Guanidinated Cytochrome *c*. II*

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ABSTRACT: Tuna heart cytochrome *c* can be fully guanidinated by reaction with *O*-methylisourea, and the product is active in the succinate oxidase system. At neutral and acid pH, the visible absorption spectra of the guanidinated and nonguanidinated protein correspond closely to one another and to those of guanidinated and nonguanidinated horse heart cytochrome *c*.

Only in the case of the oxidized forms at alkaline pH are appreciable differences observed. The two guanidinated preparations in the reduced state remain hemochromes throughout the range of pH investigated (pH 1.5–12.2), and in the oxidized form in acid solution undergo the same transition observed with unmodified material. There is little or no difference in the oxidation–reduction potentials at pH 7. It would appear that at neutral and acid pH the four preparations do not differ in the type of groups coordinated to the heme iron. If these are, as generally held, two nitrogenous side-chain groups, the simplest interpretation consistent with present evidence is that the iron is bound to histidine residues 18 and 26.

Modification of horse heart cytochrome *c* by guanidination of all the lysine residues results in a product which remains a hemochrome to the same low values of pH as does the unaltered molecule, and which is active in the succinate oxidase and cytochrome oxidase systems (Hettinger and Harbury, 1964). Trifluoroacetylation of all the lysine residues results in an inactive product, essentially insoluble below pH 6 and subject to deacylation at pH 10 and above, but which at pH 7–9 has a visible spectrum virtually indistinguishable from that of either guanidinated or unmodified preparations (Fanger and Harbury, 1965). If it is assumed that the iron is bound to two nitrogenous side-chain groups of the protein, the simplest interpretation of both sets of observations is that the coordinated groups in horse heart cytochrome *c* at neutral and acid pH are not a histidine residue and a lysine residue (Margoliash *et al.*, 1959; Margoliash, 1962), but two histidine residues (Theorell and Åkeson, 1941).

There are in horse heart cytochrome *c* a total of three histidine residues, located at positions 18, 26, and 33 from the amino terminus (Margoliash *et al.*, 1961). Cytochrome *c* from tuna heart, on the other hand, has histidine residues only in positions 18 and 26 (Kreil, 1963). Thus, were it established that this protein is a diimidazole complex at physiologic pH, its iron-coordinated residues would be fully defined. The only data bearing on the ligand groups of a fish heart cytochrome *c* (salmon) have, however, been taken to indicate that these are not both imidazole groups (Paléus, 1954), and, in the absence of further information, the type of coordination complex represented by the tuna molecule has been left an open question (Kreil, 1963).

If the iron of tuna heart cytochrome *c* at physiologic pH were in fact not coordinated to two histidine residues, but to a histidine residue and a lysine residue, this should find expression in the response of the molecule to amino group modification procedures. We report here on the effects of guanidination.

Materials and Methods

Materials. Tuna hearts (Pacific Albacore; *Thunnus germo*) were supplied in frozen form by the Van Camp Sea Food Co., Port of Long Beach, Calif.[1] Cytochrome *c* was extracted as outlined by Kreil (1963) and then purified chromatographically (Margoliash, 1957). The product had an iron content of 0.43%.

Horse heart cytochrome *c* was obtained from the Sigma Chemical Co. (Type III, iron content 0.43%). Guanidinated horse heart cytochrome *c* and a heme hexadecapeptide from horse heart cytochrome *c* were materials prepared previously (Hettinger and Harbury, 1964). Partially succinylated horse heart cytochrome *c* was prepared as described by Takemori *et al.* (1962). Estimates based on electrophoretic mobility indicated about a fourth of the lysine residues were in modified form. *O*-Methylisourea hydrochloride, synthesized as described by Kurzer and Lawson (1954) and stored at −15° in evacuated ampoules (Klee and Richards, 1957), was the same sample used in earlier work (Hettinger and Harbury, 1964).

