Structural Location of the Tyrosyl and Tryptophanyl Residues of Tuna Heart Cytochrome *c**

Earle Stellwagen† and Eleanor B. McGowan‡

ABSTRACT: Spectrophotometric titration and solvent perturbation measurements both indicate that three of the five tyrosyl residues of tuna heart ferricytochrome *c* are exposed. However, four tyrosyl residues, located at positions 46, 48, 67, and 74, are iodinated under conditions which do not cause a gross change in the protein structure. Spectrophotometric titration of the iodinated protein indicates that the completely unreactive tyrosyl residue at position 97 is buried in the native protein. Assuming that tuna heart and horse heart ferricytochrome *c* have homologous conformations, it is proposed that the other buried residue is tyrosyl 48. Analysis of the spectrophotometric titration of the tyrosyl residues of tuna ferrocytochrome *c* indicates that all five tyrosyl residues are buried in the reduced protein at neutral pH. Titration of the first three tyrosyl residues in ferrocytochrome *c* coincides with the oxidation of the heme iron in the pH range from 11 to 12. The remaining two tyrosyl residues are titrated during the gross unfolding of the protein structure in the pH region 12 to 13. Assuming the structural location of the invariant tryptophanyl residue at position 59 is identical in both the horse heart and the tuna heart proteins, solvent perturbation measurements indicate that tryptophanyl residue 33 is exposed in tuna heart ferricytochrome *c*.

Two of the four tyrosyl residues of horse heart ferricytochrome *c* are exposed to the solvent, as shown by solvent perturbation (Stellwagen and Van Rooyan, 1967; Herskovits, 1969) and chemical modification (Cronin and Harbury, 1965; Stellwagen and Van Rooyan, 1967) measurements. Analysis of the iodinated protein indicates that the exposed tyrosyl residues are located at positions 67 and 74 in the amino acid sequence (McGowan and Stellwagen, 1970). The amino acid sequence of tuna heart cytochrome *c* contains four tyrosyl residues in the same positions as in the horse heart protein (residues 48, 67, 74, and 97) plus a fifth tyrosyl residue at position 46 (Kreil, 1963, 1965) replacing a phenylalaninyl residue in the horse heart protein. In this report the properties of the four common tyrosyl residues in these two homologous proteins are compared, and the structural environment of the fifth tyrosyl residue in the tuna protein is investigated.

Materials and Methods

Materials. Hearts from yellowfin tuna were kindly provided by Westgate-California Foods, Inc., San Diego, Calif., and by Ralston-Purina Co., Van Camp Sea Food Division, Terminal Island, Calif. Tuna heart ferricytochrome *c* was prepared by the procedure described by Margoliash and Walasek (1966). The purified protein had less than 2% reactivity with CO, an $A_{550(red)}/A_{280(oxid)}$ of 1.02, and visible absorbance maxima ratios for both ferri- and ferrocytochrome *c* which varied from the corresponding values for horse heart cytochrome *c* by no more than 2%.

Methods. Procedures for the measurement of spectrophotometric titration of the tyrosyl residues (Stellwagen, 1964), solvent perturbation difference spectra (Stellwagen and Van Rooyan, 1967), iodination, sequence analysis, and electron transport activity using pig heart NADH–cytochrome *c* reductase (McGowan and Stellwagen, 1970), have been described previously.

Results

Spectrophotometric Titrations. In the presence of 9.4 M urea, a solvent which unfolds the native conformation of horse heart ferricytochrome *c* (Myer, 1968; Stellwagen, 1968), the $\Delta\epsilon_{243}$ for tuna heart ferricytochrome *c* describes a reversible sigmoidal dependence on pH over the pH range 7 to 13.5 having an apparent pK of 10.9 and a total $\Delta\epsilon_{243}$ of 57 mM^{-1} cm^{-1}. Assuming that all five tyrosyl residues are exposed in this solvent, the $\Delta\epsilon_{243}$ per tyrosyl residue is 11 mM^{-1} cm^{-1}, a value within the range observed for the phenolic ionization of tyrosine, *N*-acetyltyrosine, and tyrosyl residues (Hermans, 1962; Paiva and Paiva, 1962; Donovan, 1964). The apparent pK observed for the ionization of the phenolic hydroxyl groups of tuna heart ferricytochrome *c* is identical with that reported for ribonuclease in the presence of 8 M urea (Blumenfeld and Levy, 1958). It was concluded, therefore, that the exposed heme moiety of tuna cytochrome *c* does not interfere with the spectrophotometric titration of the phenolic hydroxyl groups of the five tyrosyl residues.

