Reactivity of Individual Tyrosyl Residues of Horse Heart Ferricytochrome *c* toward Iodination*

Eleanor B. McGowan† and Earle Stellwagen‡

ABSTRACT: Horse heart ferricytochrome *c* was iodinated with an eightfold molar excess of KI_3 for 10 min at pH 9.5 and 2°. Although this treatment does not change the gross conformation of the protein, changes in the physical, chemical, and biological properties of the heme moiety indicate that the heme crevice is opened. Using cyanogen bromide and trypsin, the iodinated protein was cleaved to isolate each tyrosyl residue on a separate peptide. Each peptide was titrated spectrophotometrically at three wavelengths over the pH range 4.6 to 13.0 to determine the number of tyrosyl, monoiodotyrosyl, and diiodotyrosyl residues. Only tyrosyl residues at position 67 and position 74 in the amino acid sequence of the native protein are iodinated significantly.

Only two of the four tyrosyl residues of native horse heart ferricytochrome *c* are exposed to the solvent as indicated by chemical reactivity (Cronin and Harbury, 1965; Stellwagen and Van Rooyan, 1967), solvent perturbation (Stellwagen and Van Rooyan, 1967; Herskovits, 1969), and spectrophotometric titration (Rupley, 1964; Stellwagen and Van Rooyan, 1967). Changes in apparent p*K* (Stellwagen, 1964) and chemical reactivity (Ulmer, 1966) of these two tyrosyl residues following reduction of the heme iron suggests that they are not exposed in ferrocytochrome *c*. The following communication describes the iodination of ferricytochrome *c* and the identification of the positions of the tyrosyl residues significantly iodinated.

Materials and Methods

Materials. Horse heart cytochrome *c*, type VI, lot 75 B-7160, was obtained from the Sigma Chemical Co. The protein was converted into ferricytochrome *c* by treatment with excess ferricyanide. The oxidant was removed by exclusion chromatography using Sephadex G-25. Cyanogen bromide was purchased from the Aldrich Chemical Co. Phosphocellulose was obtained from Bio-Rad Laboratories. NADH–cytochrome *c* reductase, type I, from pig heart, 3-iodo-L-tyrosine, and 3,5-diiodotyrosine were obtained from the Sigma Chemical Co. Trypsin treated with L-(1-tosylamido-2-phenyl)-ethyl chloromethyl ketone was purchased from the Worthington Biochemical Corp. Bromoacetic acid was obtained from the Eastman Kodak Co.

Iodination. Solutions of ferricytochrome containing 10 mg/ml of protein in 0.5 M glycine, pH 9.5, or in 0.5 M potassium phosphate, pH 7.0, were reacted at 2° with the desired volume of KI_3 solution prepared as described by Gruen *et al.* (1959). The KI_3 solution was added in 8 equal increments at 20-sec intervals. The protein solution was rapidly stirred and maintained at 2° during the addition of KI_3 solution and for a period of 2 to 20 min after the initial addition of reagent. The reaction was terminated by exclusion chromatography using a Sephadex G-25 column equilibrated and developed with 0.1 M KCl. The iodinated protein was then dialyzed against distilled water and lyophilized. The iodination of the unfolded protein was performed in 9.4 M urea–0.3 M glycine, pH 9.5, at 25°.

Peptide Cleavage. Native or iodinated protein was cleaved with CNBr using a modification of the procedure described by Holmgren and Reichard (1967). The protein was dissolved in 63% formic acid–37% water (v/v) to make a solution containing 10 mg of protein/ml. Solid CNBr was added to give a 520 molar excess of CNBr to protein and the reaction was allowed to proceed for 18–24 hr at room temperature, after which it was terminated by addition of 10 volumes of water. The diluted reaction mixture was frozen and the volatile components removed by lyophilization.

Biological Activity. The electron transport activity of native and iodinated cytochrome *c* was measured spectrophotometrically using a NADH–cytochrome *c* reductase assay procedure. The assay solution containing 0.08–0.12 μmole of cytochrome *c*, 1.31 μmoles of NADH, and 40 μmoles of potassium phosphate in 1.0 ml was adjusted to pH 8.5 and 25°. The reaction was initiated by addition of 10 μg of reductase and the change in absorbance at 550 mμ was recorded for at least 5 min. The ΔA_{550} per min per mg of protein for the native and iodinated proteins were compared by designating the rate for the native protein as 100%.

Other Measurements. Chemical reactivity, amino acid composition, viscosity, spectrophotometric, and pH measurements were performed as described previously (Stellwagen, 1968). The extent of carboxymethylation of histidyl and methionyl residues was measured after reaction with 0.2 M bromoacetate at pH 6.9 and room temperature for 4 days

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† 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, Massachusetts.

