Pseudomonas Cytochrome *c*. I. Effect of Modification of the Amino Groups*

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ABSTRACT: *Pseudomonas* cytochrome *c* (551), which contains only one histidine residue, remains a hemochrome to very low values of pH. Guanidination of the lysine residues, followed by acetylation of the α-amino group, does not alter this feature. Hemochrome-type spectra are exhibited also by a derivative in which all the amino groups are trifluoroacetylated (experiments at pH 7). The simplest interpretation is that, at neutral and acid pH, the heme iron is not bound to an amino group, and that the central coordination complex of this molecule corresponds to neither of the two possibilities—coordination of the iron to two histidine residues, or, alternatively, coordination to a histidine residue and a lysine residue—that have commonly been considered for the cytochromes *c*.

Structure–function studies of the cytochromes *c* have thus far been limited largely to molecules of the "mammalian type."[1] However, as sequence information becomes available for an increasing number of cytochromes *c* not in this category, opportunities will present themselves for the selection of a broader group of test systems, certain of which may offer special experimental advantages.

In the present study, which concerns the nature of the ligand groups, use has been made of a distinctive feature of *Pseudomonas* cytochrome *c*.[2] It has long been held that the heme iron of the cytochromes *c* is coordinated to two nitrogenous groups of the protein, these having been postulated to be the side chain groups of two histidine residues (Theorell and Åkeson, 1941), or of a histidine residue and a lysine residue (Margoliash *et al.*, 1959; Margoliash, 1962). However, *Pseudomonas* cytochrome *c*, in contrast to the other cytochromes *c* thus far examined, contains just one histidine residue (Ambler, 1963a,b). Of the proposed structures, the first is thus untenable in this instance, and the choice is narrowed to: (a) the second possibility, involving the coordination of an amino group; and (b) structures different from those considered to date.

Were an amino group coordinated to the iron, this should be discernible by the application of amino group modification procedures. We deal here with results of trifluoroacetylation and guanidination–acetylation of the molecule.

Materials and Methods

Materials. Pseudomonas fluorescens was grown from a culture kindly provided us by Dr. N. O. Kaplan. The conditions for growth and the methods for extraction and purification of the cytochrome *c* were those given by Ambler (1963a).

Guanidination, trifluoroacetylation, and dinitrophenylation were performed as described previously (Hettinger and Harbury, 1964; Fanger and Harbury, 1965). Conditions for acetylation were patterned after those given by Fraenkel-Conrat *et al.* (1949). Guanidinated cytochrome *c* (25 mg) in 0.6 ml of half-saturated sodium acetate was treated at 0° with 60 μl of acetic anhydride, added in three portions, with shaking, over a period of 40 min. After another 30 min, the product was dissolved in 2.4 ml of 0.1 M phosphate buffer of pH 7.5, desalted by gel filtration (Sephadex G-25), and lyophilized.

Amino Acid Analysis. Samples were hydrolyzed with constant-boiling hydrochloric acid in evacuated ampoules at 110°. For compounds other than dinitrophenyl (DNP) derivatives, the period of hydrolysis was 24 hr. For DNP derivatives, it was 16 hr. Quantitative analyses were obtained with a Beckman-Spinco Model 120B automatic amino acid analyzer (Spackman *et al.*, 1958). Qualitative analysis of DNP-amino acids was performed by paper chromatography (Fraenkel-Conrat *et al.*, 1955).

Spectrophotometry. Absorption spectra were recorded with Cary Model 11 and Bausch and Lomb Spectronic 505 spectrophotometers. Measurements were made under anaerobic conditions in a closed system fitted with a cuvet, electrodes for pH determinations, and microburets for the addition of titrants.[3] Samples were oxidized with potassium ferricyanide and reduced with sodium dithionite.

* From the Department of Biochemistry, Yale University, New Haven, Connecticut. *Received September 27, 1966.* This work was supported by grants from the National Institutes of Health (GM-07317) and the National Science Foundation (GB-1556). Brief reports have been presented (Harbury *et al.*, 1965; Hettinger *et al.*, 1966; Harbury, 1966).

[1] For a definition of this term, *cf.* Margoliash (1962).

[2] The preparation which has been referred to as *Pseudomonas* cytochrome *c*-551 (Ambler, 1963a,b; Horio *et al.*, 1960).

[3] Y. P. Myer and H. A. Harbury, to be published.

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

Amino Acid	Residues/Mole of Protein[a]	
	Cytochrome *c*[b]	Guanidinated Cytochrome *c*[c]
Lysine	7.9 (8)	0.14
Histidine	1.0 (1)	0.8
Arginine	1.0 (1)	0.9
Homoarginine	0 (0)	7.9
Aspartic acid	8.0 (8)	8.1
Threonine	2.0 (2)	2.1
Serine	2.8 (3)	2.8
Glutamic acid	10.1 (10)	10.0
Proline	5.4 (6)	5.6
Glycine	6.9 (7)	7.0
Alanine	13.1 (13)	13.1
Half-cystine[d]	1.3 (2)	1.3
Valine	6.4 (7)	6.1
Methionine[e]	1.8 (2)	1.6
Isoleucine	2.6 (3)	2.5
Leucine	3.9 (4)	3.9
Tyrosine	0.8 (1)	0.8
Phenylalanine	1.6 (2)	1.7

[a] No corrections were made for loss on hydrolysis. [b] Literature values (Ambler, 1963a) in parentheses. [c] Sample treated with *O*-methylisourea for 100 hr. [d] Thioether bridges not cleaved prior to hydrolysis. [e] Includes methionine sulfoxides.