The spectrophotometric titrations of native tuna heart and horse heart ferricytochrome *c* at 243 mμ over the pH

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† U. S. Public Health Service Career Development Awardee, Grant 1-K03-GM08737, from the Institute of General Medical Sciences; to whom to address inquires.

‡ Predoctoral Trainee, U. S. Public Health Service Training Grant 5-T01-GM00550 from the Institute of General Medical Sciences. Taken in part from a dissertation submitted by Eleanor B. McGowan in partial satisfaction of the requirements for the degree of Doctor of Philosophy, University of Iowa, 1969. Present address: Graduate Department of Biochemistry, Brandeis University, Waltham, Mass.

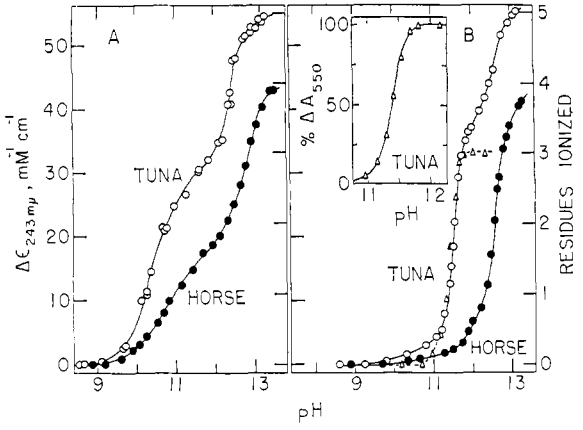

FIGURE 1: Spectrophotometric titrations of tuna heart and horse heart cytochrome *c:* (A) ferricytochrome *c;* (B) ferrocytochrome *c*. The protein was reduced with sodium dithionite and the reducing agent was removed by gel filtration just prior to spectrophotometric titration. Insert: the autoxidation of ferrocytochrome *c* as indicated by the loss of absorbance at 550 mμ measured within 1 min after adjustment of the pH of the solution. All titrations were performed at room temperature in 0.2 M KCl using protein concentrations ranging from 0.01 to 0.02 mM: (○) tuna heart cytochrome *c;* (●) horse heart cytochrome *c*. The triangles and dashed line in the major portion of Figure 1B represent normalization of the pH dependence of autoxidation of tuna ferrocytochrome *c* with the $\Delta\epsilon_{243}$ accompanying the ionization of three tyrosyl residues.

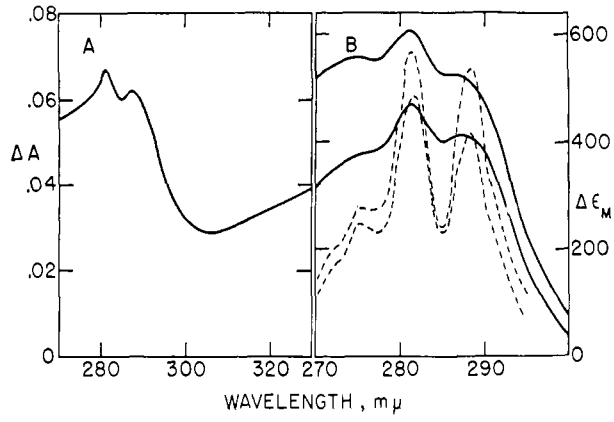

FIGURE 2: Ultraviolet solvent perturbation difference spectra in 20% ethylene glycol: (A) tuna heart ferricytochrome *c*, 7.97 × 10^{-2} mM in 0.1 M phosphate buffer, pH 7.0; (B) —, tuna heart ferricytochrome *c* corrected for heme perturbation. The upper spectrum was calculated by subtraction of a base line obtained by extrapolation of the difference spectrum observed from 310 to 330 mμ through the region from 270 to 300 mμ (Herskovits, 1969). The lower spectrum was obtained by subtraction of a flat base line equivalent to the difference spectrum observed at 310 mμ (Stellwagen and Van Rooyan, 1967). – – –, spectra of model mixtures shifted 3 mμ to lower wavelengths. The upper model spectrum is equivalent to 3 tyrosyl and 1.6 tryptophanyl residues while the lower model spectrum is equivalent to 3 tyrosyl and 1.0 tryptophanyl residues.

range 8–13 are compared in Figure 1A. The total $\Delta\epsilon_{243}$ observed is that expected for the number of tyrosyl residues in each protein, indicating that the $\Delta\epsilon_{243}$ due to the unfolding of tuna ferricytochrome *c* at high pH is negligible, as was observed for the horse heart protein (Stellwagen, 1964). While both titration curves are bimodal, the steps in the tuna titration curve are more apparent. The initial step of the tuna titration curve is equivalent to that expected for three exposed tyrosyl residues having apparent p*K* values of 10.5. The steepness of the second step of the tuna titration curve suggests that the ionization of these two tyrosyl residues, having apparent p*K* values of 12.4, results from the cooperative unfolding of the native conformation. Viscosity measurements have shown that the homologous protein horse heart ferricytochrome *c* is unfolded at high pH values with an apparent p*K* of 12.6 (Stellwagen, 1964).