Guanidination of Tuna Heart Cytochrome c. In the studies with cytochrome *c* from horse heart, no differences were seen in the extent of guanidination at-

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tainable with samples of different degree of reduction (Hettinger and Harbury, 1964). Accordingly, no attempt was made to control the oxidation state of the tuna heart samples subjected to modification. All were approximately 95% in the oxidized form. The reaction was carried out at 2° for periods of 80, 100, and 150 hr. In each experiment, the cytochrome *c* was added to a solution of *O*-methylisourea of pH 8.0–8.5, and the pH then adjusted to 11.0 with 6 M sodium hydroxide. The final concentration of cytochrome *c* was 1%, and that of *O*-methylisourea, 0.5 M. Upon conclusion of a reaction period, the solution was dialyzed against 0.05 M phosphate buffer of pH 7.0, then dialyzed against water, filtered to remove small amounts of insoluble material, and lyophilized. In the case of the reaction carried out for 150 hr, the sample was dialyzed and lyophilized at the end of 100 hr, and then was treated anew with *O*-methylisourea for an additional 50 hr.

Chromatography of the guanidinated preparations on Amberlite CG-50 yielded an elution pattern very similar to that obtained with the nonguanidinated protein. There was little or no material corresponding to fraction II (Margoliash, 1957).

Amino Acid Analysis. Samples were hydrolyzed with constant boiling hydrochloric acid in evacuated ampoules at 110° for 24 hr. Quantitative analyses (Moore *et al.*, 1958) were obtained with a Beckman-Spinco Model 120B automatic amino acid analyzer.[2]

Determination of Activity. Measurements of electron transfer activity were carried out with the use of a rat liver succinate oxidase system (Schneider and Potter, 1943). The conditions were those outlined previously (Hettinger and Harbury, 1964). To facilitate comparisons, tuna heart and corresponding horse heart preparations were assayed concurrently.

Spectrophotometry. Measurements were made under anaerobic conditions, in an apparatus operated as a closed system. Titrating agents were added, and pH and spectra were monitored continuously, without exposure of samples to the atmosphere. Spectra were recorded with Cary Model 11 and Bausch and Lomb Spectronic 505 spectrophotometers, and measurements of pH were made with a Radiometer instrument Model PHM 4c.

Oxidation–Reduction Potentiometry. E_m values are based on potentials measured at several ratios of oxidant to reductant, ranging from about 3 to 0.3, the degree of reduction being calculated from the absorption spectrum. The concentration of cytochrome *c* was 1×10^{-4} M. Methyl viologen served as the reducing agent, and 2,6-dichlorophenolindophenol, at a concentration of 3×10^{-6} M, was used as mediator. Procedures and apparatus were essentially those described previously (Harbury, 1957; Harbury and Loach, 1959).

Results

Extent of Guanidination. Reaction of the tuna heart cytochrome *c* with *O*-methylisourea for 150 hr led to a diminution in lysine content from 15.7 residues/mole to 0.29 residue/mole, and a corresponding increase in the homoarginine content of the protein. Limitation of the reaction period to 100 hr resulted in a lysine analysis of 0.39 residue/mole, and, after 80 hr of reaction, a value of 0.45 residue/mole was obtained. The modification procedure was without effect on amino acids other than lysine (Table I).

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

Amino Acid	Residues/Mole of Protein[a] Cytochrome *c*[b]	Guanidinated Cytochrome *c*[c]
Lysine	15.7 (16)	0.34
Histidine	2.0 (2)	1.9
Arginine	2.2 (2)	2.2
Homoarginine	0 (0)	15.8
Aspartic acid	10.2 (10)	10.1
Threonine	6.5 (7)	6.5
Serine	3.7 (4)	3.5
Glutamic acid	9.5 (9)	9.4
Proline	3.2 (3)	3.2
Glycine	12.7 (13)	12.6
Alanine	7.2 (7)	7.2
Half-cystine[d]	1.3 (2)	1.2
Valine	5.8 (6)	5.7
Methionine	1.9 (2)	1.9
Isoleucine	3.8 (4)	3.7
Leucine	6.0 (6)	5.9
Tyrosine	4.6 (5)	4.6
Phenylalanine	3.0 (3)	2.9

[a] The measured values were adjusted to residues per mole of protein by use of a constant multiplying factor, obtained from the following 10 amino acids upon assumption of the composition $(Lys + Homoarg)_{16}His_2Arg_2Asp_{10}Glu_9Gly_{13}Ala_7Val_6Ileu_4Leu_6$. No corrections were made for loss on hydrolysis. [b] Average of two analyses. Literature values (Kreil, 1963) in parentheses. [c] Average of two analyses. Samples (95% oxidized form) were treated with *O*-methylisourea for 100 and 150 hr. [d] Thioether bridges not cleaved prior to hydrolysis.