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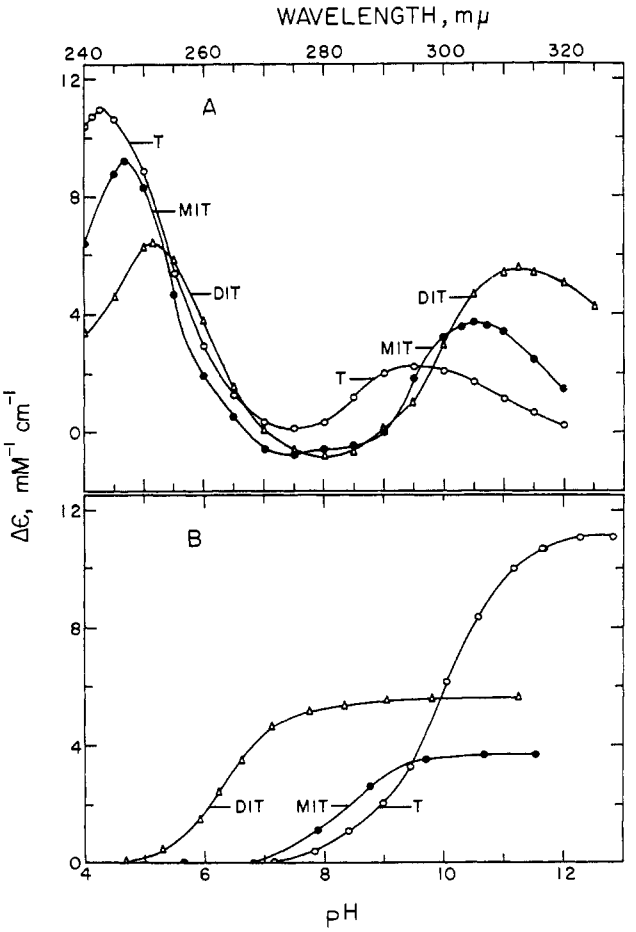

FIGURE 1: Difference spectra and titration curves of model compounds: (A) pH difference spectra. (○) Tyrosine (T), pH 12.8 *vs.* pH 4.7; (●) 3-iodotyrosine (MIT), pH 10.8 *vs.* pH 4.7; (Δ) 3,5-diiodotyrosine (DIT), pH 9.7 *vs.* pH 4.6; (B) titration curves, (○) tyrosine (T) at 243 mμ; (●) 3-iodotyrosine (MIT) at 305 mμ; (Δ) 3,5-diiodotyrosine (DIT) at 311 mμ. The concentrations of the model compounds ranged from 1 to 10 × 10^{-5} M. The solvent was 0.2 M KCl and the temperature was maintained at 25°.

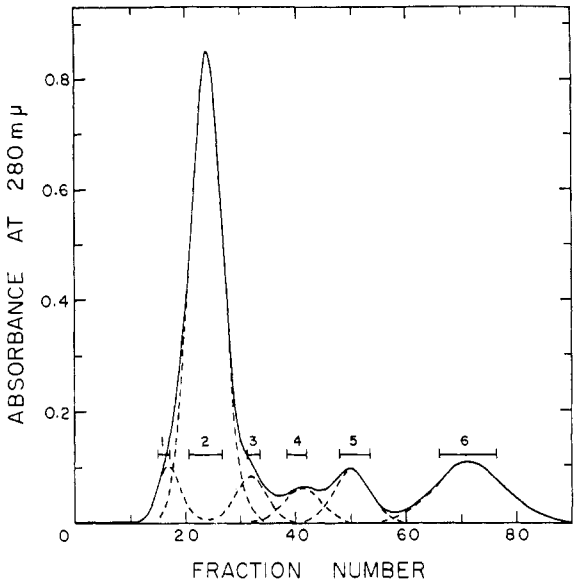

FIGURE 2: Exclusion chromatography of CNBr-hydrolyzed iodinated ferricytochrome *c*. Native ferricytochrome *c* was iodinated with an eightfold molar excess of KI_3 at pH 9.5 and 2° for 10 min, freed of the iodination reagent by gel filtration, and then subjected to CNBr cleavage. The CNBr-cleaved iodinated protein (10 mg) in 0.5 ml of 40:9:51, CH_3COOH–$HCOOH$–H_2O was applied to a 1.2 × 45 cm column of Sephadex G-50 equilibrated and developed with the same solvent at room temperature using a flow rate of 6 ml/hr. Effluent fractions containing 0.2 ml were collected: (———) absorbance of the effluent at 280 mμ; (— — —) resolution of the effluent profile into Gaussian components. Selected fractions were pooled as indicated and numbered in the order of elution.

