|---|---|
| | Native | CmMET- | Cyan- | $CmMET_2$- |
| Hydrodynamic | | | | |
| $\eta_{sp}/c$ (ml/g) | 2.6 | 2.4 | | 2.5 |
| Spectral | | | | |
| Oxidized | | | | |
| Soret, $\lambda_{max}$ (m$\mu$) | 410 | 408 | 413 | 408 |
| $\epsilon_{Soret} \times 10^{-3}$ | 106 | 106 | 133 | 96.8 |
| $\epsilon_{530} \times 10^{-3}$ | 11.1 | 11.2 | | 9.4 |
| $\epsilon_{695}$ | 820 | 820 | 135 | 135 |
| Reduced | | | | |
| Soret, $\lambda_{max}$ (m$\mu$) | 416 | 416 | | 416 |
| $\epsilon_{Soret} \times 10^{-3}$ | 129 | 129 | | 106 |
| $\epsilon_{550} \times 10^{-3}$ | 27.7 | 27.7 | | 16.5 |
| Solvent perturbation | | | | |
| $\Delta\epsilon_{288}$ | 224 | 224 | 224 | 224 |
| $\Delta\epsilon_{Soret}/\epsilon_{Soret} \times 10^3$ | 17 | 17 | 30 | 24 |

spin ferrihemoprotein complex (Lemberg and Legge, 1949), The apparent p*K*'s of the transition from low- to high-spin complexes for ferricytochrome *c*, CmMET-ferricytochrome *c*, and $CmMET_2$-ferricytochrome *c* are 2.5, 3.2, and 3.9, respectively, as shown in Figure 4.

*Solvent Perturbation Spectra.* The perturbation of the near-ultraviolet absorption spectrum of ferricytochrome *c* by 20% ethylene glycol is not increased by carboxymethylation of one or both methionyl residues or by formation of cyanferricytochrome *c*, as shown in Figure 5A. These perturbation spectra are equivalent to that expected for two exposed tyrosyl and no tryptophanyl residues (Stellwagen and Van Rooyan, 1967). Similarly,

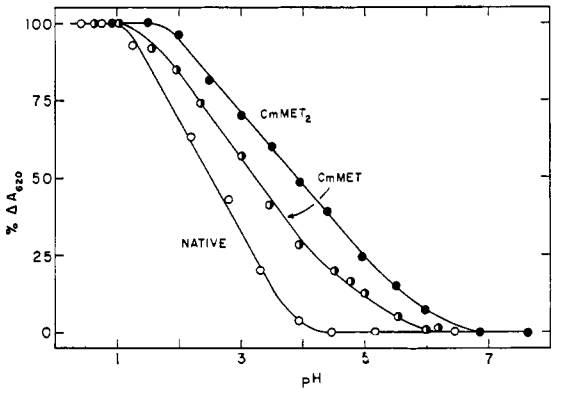


FIGURE 4: Change in the absorbance of cytochrome *c* at 620 m$\mu$ as a function of pH. (○) Ferricytochrome *c*, (◑) CmMET-ferricytochrome *c*, and (●) $CmMET_2$-ferricytochrome *c*. The solvent was 0.2 M KCl. Protein concentration ranged from 6 to 8 $\times 10^{-4}$ M.

carboxymethylation of one methionyl residue does not alter the perturbation of the Soret absorption of the heme moiety by 20% ethylene glycol. However, carboxymethylation of both methionyl residues increases the perturbation of the heme to a value intermediate between those observed for ferricytochrome *c* and cyanferricytochrome *c*, as shown in Figure 5B and Table I.

*Titration of Tyrosyl Residue.* Formation of cyanferricytochrome *c* or carboxymethylation of one or both methionyl residues lowers the apparent p*K* values of the phenolic hydroxyl groups of at least three of the four tyrosyl residues of ferricytochrome *c*. However, the lowered p*K* values are not equivalent to the apparent p*K* value observed for the four exposed tyrosyl residues in a tryptic digest of ferricytochrome *c*, as shown in Figure 6.

*Viscosity Measurements.* The reduced viscosity of ferricytochrome *c* is not increased by carboxymethylation of one or both methionyl residues as shown in Table I.