In our earlier experiments with preparations from horse heart, reaction periods of 100–120 hr were found to result in lysine contents of some 0.1 residue/mole. Full modification may thus be attained somewhat more slowly with the protein from tuna than with that from horse. There has, however, been no need, for the purposes of the present work, to bring the residual lysine content below the level of 0.3 residue/mole obtained in 100–150 hr, and detailed studies of rates or of the effects

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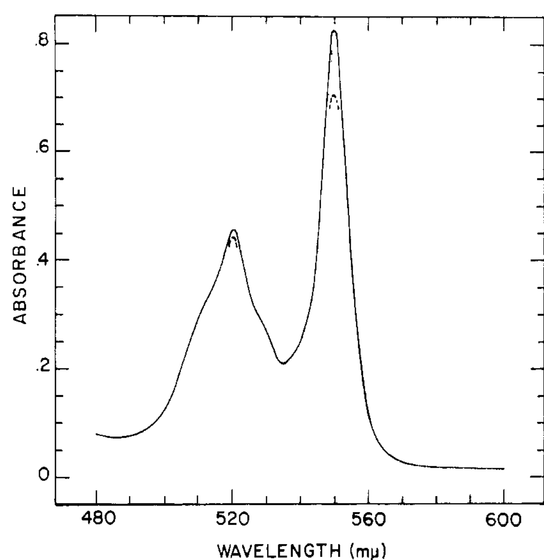

FIGURE 1: Spectra of guanidinated tuna heart cytochrome *c*, reduced form. Concentration, 5.4×10^{-6} M; 30°; 5-cm cuvet. Solid line, pH 7.10; broken line, pH 1.63.

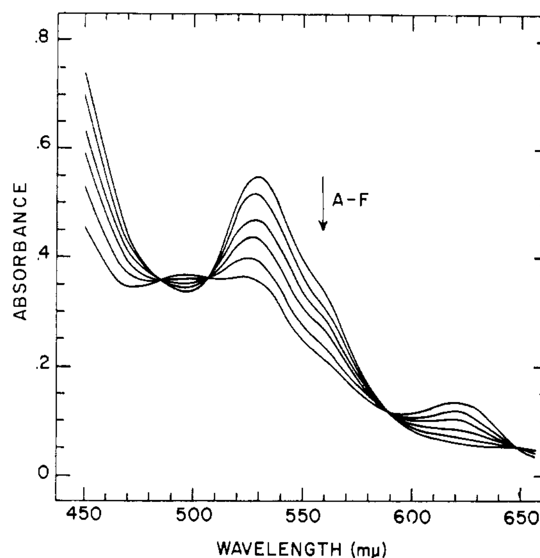

FIGURE 2: Spectra of guanidinated tuna heart cytochrome *c*, oxidized form. Concentration, 9.5×10^{-6} M; 30°; 5-cm cuvet. A, pH 6.91; B, pH 3.38; C, pH 2.72; D, pH 2.44; E, pH 2.11; F, pH 1.52.

of periods of treatment greater than 150 hr have not been undertaken.

Activity. The results of assays with the rat liver succinate oxidase system are summarized in Table II.

TABLE II: Effects of Cytochrome *c* and Cytochrome *c* Derivatives on Succinate Oxidase System of Rat Liver.

Sample	Oxygen Uptake (μl min⁻¹) Sample Concn (μM) 1.5	3.0	9.0
Tuna	2.2	3.7	5.4
Tuna, guanidinated	3.2	4.8	5.6
Horse	2.2	3.7	5.6
Horse, guanidinated	3.7	5.0	5.1
Horse, succinylated[a]			0
Hexadecapeptide			0

[a] Partially; about a fourth of the lysine residues in modified form.