(Stellwagen, 1968). Protein concentrations were determined spectrophotometrically using the extinction coefficients of Margoliash and Frohwirt (1959). Peptide concentrations were calculated by amino acid analysis.

Results

Model Compounds. The ultraviolet difference spectra resulting from the ionization of the phenolic hydroxyl groups of tyrosine, 3-iodotyrosine, and 3,5-diiodotyrosine exhibit maxima at 243 and 295 mμ, 247 and 305; and 252 and 312, respectively, as shown in Figure 1A. Spectrophotometric titration curves for these three compounds exhibit pK_{app} values of 10.0, 8.3, and 6.3, respectively, as shown in Figure 1B. Similar pK_{app} and $\Delta\epsilon_{max}$ values have been reported (Hermans, 1962; Edelhoch, 1962; Di Sabato, 1965). These differences in pK_{app} and $\Delta\lambda_{max}$ allow mixtures of these compounds to be resolved into the concentration of each component using spectrophotometric titration data obtained at three different wavelengths. In this study the concentration of each of these residues in a mixture of peptides was calculated from spectrophotometric titrations at 243, 305, and 325 mμ using $\Delta\epsilon$ (mM^{-1} cm^{-1}) values of 11.0, 1.7, and 0.0 for a tyrosyl residue, 8.0, 3.8, and 0.5 for a monoiodotyrosyl residue, and 4.0, 4.7, and 4.4 for a diiodotyrosyl residue, respectively.

Iodination. Native ferricytochrome *c* was reacted with an eightfold molar excess of KI_3 for 10 min at pH 9.5 and 2°. After removal of the iodination reagent by gel filtration, the ultraviolet difference spectrum produced by comparing the absorbance of the iodinated protein in 0.2 M KCl at pH 7.75 and pH 4.40 exhibited maxima at 255 and 315 mμ, characteristic of diiodotyrosine. As the pH of iodinated protein was raised stepwise from pH 7.55 to pH 13.1, the maxima in the difference spectra relative to pH 4.40 shifted to lower wavelengths, indicating the presence of monoiodotyrosyl and/or tyrosyl residues. Iodination of the unfolded protein in 9.5 M urea at pH 9.5 with a 20-fold molar excess of KI_3 for 10 min and subsequent removal of the urea and iodination reagent by gel filtration, produced pH difference spectra having maxima at 255 and 317 mμ at all pH values between pH 7.7 to 13.1 compared with the same solution at pH 4.8. These difference spectra describe a smooth titration curve having a pK_{app} of 7.1 and a $\Delta\epsilon$ of 22.4 mM^{-1} cm^{-1}, equivalent to that expected for the ionization of 4.1 diiodotyrosyl residues per molecule of protein.

Sequence Analysis. Complete reaction of the methionyl residues of horse heart cytochrome *c* with CNBr should

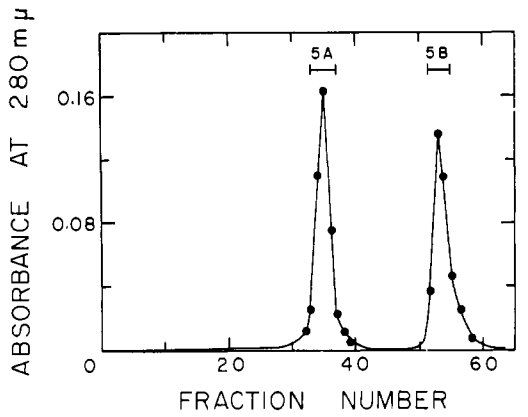

FIGURE 3: Ion-exchange chromatography of a tryptic digest of fraction 5. A solution (1 ml) containing 2 mg of the material of fraction 5 was treated for 24 hr at room temperature in 0.03 M Tris buffer–0.003 M $CaCl_2$, pH 8.0, with 2% (w/w) L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone trypsin added after 0, 8, and 16 hr of reaction. The pH of the reaction mixture was lowered to pH 6.0 with HCl and the digest applied to a 0.9 × 16 cm column of phosphocellulose equilibrated and developed with 0.02 M phosphate buffer, pH 6.0. Effluent fractions containing 0.6 ml were collected. Fractions 33–37 and 52–55 were pooled separately, and designated fractions 5A and 5B, respectively.