In contrast to the titration curve of the tyrosyl residues of horse heart ferrocytochrome *c*, the titration curve of tuna heart ferrocytochrome *c* is bimodal with apparent p*K* values of 11.5 and 12.5, as shown in Figure 1B. Titration in the presence of a 200-fold molar excess of $NaBH_4$ selectively eliminates the tyrosyl ionization which occurs in the pH region between 10 and 11. The two steps of the bimodal titration curve are restricted to narrow pH ranges, suggesting two sequential cooperative events. As shown in the insert of Figure 1B, autoxidation of ferrocytochrome *c* occurs in the same range as the initial step of the tyrosyl titration curve. The autoxidation curve can be fit with the titration curve for three, but not four, tyrosyl residues as shown in Figure 1B. The titration curve for the remaining two tyrosyl residues is identical with the titration curve of the two buried tyrosyl residues of tuna heart ferricytochrome *c* as would be expected if both forms of the protein are in the oxidized form at pH 12 and above. It was concluded, therefore, that all five tyrosyl residues in tuna heart ferrocytochrome *c* are buried and that ionization of three of the five residues is coincident with autoxidation of the heme iron.

Solvent Perturbation. The ultraviolet solvent perturbation difference spectrum of native tuna ferricytochrome *c* in 20% ethylene glycol exhibits maxima at 281.5 and 288.5 mμ as shown in Figure 2. Maxima at these wavelengths do not correspond to the maxima observed for the solvent perturbation difference spectra of the model chromophores, *N*-acetyltyrosine ethyl ester, *N*-acetyltryptophan ethyl ester, or combination thereof, in 20% ethylene glycol (Herskovits and Sorensen, 1968). Therefore, in order to fit a model mixture to the observed solvent perturbation difference spectrum for tuna cytochrome *c*, the $\Delta\lambda_{max}$ of either or both model chromophores must be shifted. While numerous combinations approximate the observed protein spectrum, the closest fit is obtained by shifting the perturbation difference spectrum of both model chromophores 3 mμ toward lower wavelengths. If it is assumed that the solvent perturbation difference spectrum of the heme moiety in the region 270–300 mμ can be estimated by extrapolation of the perturbation observed at wavelengths greater than 300 mμ as proposed by Herskovits (1969), the observed protein spectral perturbation is that expected for 1.6 exposed tryptophanyl and 3 exposed tyrosyl residues. However, if it is assumed that the heme perturbation is constant over the region 270–310 mμ as proposed by Stellwagen and Van Rooyan (1967), the observed spectrum is that expected for 1 exposed tryptophanyl and 3 exposed tyrosyl residues.

Iodination. Tuna ferricytochrome *c* was reacted with increasing concentrations of KI_3 and the extent of formation of diiodotyrosyl residues measured by spectrophotometric

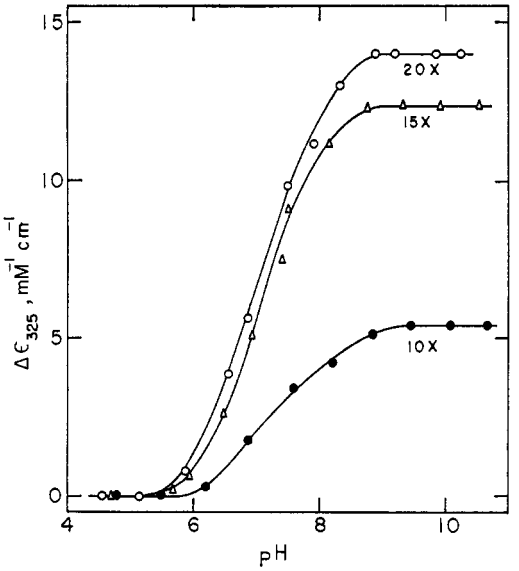

FIGURE 3: Spectrophotometric titrations of iodinated tuna heart ferricytochrome *c* at 325 mμ. The protein was reacted with the indicated molar excess of KI_3 at pH 9.5 and 2° for 10 to 17.5 min. The protein was freed of the iodination reaction mixture by gel filtration and titrated at room temperature in 0.2 M KCl using the protein solution adjusted to pH 4.5 as the reference solution. Protein concentrations for titration varied from 1.15 to 2.13 × 10^{-2} mM.