Guanidinated tuna cytochrome *c* was found to be fully as active as the nonguanidinated molecule and, in fact, over the lower part of the concentration range covered, stimulated succinate oxidation more markedly than did the unmodified protein. At a concentration of 1.5×10^{-6} M, guanidinated tuna heart cytochrome *c* yielded a rate of oxygen uptake 46% greater than that obtained with nonguanidinated material. At 3×10^{-6} M, the difference was 30%, and at 9×10^{-6} M it was 4%. Unmodified tuna cytochrome *c* stimulated oxygen uptake to the same degree as did horse heart cytochrome *c*.

The activity of the guanidinated preparations[3] contrasted sharply with the results obtained with partially succinylated horse heart cytochrome *c* (*cf.* also Takemori *et al.*, 1962) and a heme hexadecapeptide from horse heart cytochrome *c*. Neither of these compounds gave a rate of oxygen uptake exceeding that of the blank.

Spectra and Potentials. The Soret and visible absorption spectra of guanidinated and nonguanidinated tuna heart cytochrome *c* at neutral and acid pH were found to correspond closely to those of horse heart cytochrome *c*. In Table III are listed some molar absorptivity values, calculated from single measurements and thus approximations only, but which serve adequately to illustrate the degree of concordance observed. Reduced tuna cytochrome *c*, whether guanidinated or nonguanidinated, remained a hemochrome to the lowest values of pH investigated (Figure 1). Guanidinated and nonguanidinated tuna cytochrome *c* in the oxidized state displayed, at acid pH, the same change in spectrum seen with the protein from horse heart (midpoint pH 2.5). A representative family of curves is shown in Figure 2.

The agreement between guanidinated and nonguanidinated samples, as well as between preparations from tuna and horse, was found to extend also to the oxidation–reduction potentials measured at pH 7. As indicated in Table III, the E_{m7} values of the modified and unmodified systems are all within 0.01 v of one another.

[3] For earlier work with partially guanidinated cytochrome *c*, *cf.* Takahashi *et al.* (1958) and Takemori *et al.* (1962).

TABLE III: Oxidation–Reduction Potentials and Molar Absorptivities[a] of Cytochrome *c* and Guanidinated Cytochrome *c*, pH 6.85.[b]

Cytochrome *c*	E_m (v)	Molar Absorptivity[a] (M^{-1} cm^{-1}) 409 mμ (ox)	530 mμ (ox)	415 mμ (red)	520 mμ (red)	550 mμ (red)
Horse	+0.254	1.09×10^5	1.17×10^4	1.44×10^5	1.68×10^4	2.99×10^4
Horse, guan.	+0.245	1.04×10^5	1.12×10^4	1.37×10^5	1.63×10^4	2.84×10^4
Tuna	+0.250	1.09×10^5	1.16×10^4	1.41×10^5	1.67×10^4	3.05×10^4
Tuna, guan.	+0.247	1.08×10^5	1.19×10^4	1.40×10^5	1.70×10^4	3.05×10^4

[a] Approximate only, see text. Based on iron content. [b] Phosphate buffer (0.1 M), 30°.

At alkaline pH, the oxidized form of guanidinated tuna cytochrome *c*, like that of guanidinated horse heart cytochrome *c*, displayed spectra differing significantly from those of the nonguanidinated compound (Hettinger and Harbury, 1964). A detailed analysis has not yet been undertaken.

Other Observations. Guanidinated preparations exhibited a greater susceptibility to denaturation than did unmodified samples. Heating to 100° for 1 min caused complete precipitation. The same treatment was without visible effect on unmodified cytochrome *c*.

The rate of autoxidation of guanidinated samples, as gauged by the catalysis of ascorbic acid oxidation, was about the same as that of nonguanidinated material.

Discussion

It generally has been assumed that one of the iron-coordinated groups of horse heart cytochrome *c* is contributed by histidine residue 18, located next to one of the thioether bridges to the prosthetic group (Tuppy and Paléus, 1955; Margoliash *et al.*, 1961), and thus presumably in an especially favorable position for such binding. In models constructed of a heme undecapeptide segment of the molecule, complex formation is easily effected (Ehrenberg and Theorell, 1955; Margoliash *et al.*, 1959), and comparative studies of histidine-free and histidine-containing heme octapeptide derivatives have demonstrated conclusively that, at least at the small-peptide level, the proposed coordination indeed takes place (Myer and Harbury, unpublished data; Harbury *et al.*, 1962).