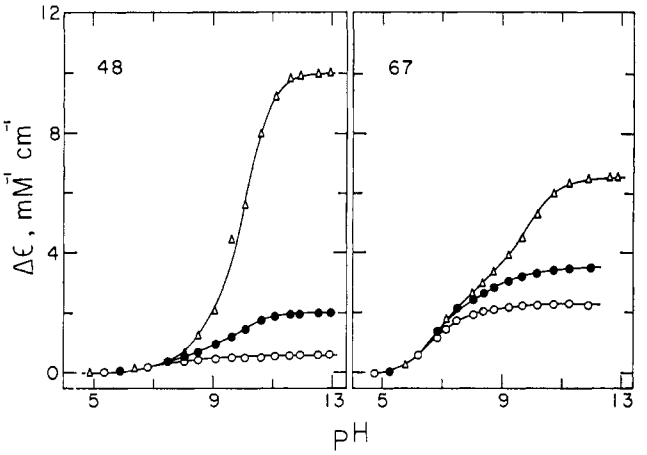

FIGURE 4: Spectrophotometric titrations of fraction 2A containing tyrosyl 48 and fraction 5A containing tyrosyl 67: (○) 325 mμ, (●) 305 mμ, (Δ) 243 mμ. All titrations were performed at room temperature in 0.2 M KCl. The concentration of peptide ranged from 0.08 to 0.13 mM.

cleave the protein into three fragments: a hemopeptide consisting of residues 1–65 including tyrosyl 48, a peptide containing residues 66–80 including tyrosyl 67 and tyrosyl 74, and a peptide containing residues 81–104 including tyrosyl 97. The differences in the sizes of these peptides should facilitate their separation by exclusion chromatography. The elution profile of CNBr-cleaved iodinated ferricytochrome *c* from Sephadex G-50 can be resolved into six fractions as shown in Figure 2. Native ferricytochrome *c* treated with CNBr and chromatographed in the same manner displays an elution profile similar to that shown in Figure 2 except that the relative amount of fraction 6 is less. The amino acid compositions of fractions 2, 4, and 5 correspond with those expected for the peptides containing residues 1–65, 81–104, and 66–80, respectively, as shown in Table I. Fraction 2 appears to dominate the elution profile because it contains the heme moiety which absorbs strongly at 280 mμ. The amino acid compositions of fractions 1 and 3, which are not shown, correspond to those expected for peptides containing residues 1–80 and 66–104, respectively, indicating that these peptides resulted from single cleavages of the protein by CNBr. Fraction 6, which was brown in color, was insoluble in water and dilute HCl and contained primarily glutamic acid, threonine, and leucine in a ratio of 2:1:1.

Fraction 5, containing both tyrosyl 67 and tyrosyl 74, was cleaved further with trypsin and the tryptic digest was resolved into two fractions by ion-exchange chromatography, as shown in Figure 3. The amino acid compositions of these two fractions, designated 5A and 5B, correspond to those expected for peptides containing residues 66–72 and 74–79, respectively, as shown in Table I.

Since changes in the absorbance of the heme moiety of the hemopeptide interfere with the spectrophotometric titration of tyrosyl 48, fraction 2 was cleaved further by treatment with trypsin. The pH of the tryptic digest was lowered to pH 5.2 with HCl and the solution was applied to a 0.9 × 16 cm column of phosphocellulose equilibrated and developed with 0.02 M acetate, pH 5.2. The red hemopeptide remained at the top of the phosphocellulose column throughout the elution procedure. Only one boundary absorbing at 280 mμ was eluted. The amino acid composition of this fraction, designated 2A, corresponded with that expected for a mixture of peptides containing residues 1–5, 9–13, 40–53, and 61–65, as shown in Table I. In some experiments this mixture of peptides was resolved further by applying it to a 1 × 16 cm column of DEAE-cellulose equilibrated and developed with the same solvent, 0.02 M acetate, pH 5.2. Fractions containing material absorbing at 280 mμ were pooled. The amino acid composition of this material, designated fraction 2AP, corresponded to that expected for the peptide containing residues 40–53. The spectrophotometric titration characteristics of fractions 2A and 2AP were equivalent.

The four fractions 2A, 5A, 5B, and 4 containing tyrosyls 48, 67, 74, and 97, respectively, were titrated spectrophotometrically at 243, 305, and 325 mμ over the pH range 4.5–12.0. Typical titration curves for each peptide are shown in Figures 4 and 5. The concentration of tyrosyl, monoiodotyrosyl, and diiodotyrosyl residues in each peptide was calculated from these titration curves using the extinction coefficients of the model compounds. The results are given in Table II.

The iodination reaction was also performed at pH 7.0. However, in order to obtain the same extent of reaction, it was necessary to increase both the concentration of the iodination reagent and the reaction time. At pH 7.0 the concentration of the tyrosyl anion, the reactive species in iodination (Mayberry *et al.*, 1964), is substantially less than at pH 9.5. Ferricytochrome *c* iodinated with a 16-fold molar excess of KI_3 for 20 min at pH 7.0 and 2° was cleaved, fractionated, and titrated as described above. The extent of iodination of the individual tyrosyl residues at pH 7.0 was similar to that observed for iodination at pH 9.5 as shown in Table II.