## Discussion

Many of the conformational features of ferricytochrome *c* are preserved upon formation of cyanferricytochrome *c*. The gross shape of the protein indicated by viscosity measurements (Table I) remains the same. Only a slight change is observed in the far-ultraviolet Cotton effect (Myer and Harbury, 1965), suggesting that the asymmetric environments of the contributing chromophores are largely unaltered. Although the apparent p*K* values of the phenolic hydroxyl groups of tyrosyl residues are shifted toward lower values by 0.1–0.5 pH unit (Figure 6), there is no increase in the num-

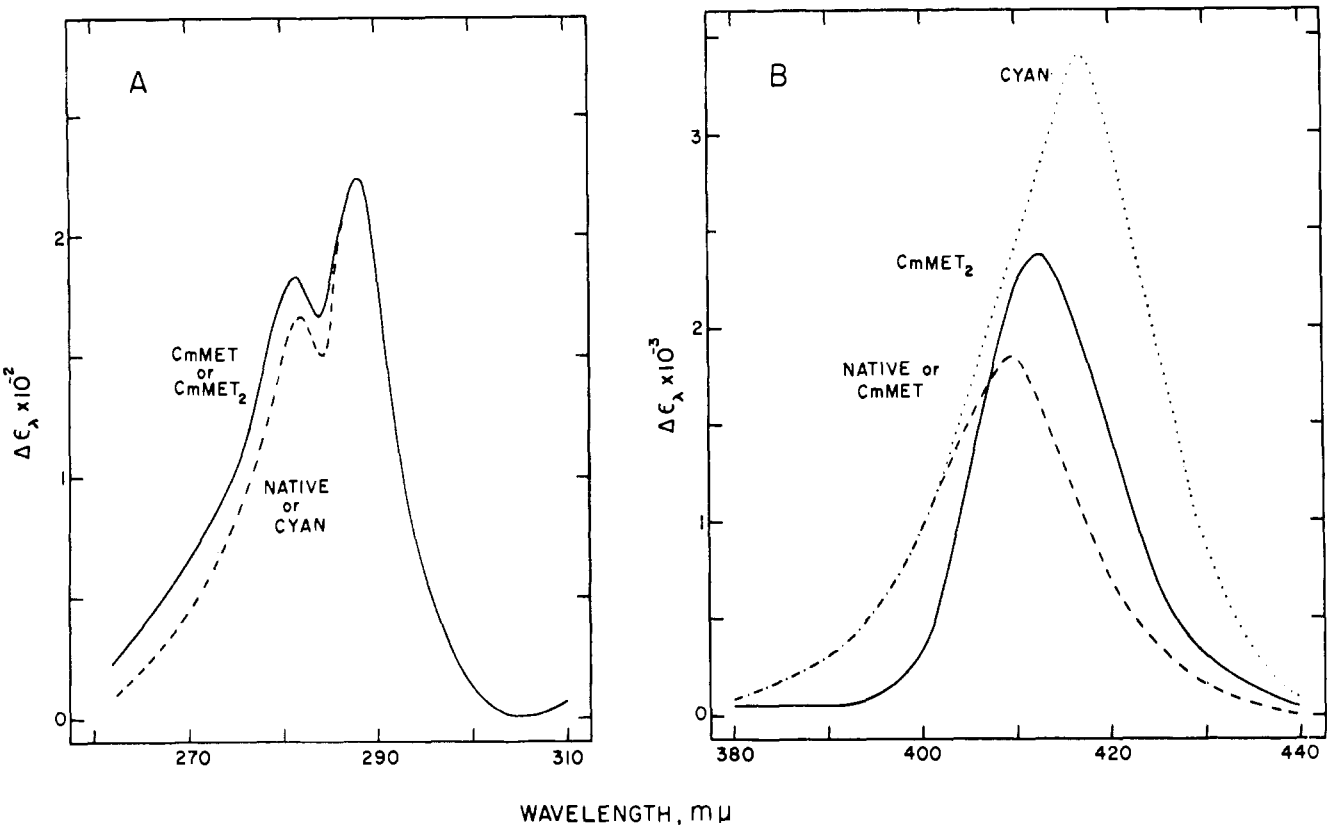


FIGURE 5: Solvent perturbation difference spectra of cytochrome *c* in 20% ethylene glycol. (A) – –, ferricytochrome *c* or cyanferricytochrome *c*; ——, CmMET-ferricytochrome *c* or $CmMET_2$-ferricytochrome *c*. All perturbation difference spectra were normalized to a $\Delta\epsilon_{310}$ of zero. (B) – – –, ferricytochrome *c*, or CmMET-ferricytochrome *c*; ——, $CmMET_2$-ferricytochrome *c*; · · · ·, cyanferricytochrome *c*. The protein solvent was 0.2 M KCl–0.1 M phosphate buffer (pH 7.0). Protein concentrations ranged from 1 to 10 × $10^{-5}$ M.