TABLE II: Amino Acid Analysis of DNP-Proteins.

Amino Acid	Sample Dinitrophenylated (residues/mole of protein)	
	Pseudomonas Cytochrome *c*	Trifluoroacetylated *Pseudomonas* Cytochrome *c*
Lysine	0.6	8.0
Histidine	0.1	0.1
Arginine[a]	1.0	1.0
ε-DNP-Lysine	7.5	0

[a] Taken as reference.

Results

Table I gives the amino acid compositions found before and after treatment of the protein for 100 hr with *O*-methylisourea. The reaction resulted in a decrease in the lysine content, from 7.9 to 0.14 residues/mole, and a corresponding increase in the homoarginine content. All the lysine residues were thus modified.

Dinitrophenylation and acid hydrolysis of the guanidinated protein led to the formation of DNP-glutamic acid in yields approximately equal to those obtained in the case of the nonguanidinated preparation (paper chromatography). The reaction with *O*-methylisourea was thus largely without effect on the α-amino group of the molecule. However, this group was easily blocked by treatment of the guanidinated preparation with acetic anhydride. Upon dinitrophenylation and hydrolysis of the guanidinated–acetylated product, no DNP-glutamic acid could be detected.

A derivative with no free amino groups was prepared also by trifluoroacetylation of the *Pseudomonas* molecule. As shown in Table II, dinitrophenylation of this derivative yielded no ε-DNP-lysine, in contrast to a value of 7.5 residues of ε-DNP-lysine/mole of protein obtained upon dinitrophenylation of the nontrifluoroacetylated molecule; and, upon paper chromatography, no DNP-glutamic acid was observed.

Figure 1 shows that the visible absorption spectra of the trifluoroacetylated and guanidinated–acetylated preparations at pH 7 are very similar to those of the unmodified molecule. In both oxidation states, the derivatives remain typical hemochromes.[4] Furthermore, the guanidinated–acetylated molecule retains its hemochrome character to the same low values of pH as does the untreated protein.[5] Reduced *Pseudomonas* cytochrome *c*, whether its amino groups were free or guanidinated–acetylated, was found still to be a hemochrome at pH 1.5, the lower limit of the pH range investigated. Oxidized *Pseudomonas* cytochrome *c*, guanidinated–acetylated or unmodified, exhibited a change in spectrum to the high-spin type only at the low values of pH where such a transition occurs in the case of the "mammalian-type" cytochromes *c* (Figure 2).

Discussion

All cytochromes *c* thus far studied remain hemochromes to very low values of pH. In the case of the oxidized form, conversion to the high-spin state occurs with a midpoint of change about four units below the range of p*K* values usually assigned to the imidazole groups of proteins, and some eight units below the p*K* values for ε-amino groups. Even larger differences hold for the systems in the reduced form.

If the hemochrome character of *Pseudomonas*

[4] The reduced guanidinated–acetylated preparation exhibited a small band near 580 mμ (Figure 1). This band occurred also in the spectrum of guanidinated *Pseudomonas* cytochrome *c* not subjected to acetylation (not shown), but has not been seen in the spectra of guanidinated horse or tuna cytochrome *c* (Hettinger and Harbury, 1964, 1965).

[5] The trifluoroacetylated derivative is insufficiently soluble at low pH.