Properties of the Iodinated Protein. Some physical, chemical, and biological properties of native and iodinated cytochrome *c* are compared in Table III. The absorption band at 695 mμ,

TABLE I: Amino Acid Analyses.[a]

	Ferricytochrome *c* Residues/Molecule			CNBr-Hydrolyzed Iodinated Ferricytochrome *c* Residues/Molecule						Tryptic Digest Fraction 2 Residues/Molecule		Tryptic Digest Fraction 5 Residues/Molecule			
				Peptide 1–65	Fraction 2	Peptide 66–80	Fraction 5	Peptide 81–104	Fraction 4	Peptides 1–5, 9–13, 40–53, 61–65	Fraction 2A	Peptide 66–72	Fraction 5A	Peptide 74–79	Fraction 5B
	Theory	Native	Iodinated												
Asp	8	8.0	8.0	5	5.0	1	1.0	2	2.0	3	3.0	1	1.0	0	0
Thr	10	9.3	9.3	7	6.4	1	0.9	2	1.7	4	4.1	0	0	1	0.7
Ser	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Glu	12	11.9	11.7	7	7.2	2	2.0	3	2.8	5	4.3	2	2.1	1	1.0
Pro	4	3.9	4.0	2	2.1	2	1.9	0	0	1	1.1	1	1.0	1	1.0
Gly	12	12.1	12.3	10	10.0	1	1.1	1	0.9	3	3.4	0	0	1	0
Ala	6	5.9	5.8	3	3.0	0	0	3	2.8	2	1.9	0	0	0	0
Cys	2	1.6	1.5	2	1.5	0	0	0	0	0	0	0	0	0	0
Val	3	2.9	2.9	3	2.7	0	0	0	0	2	1.5	0	0	0	0
Met	2	1.5	1.4	1	0	1	0	0	0	1	0	0	0	0	0
Ile	6	5.5	5.6	2	1.9	1	0.9	3	2.6	1	0.4	0	0	1	0.7
Leu	6	5.8	5.9	3	2.9	1	0.9	2	1.9	1	1.0	1	0.9	0	0
Tyr	4	3.5	2.0	1	0.7	2	1.0	1	0.8	1	0.6	1	0.6	1	0.6
Phe	4	3.8	3.6	3	2.6	0	0	1	0.8	1	1.0	0	Na	0	0
Trp	1	Na	Na	1	Na	0	Na	0	Na	0	Na	0	Na	0	Na
Lys	19	19.0	19.0	11	11.0	3	Na	5	5.0	3	Na	1	Na	1	Na
His	3	2.8	2.7	3	2.8	0	Na	0	0	0	Na	0	Na	0	Na
Arg	2	1.9	1.9	1	0.8	0	Na	1	0.9	0	Na	0	Na	0	Na

[a] Native ferricytochrome *c* was iodinated with an eightfold molar excess of KI_3 for 10 min at pH 9.5 and 2°. The iodinated protein was treated with CNBr and then subjected to exclusion chromatography. The fraction designations refer to those defined in Figures 2 and 3. All samples were hydrolyzed in 6 N HCl for 24 hr at 110 ± 1°. The numbers of acidic and neutral amino acid residues per molecule of protein or peptide were calculated relative to the expected number of aspartyl residues while the numbers of basic amino acid residues per molecule were calculated relative to the expected number of lysyl residues. In the analysis of fraction 5B the amino acid composition was related to the expected number of glutamyl residues since no aspartyl residues are present in this peptide. No corrections were made for destruction of amino acids during acid hydrolysis or for incomplete hydrolysis of peptide bonds. The numeral 0 indicates there was no detectable deviation in the base line at the appropriate effluent volume. Na, not analyzed.

TABLE II: Iodination of Tyrosyl Residues.[a]

pH	Residue	Sequence Position 48	67	74	97
		Moles of Residue/Mole of Protein			
9.5	Tyrosyl	0.9 ± 0.1	0.1 ± 0.1	0.2 ± 0.1	1.0 ± 0.0
	Monoiodotyrosyl	0	0.2 ± 0.1	0.5 ± 0.0	0
	Diiodotyrosyl	0.1 ± 0.1	0.7 ± 0.2	0.3 ± 0.1	0
7.0	Tyrosyl	0.8 ± 0.0	0.3 ± 0.2	0.1 ± 0.1	1.0 ± 0.0
	Monoidotyrosyl	0	0.2 ± 0.1	0.2 ± 0.0	0
	Diiodotyrosyl	0.2 ± 0.0	0.5 ± 0.1	0.7 ± 0.1	0