titration at 325 mμ over the pH range 5–10. Typical titrations, shown in Figure 3, had apparent p*K* values from 7.0 to 7.3, characteristic of diiodotyrosyl residues (Gruen *et al.*, 1959; Donovan, 1963). Using a $\Delta\epsilon$ of 4.4 mM^{-1} cm^{-1} for the phenolic ionization of diiodotyrosine (McGowan and Stellwagen, 1970), 1.4 ± 0.2, 2.8 ± 0.1, and 3.3 ± 0.1 tyrosyl residues of tuna ferricytochrome *c* were diiodinated by reaction with 10-, 15-, and 20-fold molar excesses of KI_3, respectively.

In order to locate the positions of the iodinated tyrosyl residues in the amino acid sequence, the iodinated protein was cleaved with cyanogen bromide and trypsin and the

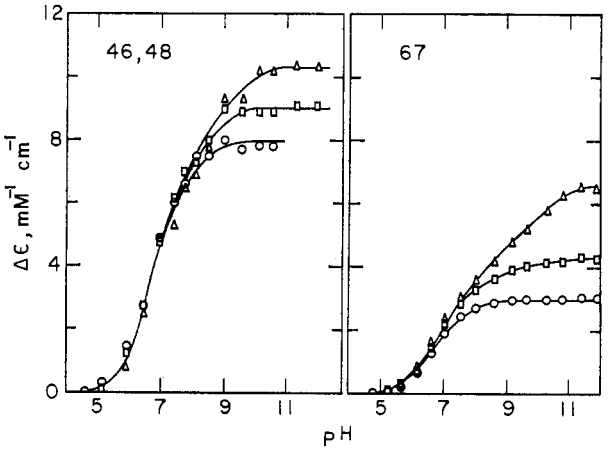

FIGURE 4: Spectrophotometric titrations of peptide 40–53 containing tyrosyls 46 and 48 and peptide 66–72 containing tyrosyl 67: (O) 325 mμ, (□) 305 mμ, (△) 243 mμ. All titrations were performed at room temperature in 0.2 M KCl. The concentration of peptide ranged from 1.03 to 1.42 × 10^{-2} mM.

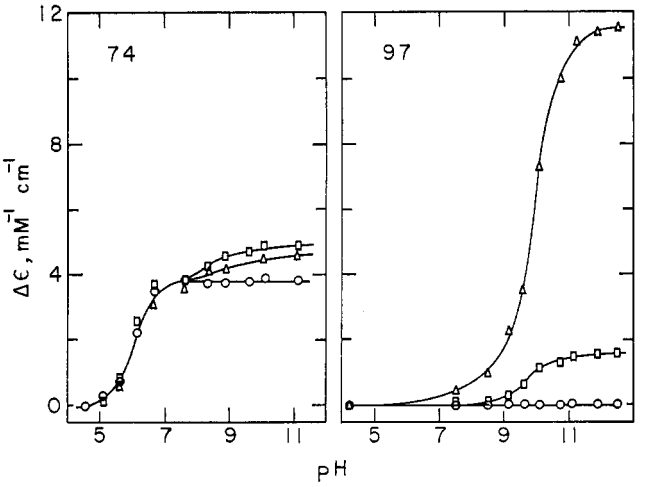

FIGURE 5: Spectrophotometric titrations of peptide 74–79 containing tyrosyl 74 and peptide 81–104 containing tyrosyl 97: (O) 325 mμ, (□) 305 mμ, (△) 243 mμ. All titrations were performed at room temperature in 0.2 M KCl. The concentration of peptide ranged from 1.65 to 2.21 × 10^{-2} mM.

tyrosyl peptides were purified and titrated at 243, 305, and 325 mμ over the pH range 5–12 as described by McGowan and Stellwagen (1970). While this procedure does not isolate tyrosyls 46 and 48 of tuna cytochrome *c* on separate peptides, all attempts to specifically cleave one of the peptide bonds separating these two residues were unsuccessful. Typical titration curves of peptides containing tyrosyls 46 and 48, 67, 74 and 97 are shown in Figures 4 and 5. Using the extinction coefficients for tyrosine, monoiodotyrosine, and diiodotyrosine at these wavelengths (McGowan and Stellwagen, 1970) the titration curves for the peptides obtained from tuna cytochrome *c* iodinated with 10-, 15-, and 20-fold molar excesses of KI_3 were resolved into the contents of each of these residues, with the results shown in Table I.