ber of tyrosyl residues which can be perturbed by ethylene glycol (Figure 5A). Two of the three histidyl residues continue to be unreactive with bromoacetate (Stellwagen, 1966; Harbury, 1966b) and the single tryptophanyl residue remains buried as indicated by solvent perturbation measurements (Figure 5A).

However, changes are observed in the environment of the heme moiety upon formation of cyanferricytochrome *c*. The heme moiety becomes more exposed, as indicated by an increase in the solvent perturbation of the Soret absorbance (Figure 5B). As first observed by Horecker and Kornberg (1946) and seen in Figure 3, the absorption band at 695 mμ disappears upon coordination of cyanide. Eaton and Hochstrasser (1966) have reported that the transition of this band is polarized approximately perpendicular to the plane of the porphyrin ring, suggesting the promotion of an electron from a nonporphyrin ligand into an iron–porphyrin orbital. If this interpretation is correct, substitution of a protein ligand would be expected to alter this absorption band. And finally, a formerly buried methionyl residue becomes chemically reactive upon formation of cyanferricytochrome *c*. These observations are consistent with the view that cyanide replaces methionyl residue 80, whose sulfur atom serves as a ligand for the heme iron of ferricytochrome *c*.

Carboxymethylation of methionyl 80 in cyanferricytochrome *c* would be expected to prevent the reversal of the altered properties of the protein following removal of the cyanide. As shown in Figures 3 and 5 and in Table I, the gross shape, absorption spectra, and structural environment of the heme moiety and the tyrosyl and tryptophanyl residues of ferricytochrome *c* are virtually unaltered by carboxymethylation of a single methionyl residue, residue 65 (Ando *et al.*, 1965; Harbury, 1966a,b), and a single histidyl residue, residue 33 (Matsubara *et al.*, 1965; Harbury, 1966a,b). Changes are observed in the apparent p*K* of the spectral transition at 620 mμ (Figure 4) to a high-spin complex and in the apparent p*K* of the abnormal tyrosyl residues (Figure 6). These changes probably reflect an increased susceptibility of the carboxymethylated protein to acidic and basic denaturation rather than a change in the specific structural environments of the heme moiety and the buried tyrosyl residues, respectively.

Carboxymethylation of the second methionyl residue likewise does not alter the gross shape or the structural environment of the buried tyrosyl, histidyl, or tryptophanyl residues. Changes are observed, however, in the spectral properties of the heme. The exposure of the heme moiety is increased to a value intermediate between that observed for CmMET-ferricytochrome *c* and cyanferricytochrome *c*, as shown in Figure 5B and Table I. The location of the maxima in the visible absorption spectra are unaltered by carboxymethylation of the second methionyl residue, although the extinc-

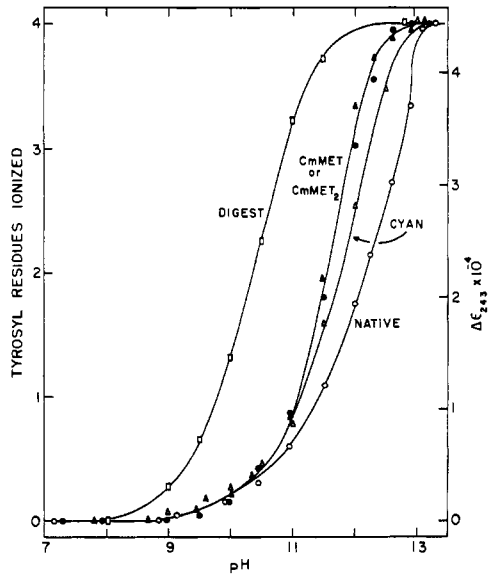


FIGURE 6: Spectrophotometric titrations of the tyrosyl residues of cytochrome *c*. (○) Ferricytochrome *c*, (△) cyanferricytochrome *c*, (●) CmMET-ferricytochrome *c*, (▲) $CmMET_2$-ferricytochrome *c*, and (□) tryptic digest of ferricytochrome *c*, from Stellwagen (1964). The solvent was 0.2 M KCl. Protein concentrations ranged from 1 to 2 × $10^{-5}$ M.

tions measured at these wavelengths are reduced by 10–40% (Table I). However, the absorption band at 695 mμ, which is present in CmMET-ferricytochrome *c* but lost upon formation of cyanferricytochrome *c*, also disappears upon carboxymethylation of both methionyl residues.