[a] The protein was iodinated at 2° for 10 min (pH 9.5) using an eightfold molar excess of KI_3 in 0.5 M glycine buffer; or for 20 min (pH 7.0) using a 16-fold molar excess of KI_3 in 0.5 M phosphate buffer. The variations represent the range of values observed in two (pH 7) or three (pH 9.5) separate iodination experiments.

characteristic of native ferricytochrome *c*, is absent in iodinated ferricytochrome *c*; the remaining absorbance represents end absorbance from a neighboring band. The maximum in the Soret solvent perturbation difference spectrum occurs at 410 for the native protein and 408 mμ for the iodinated protein. Removal of the reductant, sodium hydrosulfite, from solutions of iodinated cytochrome *c* by exclusion chromatography resulted in rapid oxidation of the heme iron, in contrast to the relative stability of native ferrocytochrome *c* under similar conditions.

In order to estimate the pK_{app} of the unmodified tyrosyl residues, ferricytochrome *c* iodinated at either pH 9.5 or 7.0 was titrated spectrophotometrically at 243 mμ with the results shown in Figure 6. Subtracting the $\Delta\epsilon_{243\,m\mu}$ value predicted for the ionization of the iodinated tyrosyl residues in each sample gives a common curve equivalent to that expected for the ionization of two tyrosyl residues with pK_{app} values of 12.2.

TABLE III: Comparison of the Properties of Native and Iodinated Cytochrome *c*.[a]

Measurement	Ferricytochrome *c* Native	Ferricytochrome *c* Iodinated	Ferrocytochrome *c* Native	Ferrocytochrome *c* Iodinated
Absorbance				
Soret, λ_{max}, mμ	410	407	416	417
Soret, ε, $mM^{-1}\,cm^{-1}$	106	113	129	138
α, λ_{max}, mμ	528	528	550	551
α, ε, $mM^{-1}\,cm^{-1}$	11.2	9.8	27.7	24.5
ϵ_{695}, $M^{-1}\,cm^{-1}$	810	150		
Heme perturbation, $\Delta\epsilon/\epsilon_{Soret}$, ±0.002	0.019	0.028		
Reduced viscosity, ml/g, ±0.2	2.6	2.8		
Reactive histidyl residues/molecule of protein, ±0.1	1.0	0.9		
Reactive methionyl residues/molecule of protein, ±0.1	0.9	1.5		
Rate of reduction by NADH–cytochrome *c* reductase, ±2%	100%	40%		

[a] The protein was iodinated with an eightfold molar excess of KI_3 at pH 9.5 and 2° for 10 min. All spectral measurements were made in 0.1 M phosphate buffer at pH 7.0. The variations represent the range of values observed for two or three separate iodinated preparations. The characteristics of the Soret and α bands of the native protein were obtained from Margoliash and Frohwirt (1959).

Discussion

As shown in Table II, only tyrosyls 67 and 74 are iodinated significantly at both pH 7.0 and 9.5. The similarity of the

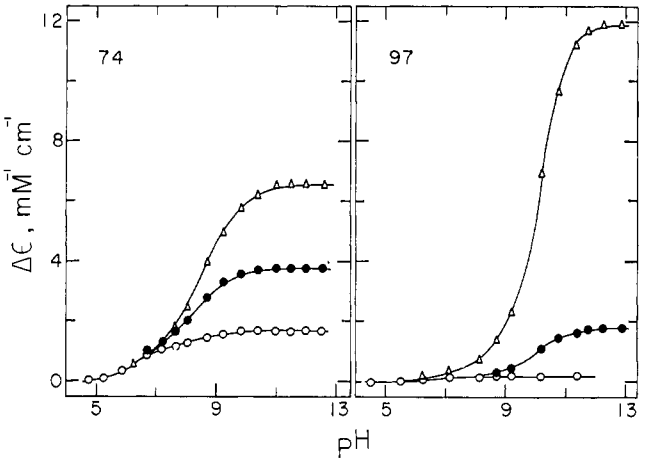

FIGURE 5: Spectrophotometric titrations of fraction 5B containing tyrosyl 74 and fraction 4 containing tyrosyl 97: (○) 325 mμ, (●) 305 mμ, (Δ) 243 mμ. All titrations were performed at room temperature in 0.2 M KCl. The concentration of peptide ranged from 0.04 to 0.10 mM.