The greater uncertainty has concerned the identity of the group in coordination position 6. If the ε-amino group of lysine is considered ruled out at neutral pH (Hettinger and Harbury, 1964; Fanger and Harbury, 1965), and the usual assumption is made that the iron is bound to two nitrogenous side-chain groups, the choice in effect is between the remaining histidine residues of the molecule, residues 26 and 33. The fact that histidine 33 is not present in all "mammalian-type" cytochromes *c* (Kreil, 1963) would seem to favor histidine 26. Implicit in this, however, is the assumption that the coordinated residues in "mammalian-type" cytochromes *c* are in all instances the same, a point on which there is but limited evidence. Most studies dealing with the nature of the central coordination complex have been restricted to cytochrome *c* from horse or beef heart, and few data are available for preparations from other sources. Among the more relevant results are those of some comparative acid–base titrations of beef, chicken, and salmon cytochromes *c*, and these data have, in fact, been interpreted as indicating that one of the ligands in salmon cytochrome *c* differs from the groups coordinated in the other two proteins (Paléus, 1954).

Tuna heart cytochrome *c* contains, like salmon cytochrome *c* (Paléus, 1954), just two histidine residues and, since in this case a complete sequence analysis is available (Kreil, 1963), was chosen for the comparative guanidination experiments reported here. The histidine residues occur in positions 18 and 26, position 33 being taken by a tryptophan residue.

This substitution of tryptophan for histidine is, for the purposes of the present study, the most important difference in the sequences of horse and tuna cytochromes *c*, but it is not the only one. The two proteins differ in some 20 positions, about one in five, and, though the net charge at physiologic pH remains the same, 8 of the substitutions involve a change in charge type, and 17 a change in functional side-chain group. There could thus be significant dissimilarities in properties quite unrelated to the difference in histidine content, and it is fortunate that these did not present themselves to a degree sufficient to be a problem. The only effect noted was a small difference in the apparent rates of reaction with *O*-methylisourea.

The principal results of guanidination are strikingly similar for the two proteins. As in the case of horse heart cytochrome *c*, all lysine residues of tuna cytochrome *c* can be converted to homoarginine. The fully guanidinated product is active in the succinate oxidase system, and its Soret and visible spectra at neutral and acid pH, as well as its oxidation–reduction potential at pH 7, differ little if at all from those of the unmodified protein. Whatever the role of the lysine residues, there is no indication that the fundamental properties of the molecule at physiologic pH or below depend

specifically upon the presence of amino groups.

The most straightforward interpretation, for tuna as for horse heart cytochrome *c*, is that lysine is not, at neutral and acid pH, one of the groups coordinated to the heme iron. There are, of course, alternative possibilities, for example, that lysine does contribute one of the iron-coordinated groups, but that this function is assumed by homoarginine without loss of activity or change in the potentials and spectra recorded, but such constructions seem unlikely and receive little support from other recent observations. Thus, relevant to the illustration used, it has been found that not only the guanidinated derivative of horse heart cytochrome *c*, but also fully trifluoroacetylated preparations retain, at neutral pH, the hemochrome spectrum characteristic of the parent molecule (Fanger and Harbury, 1965).

Two structures for the central coordination complex of cytochrome *c* have received serious consideration over the years. According to the one view, the iron is bound to two imidazole groups of the protein (Theorell and Åkeson, 1941; Paul, 1951). According to the other, the ligands are an imidazole group and an amino group (Margoliash *et al.*, 1959; Margoliash, 1962; Butt and Keilin, 1962). The results for the protein from tuna heart, like those for horse heart cytochrome *c*, clearly weigh against the second hypothesis, but can be accounted for very simply in terms of the first. In the absence of evidence supporting some third possibility, the more likely structure for both molecules at physiologic pH would appear thus to be one involving coordination of the heme iron to two histidine residues. These would have to be, in the case of the molecule from tuna heart, residues 18 and 26, and presumably the same two histidine residues would be the ones operative in horse heart cytochrome *c*.

Added in Proof

Further studies have raised the possibility that coordination of methionine may occur among the cytochromes *c* (Harbury *et al.*, 1965).

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