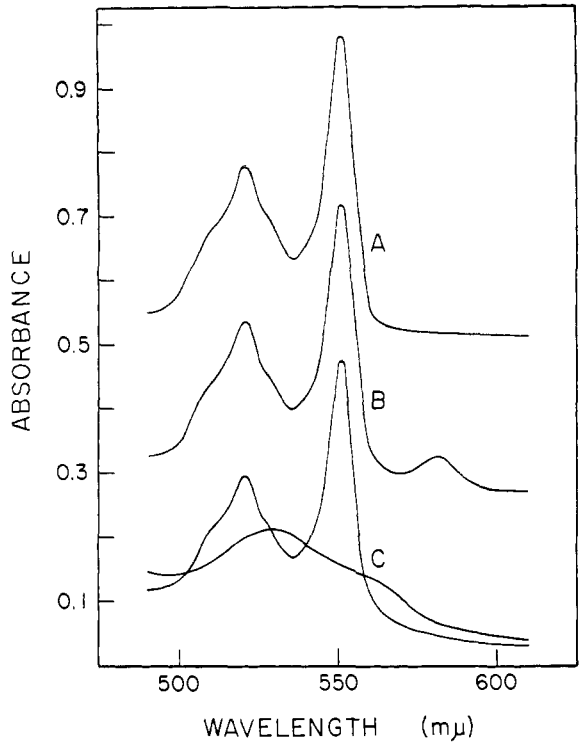

FIGURE 1: Spectra of *Pseudomonas* cytochrome *c* and its trifluoroacetylated and guanidinated–acetylated derivatives. Concentration ≅ 1.7 × 10^{-5} M; pH 7.0; temperature 22°; 1-cm cuvet; curves A and B displaced 0.4 and 0.2 absorbance unit, respectively. (A) *Pseudomonas* cytochrome *c*, reduced form; (B) guanidinated–acetylated *Pseudomonas* cytochrome *c*, reduced form; (C) trifluoroacetylated *Pseudomonas* cytochrome *c*, reduced form; and unmodified, guanidinated–acetylated, and trifluoroacetylated *Pseudomonas* cytochrome *c*, oxidized form.

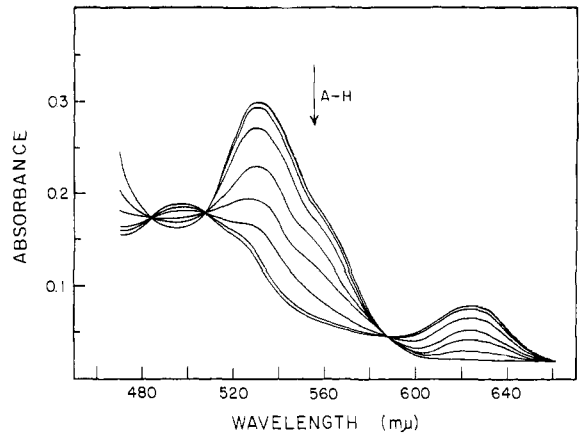

FIGURE 2: Spectra of guanidinated–acetylated *Pseudomonas* cytochrome *c*, oxidized form. Concentration ≅ 5 × 10^{-6} M; temperature 30°; 5-cm cuvet. (A) pH 4.1; (B) pH 3.0; (C) pH 2.6; (D) pH 2.4; (E) pH 2.2; (F) pH 2.0; (G) pH 1.8; and (H) pH 1.6.

cytochrome *c*, with its single histidine residue, is construed to be indicative of the coordination of an amino group, the retention of this structure to low pH would be more easily accounted for, in terms of the cooperative effects required, were the ligand in question the α-amino group rather than an amino group of a lysine residue. In many of the "mammalian-type" cytochromes *c*, there is an acetyl group at the amino terminus of the chain, but in *Pseudomonas* cytochrome *c* the α-amino group is free, and thus potentially available for such a role. However, trifluoroacetylation of the α- as well as all eight ε-amino groups results in a derivative which remains a hemochrome at neutral pH, and if the lysine residues are guanidinated and the α-amino group acetylated, to yield a derivative soluble in acid solution, it is found that the hemochrome character is retained to the same low values of pH as in the case of the unmodified system.

Although it will be desirable to have additional evidence that the ligand groups in the modified preparations are the same ones operative prior to modification, the simplest interpretation of these findings is that, at neutral and acid pH, coordination of an amino group to the heme iron does not occur in this molecule. This parallels the inference drawn previously in relation to horse and tuna cytochrome *c*. In the more detailed studies of these two proteins, it was found that guanidination of the amino groups is compatible with the retention of electron-transfer activity in the succinate oxidase and cytochrome oxidase systems, and is without effect not only on the hemochrome spectrum, but also on the oxidation–reduction potential and optical rotatory dispersion pattern at neutral pH (Hettinger and Harbury, 1964, 1965; Myer and Harbury, 1965).

Taken alone, the results of the amino group modification studies of horse and tuna cytochrome *c* allow the possibility that the heme iron is coordinated to two histidine residues. However, recent sequence determinations by Heller and Smith (1965, 1966) and by Stewart *et al.* (1966) have shown that although each of the "mammalian-type" cytochromes *c* thus far studied contains two or more histidine residues, only one of these, histidine 18, is common to all. If it is assumed that the ligand groups in the "mammalian-type" molecules are invariant both in nature and position, it follows that the number of coordinated histidine side chains cannot exceed one (Heller and Smith, 1965, 1966; Stewart *et al.*, 1966). That, of course, is necessarily so in the case of *Pseudomonas* cytochrome *c*, and evidence against amino group coordination in this protein can be reconciled with the concept of binding to two nitrogenous ligands only by turning to some group not previously considered a likely candidate for such a role. Obviously, there remain in this connection opportunities for further inquiry. However, the question might well also be asked whether the basic premise of coordination to two nitrogenous protein

groups is in fact a valid one (Harbury *et al.*, 1965; Heller and Smith, 1965, 1966). Although such a structure is consistent with the results of many experimental observations, the evidence in its support is not conclusive, and there are other possibilities. Experiments dealing with one of these, the coordination of a methionine residue, are summarized in an accompanying paper (Fanger *et al* 1967).

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