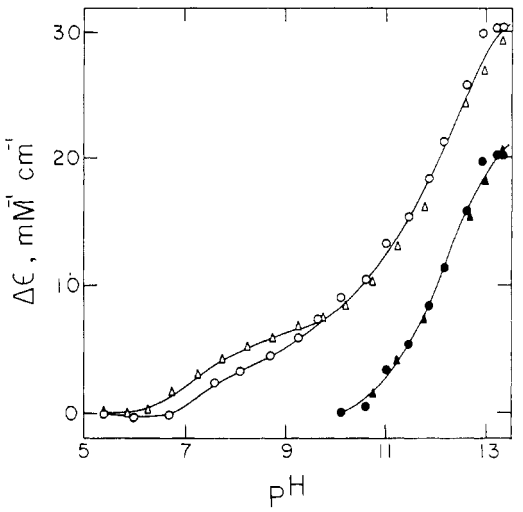

FIGURE 6: Spectrophotometric titration of iodinated ferricytochrome *c*: (○) iodinated with an eight-fold molar excess of KI_3 for 10 min at pH 9.5; (●) values remaining after subtraction of a $\Delta\epsilon_{243\ m\mu}$ of 10.0 mM^{-1} cm^{-1} expected for the ionization of 0.7 monoiodotyrosyl and 1.1 diiodotyrosyl residues; (△) iodinated with a 16-fold molar excess of KI_3 for 20 min at pH 7.0; (▲) values remaining after subtraction of a $\Delta\epsilon_{243}$ of 8.8 mM^{-1} cm^{-1} expected for the ionization of 0.4 monoiodotyrosyl and 1.4 diiodotyrosyl residues. All titrations were performed at room temperature in 0.2 M KCl. The concentration of protein ranged from 7.5 to 19.3 μM.

reactivity of each of the four tyrosyl residues at both pH values indicates that the conformational transition observed to occur between pH 7.5 and 10.5 (Greenwood and Palmer, 1965) probably does not involve gross changes in the structural environments of the tyrosyl residues. However, the predominance of a monoiodotyrosyl residue at position 74 after iodination at pH 9.5 but not at pH 7.0 suggests that tyrosyl 74 may be in a more hydrophobic environment at pH 9.5 (Mayberry and Hockert, 1970).

Spectrophotometric titrations of ferricytochrome *c* (Stellwagen, 1964; Rupley, 1964) indicate that the four tyrosyl residues have pK_{app} values of 10.1 ± 0.1, 11.2 ± 0.3, 12.5 ± 0.2, and 12.8 ± 0.3. The ionization of the latter two tyrosyl residues is coincident with gross unfolding of the native conformation. Analysis of the spectrophotometric titration of the iodinated protein at 243 mμ, Figure 6, indicates that the unmodified tyrosyl residues have pK_{app} values of about 12.2. Since the buried tyrosyl residues in the native protein have pK_{app} of 12.7 ± 0.4, these residues are probably not iodinated. It is proposed, therefore, that tyrosyl residues 67 and 74 have pK_{app} values of 10.1 ± 0.1 and 11.2 ± 0.3, or *vice versa*, and that these two tyrosyl residues are involved in the conformational transition accompanying reduction of the heme iron.

The data collected in Table III indicate that no gross conformational change accompanies the iodination of ferricytochrome *c* at pH 9.5. However, the local folding about the heme moiety has been loosened as evidenced by a greater exposure of the heme moiety to the solvent, a loss of the 695-mμ absorbance band, the chemical reactivity of the methionyl 80 which is a ligand for the heme iron (Harbury *et al.*, 1965; McDonald *et al.*, 1969), reduction in the efficiency of electron transport, and a greatly increased rate of autoxidation of the reduced iodinated protein. Changes in the rate of autoxidation, CO binding affinity, and catalytic activity of cytochrome *c* following limited iodination at pH 7.0 and 2° have been reported by Ishikura *et al.* (1959). The magnitude of many of these changes are comparable to those observed when cyanide replaces one of the protein ligands to the heme iron (Stellwagen, 1968).

The tyrosyl residues located at positions 48, 67, 74, and 97 are invariant residues in the sequences of cytochrome *c* of 22 species, except for the conservative substitution of tyrosyl 97 by a phenylalanyl residue in the sequence of *Neurospora crassa* cytochrome *c* (Dayhoff and Eck, 1968). Such persistence implies, but does not prove, a functional role for an aromatic ring located in these positions. Narita *et al.* (1968) have reported that iodination of *Candida krusei* ferricytochrome *c* results in the iodination of tyrosyls 73 and 80, which correspond to tyrosyls 67 and 74 in the sequence of mammalian cytochrome *c*. However, Skov *et al.* (1969) have reported that tyrosyls 48 and 67 of horse heart ferricytochrome *c* are nitrated by tetranitromethane at pH 8.0. The reasons for the differences in the reactivity of tyrosyls 48 and 74 with tetranitromethane and KI_3 are not known.