The spectrum of $CmMET_2$-ferricytochrome *c* in the neutral pH range is characteristic of a low-spin ferrichrome complex, indicating that the solvent does not provide a ligand for the heme iron. Therefore, the protein must provide the ligands for the fifth and sixth coordination positions of the heme iron of $CmMET_2$-ferricytochrome *c*. Since both the sulfur atom and the heme iron are positively charged in the carboxymethylated protein, the resultant electrostatic repulsion would be expected to prevent formation of a coordinate–covalent bond between these atoms. However, it can be demonstrated using Corey–Pauling–Koltun atomic models that the ionized carboxyl of the carboxymethyl group can be placed adjacent to the sulfur atom. Such a configuration would largely neutralize the formal positive charge on the sulfur atom and perhaps allow the formation of a CmMET-heme iron coordinate–covalent bond. Alternatively, an oxygen atom of the carboxymethyl group may be coordinated with the heme iron in $CmMET_2$-ferricytochrome *c*. It is also possible that an atom proximal to the heme in native ferricytochrome *c* would shift its position to coordinate with the heme iron in $CmMET_2$-ferricytochrome *c*. Although the ligand cannot be identified from the present study, the increased perturbation of the heme moiety in $CmMET_2$-ferricytochrome *c* and the absence of an absorption band at 695 mμ indicate that the geometry about the heme moiety is altered by carboxymethylation of MET 80.

As shown in Figure 2, the carboxymethylation of the second methionyl residue in ferricytochrome *c*, which is virtually complete at pH 3, precedes the loss of the 695-mμ absorption band as the pH is lowered. The disappearance of the 695-mμ absorbance parallels the appearance of the high-spin absorption band of ferricytochrome *c* at 620 mμ as shown in Figure 4, suggesting that both transitions reflect an unfolding of the protein about the heme. It is likely that the reactivity of both methionyl residues at pH 3, which has also been observed by Ando *et al.* (1965) and Tsai and Williams (1965), represents a displacement of an equilibrium which exists between the native conformation and a more unfolded form toward the latter by irreversibly carboxymethylating both exposed methionyl residues in the unfolded form.

The kinetics of the carboxymethylation of the methionyl residues of ferricytochrome *c* and cyanferricytochrome *c*, while similar, clearly are not first order as would be expected in the presence of a large excess of the alkylating agent. The reason for this is not clear, particularly in view of first-order kinetics observed for the carboxymethylation of the single histidyl residue using the same conditions (Stellwagen, 1966).

The simultaneous oxidation of both methionyl residues to their sulfoxide derivatives and the single tryptophanyl residues to its oxindole derivative with *N*-bromosuccinimide produces more drastic conformational changes (Stellwagen and Van Rooyan, 1967) than those observed here by carboxymethylation of both methionyl residues and a single histidyl residue. This may be attributed to the modification of a second buried group, the tryptophanyl residue, when *N*-bromosuccinimide is employed.

## Acknowledgment

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## References

Ando, K., Matsubara, H., and Okunuki, K. (1965), *Proc. Japan Acad. 41*, 79.

Eaton, W. A., and Hochstrasser, R. M. (1966), *in* Hemes and Hemoproteins, Chance, B., Estabrook, R. W., and Yonetani, T., Ed., New York, N. Y., Academic, p 581.

Fanger, M. W., Hettinger, T. P., and Harbury, H. A. (1967), *Biochemistry 6*, 713.

Harbury, H. A. (1966a), 151st National Meeting of the American Chemical Society, March, Pittsburgh, Pa., Abstract H30.

Harbury, H. A. (1966b), *in* Hemes and Hemoproteins, Chance, B., Estabrook, R. W., and Yonetani, T., Ed., New York, N. Y., Academic, p 391.