Ferricytochrome *c* iodinated with a 20-fold molar excess of KI_3 was titrated spectrophotometrically at 243 mμ in order to estimate the apparent p*K* of the uniodinated tyrosyl residue. A smooth titration curve was observed over the pH range from 10.0 to 13.3 having a $\Delta\epsilon$ of 14.7 mM^{-1} cm^{-1}

TABLE I: Iodination of Tyrosyl Residues.

Molar Ratio KI_3/ Protein	Sequence Position	Residue/Molecule of Protein		
		Tyr	ITyr	I_2Tyr
10	46,48	1.0	0.4	0.6
	67	0.4	0.5	0.1
	74	0.6	0.1	0.3
	97	1.0	0.0	0.0
15	46,48	0.4	0.2	1.4
20	46,48	0.0	0.3	1.7
	67	0.2	0.1	0.7
	74	0.0	0.1	0.9
	97	1.0	0.0	0.0

and an apparent p*K* of 12.0. These values are equivalent to the ionization of 1.3 buried tyrosyl residues.

Properties of the Iodinated Protein. The visible absorbance spectrum of tuna ferri- and ferrocytochrome *c* iodinated with a 20-fold molar excess of KI_3 exhibits maxima at the same wavelengths reported for the iodinated horse protein (McGowan and Stellwagen, 1970). The visible extinction coefficients of the iodinated tuna protein are equal to or no more than 5% greater than those reported for the iodinated horse protein. Iodination of both proteins results in the disappearance of the absorbance band at 695 mμ at pH 7.0.

In contrast to native tuna ferrocytochrome *c*, iodinated tuna ferrocytochrome *c* autoxidizes very rapidly upon removal of the reducing agent. Iodination also lowers the electron transport activity of tuna cytochrome *c* 60% in the NADH–cytochrome *c* reductase assay.

Discussion

While both spectrophotometric titration and solvent perturbation measurements indicate that three of the five tyrosyl residues in tuna heart ferricytochrome *c* are exposed, four tyrosyl residues are iodinated. Although iodine could penetrate the hydrophobic portions of the protein to modify a buried tyrosyl residue, measurement of the apparent p*K* values of the unmodified tyrosyl residues in iodinated horse heart ferricytochrome *c* indicate that this does not occur to an appreciable extent (McGowan and Stellwagen, 1970). Similar analysis of iodinated tuna heart ferricytochrome *c* indicates that the single uniodinated tyrosyl residue located at position 97 is one of the two buried tyrosyl residues in native tuna heart ferricytochrome *c*. Since tyrosyls 48, 67, and 74 are invariant residues in the sequences of cytochrome *c* of 26 species (Dayhoff, 1969), it is likely that their structural locations are also invariant. With this assumption, the analysis of the structural locations of the tyrosyl residues of horse heart ferricytochrome *c* (McGowan and Stellwagen, 1970) predicts that tyrosyl 48 is the other buried tyrosyl residue in native tuna heart ferricytochrome *c*. Such an assignment is strengthened by the identity of the sequences of the horse heart and tuna heart proteins between residues 63 and 88. The iodination of tyrosyl 48 in the tuna heart but not the horse heart protein may result from a conformational change caused by an increase in size and/or the phenolic ionization accompanying the iodination of neighboring exposed tyrosyl 46 in the tuna heart protein.

The marked changes in the apparent p*K* values of the three exposed tyrosyl residues upon reduction of the heme iron suggests that these residues are involved in the conformational transition accompanying changes in the oxidation state of the heme iron (Margoliash and Schejter, 1966). Similar changes in the apparent p*K* values of the exposed tyrosyl residues of horse heart (Stellwagen, 1964) and *Candida krusei* (Hamaguchi *et al.*, 1967) ferricytochrome *c* upon reduction of the heme iron have been observed.

Horse heart cytochrome *c* has a single tryptophanyl residue located at position 59 while tuna heart cytochrome *c* has two tryptophanyl residues located at positions 33 and 59 (Kreil, 1963, 1965). Assuming that the invariant residue tryptophanyl 59 has a constant conformational position in the two proteins, it is 0.0 (Stellwagen and Van Rooyan, 1967) or 0.6 (Herskovits, 1969) exposed, depending upon the evaluation of the solvent perturbation of the heme moiety in the near ultraviolet region. Thus, the exposure of 1.0 or 1.6 Trp residues in tuna heart ferricytochrome *c* indicates that tryptophanyl 33 must be exposed. The side chain of the residue at position 33 in horse heart ferricytochrome *c* is also exposed since histidyl 33 is carboxymethylated by bromoacetate (Harbury *et al.*, 1965).

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