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Properties of *Dendrostomum pyroides* Hemerythrin*

Robert E. Ferrell and G. Barrie Kitto

ABSTRACT: The nonheme iron respiratory protein hemerythrin from the sipunculid worm *Dendrostomum pyroides* has been subjected to a detailed physicochemical characterization. The protein is an octamer with a molecular weight of approximately 100,000 and dissociates to a monomer of mol wt 13,000. There are two molecules of nonheme iron bound per subunit and the native molecule combines reversibly with oxygen in a manner which involves no subunit cooperativity as determined from a Hill plot ($n = 1$), and is independent of hemerythrin concentration over a wide range of concentrations.

Immunodiffusion, amino acid analysis, and peptide mapping show that this hemerythrin is closely related to the hemerythrin of the sipunculid *Golfingia gouldii*.

The hemerythrins are a group of nonheme iron-containing, respiratory proteins found in certain members of four phyla: Sipunculoidea, Priapulida, Brachiopoda, and Annelida. A number of recent reviews of the hemerythrin literature are available (Manwell, 1960, 1964; Gihretti, 1962; Boeri, 1963).

Only the hemerythrin of the sipunculid *Golfingia gouldii* has been the subject of detailed physicochemical characterization, and to a lesser degree that of the sipunculid *Sipunculus nudus*. Klotz and his coworkers have investigated the molecular weight and dissociation properties of the hemerythrin from *G. gouldii* in a series of studies (Klotz and Keresztes-Nagy, 1963; Keresztes-Nagy and Klotz, 1963, 1965; Keresztes-Nagy *et al.*, 1965; Klapper and Klotz, 1968; Klapper *et al.*, 1966). The studies have shown the hemerythrin from this species to have a molecular weight of 107,000, and that it is composed of eight similar subunits. The molecule is reversibly dissociable and the octamer $\rightleftharpoons$ monomer equilibrium is dependent on the concentration of hemerythrin and the coordination state of the iron atoms within the molecule. The amino acid sequence of the subunit has been reported (Groskopf *et al.*, 1966a,b; Subramanian *et al.*, 1968; Klippenstein *et al.*, 1968).

The hemerythrin from *S. nudus* has been shown to bind one oxygen molecule per two nonheme iron atoms (Boeri and Ghiretti-Magaldi, 1957) and a detailed study of the oxygen binding properties of the hemerythrin from this species has been reported (Bates *et al.*, 1968). They observed only slight homotropic interaction upon oxygenation and the absence of any Bohr effect.

This paper is concerned with the physicochemical characterization of the coelomic hemerythrin from the sipunculid *Dendrostomum pyroides*, and includes iron analysis, ultracentrifugal analysis, oxygen equilibrium, amino acid composition, and peptide maps of this protein.

Materials and Methods

D. pyroides were obtained from Pacific Bio-Marine Supply Co., Venice, Calif. *G. gouldii* were from Woods Hole Biological Supply, Woods Hole, Mass. Identification of the specimens was confirmed using the key of Fisher (1952). Hemerythrin from the brachiopod *Lingula* was a gift from Dr. Bolling Sullivan, Duke University Medical School, Durham, N. C. Hydrolyzed starch was from Connaught Medical Research Laboratories, Nitroso-R-salt from Wilshire Chemical Co., Inc., and *o*-phenanthroline, *N*-ethylmaleimide were from Sigma. Sephadex G-25, G-100, and G-200 were from Pharmacia, Inc. Tosylamidoethyl chloromethyl ketone treated trypsin was from Worthington Biochemical Corp. All other chemicals were reagent grade.

Preparation of Hemerythrin. Individual specimens were bled from an incision, in the posterior end, into small centrifuge tubes. Hemerythrin-containing cells were collected by centrifugation at 2000*g* for 5 min, and the cells were washed three times by resuspension in cold sea water and centrifugation at 2000*g* for 5 min. The washed cells were suspended in 0.1 M potassium phosphate buffer (pH 7.5) and lysed by freezing and thawing. After thawing, the cell debris was removed by centrifugation at 12,000*g* for 30 min, and the supernatant hemerythrin solution was used immediately or stored at $-70°$. *Dendrostomum pyroides* hemerythrin prepared in this manner sediments as a single symmetrical peak in the ultracentrifuge and stains as a single band after starch gel electrophoresis.

Only hemerythrin-containing cells from the coelomic fluid were used in these preparations to avoid contamination from a vascular hemerythrin reported by Manwell (1963) to be present in the tentacles of certain sipunculid worms. DEAE-cellulose chromatography performed as described by Bates *et al.* (1968) showed no evidence of protein contamination, and

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