Harbury, H. A., Cronin, J. R., Fanger, M. W., Hettinger T. P., Murphy, A. J., Myer, Y. P., and Vinogradov, S. N. (1965), *Proc. Natl. Acad. Sci. U. S. 54*, 1658.

Hirs, C. H. W. (1956), *J. Biol. Chem. 219*, 611.

Horecker, B. L., and Kornberg, A. (1946), *J. Biol. Chem. 165*, 11.

Lemberg, R., and Legge, J. W. (1949), *in* Hematin Compounds and Bile Pigments, New York, N. Y., Interscience.
Margoliash, E., and Schejter, A. (1966), *Advan. Protein Chem. 21*, 113.
Matsubara, H., Ando, K., and Okunuki, K. (1965), *Proc. Japan Acad. 41*, 408.
Myer, Y. P., and Harbury, H. A. (1965), *Proc. Natl. Acad. Sci. U. S. 54*, 1391.
Neumann, N. P., Moore, S., and Stein, W. H. (1962), *Biochemistry 1*, 68.
Stellwagen, E. (1964), *Biochemistry 3*, 919.
Stellwagen, E. (1966), *Biochem. Biophys. Res. Commun. 23*, 29.
Stellwagen, E., and Van Rooyan, S. (1967), *J. Biol. Chem. 242*, 4801.
Tsai, H. J., and Williams, G. R. (1965), *Can. J. Biochem. 43*, 1409.

# Preferential Binding of Solvent Components to Proteins in Mixed Water–Organic Solvent Systems*

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ABSTRACT: The preferential interaction of lysozyme, bovine serum albumin, and insulin with one of the solvent components in water–2-chloroethanol mixtures has been investigated by the method of differential refractometry with the application of multicomponent theory. Similarly to the case found with β-lactoglobulin, as the chloroethanol contents increase, the three proteins interact preferentially first with 2-chloroethanol; then, after passing a maximum between 30 and 40 vol % of chloroethanol, this interaction decreases and is followed by a change to preferential hydration at about 60 vol %.

The preferential interaction of β-lactoglobulin A with solvent components in mixtures of water with ethylene glycol and methoxyethanol has been studied with the same technique. In these solvents, the effect is weaker than in the 2-chloroethanol system. In both systems, no significant excess binding of solvent components is detected below 30 vol %. Above this solvent composition, the organic solvent becomes progressively preferentially bound. These results are compared with those of conformational transition studies carried out in the same systems, and they are discussed in terms of the affinities of different amino acid residues for various types of media as the protein conformation is altered by the change in the medium.

When a macromolecule is dissolved in a mixed solvent (*e.g.*, water–single electrolyte or water–organic solvent), in general it will have a greater affinity for one of the solvent components and, therefore, will interact preferentially with that component over the other one. Such a preferential interaction can be detected by a variety of methods, for example, by a measurement of the buoyant behavior of the macromolecule in a density gradient (Cox and Schumaker, 1961; Vinograd and Hearst, 1962; Ifft and Vinograd, 1966), by the isopiestic measurement of vapor pressure (Hade and Tanford, 1967), by equilibrium sedimentation experiments in solvents of different densities (Schachman and Edelstein, 1966), by light-scattering measurements (Ewart *et al.*, 1946; Kay and Edsall, 1956; Read, 1960; Stauff and Mehrotra, 1961; Inoue and Timasheff, 1968a,b), by the comparison of the partial specific volume or refractive index increment before and after redistribution of solvent components across a membrane impermeable to the macromolecule (Vrij, 1959; Kielley and Harrington, 1960; Casassa and Eisenberg, 1961; Vrij and Overbeek, 1962; Noelken and Timasheff, 1967; Inoue and Timasheff, 1968b). The last method is rather simple to use if the two components of the mixed solvent have nonidentical refractive indices.

Partial specific volume (Kielley and Harrington, 1960), isopiestic (Hade and Tanford, 1967), and refractive index increment studies (Noelken and Timasheff, 1967) have shown that many proteins bind preferentially the salt component when dissolved in aqueous guanidine hydrochloride, if the reference state is chosen as that of equal molality of the salt on the two sides of the membrane. Light-scattering and refractive index increment measurements (Stauff and Mehrotra, 1961; Inoue and

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