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Ultraviolet Difference Spectroscopy of Myoglobin: Assignment of p*K* Values of Tyrosyl Phenolic Groups and the Stability of the Ferryl Derivatives†

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ABSTRACT: The ionization of tyrosyl phenolic groups of ferric myoglobins from red kangaroo, horse, and sperm whale has been studied by pH difference spectroscopy at 245 nm. As the number of tyrosyl residues in these proteins varies monotonically from one to three, respectively, we are able to make p*K* assignments for all of them. The apparent p*K* for tyrosine-146, an invariant residue in all myoglobins, is unusually high, 12.7–12.9, as this residue is in a hydrophobic region of the molecule and the tyrosyl phenolic group is hydrogen bonded to the peptide carbonyl of isoleucine-99. For the ferric cyanide and the oxy forms of the various myoglobins, this apparent p*K* is elevated by about 0.5 pH unit while in the deoxy proteins, it does not change significantly. A second tyrosine, at position 103, is found in the horse and sperm whale proteins, but not in the kangaroo protein. It has a significantly lower apparent p*K* than is observed for Tyr-146 in all the derivatives studied. A third tyrosyl residue, at position 151, is exclusive to the sperm whale protein and has an apparent p*K* of 10.3, almost equivalent to that of tyrosine in aqueous solution. The apparent p*K*s for the ferryl forms of the three myoglobins prepared with a 2-fold molar excess of H_2O_2 were also determined by pH difference spectroscopy. Although the sperm whale ferryl protein autoreduces 5 times faster than the horse or kangaroo ferryl proteins, the time resolution of the titrimetric procedure permitted the p*K* determination. As compared to the ferric forms of the proteins, it was found that the apparent p*K* for tyrosine-146 in the horse and kangaroo ferryl proteins was significantly elevated. Tyrosine-151, the residue exclusive to sperm whale myoglobin, could not be titrated at all. When the sperm whale ferryl protein was subsequently reduced to the ferric state, it was found that 85% of the optical contribution of this residue was no longer seen. Amino acid analysis showed that one of the three tyrosyl residues was lost. No analogous loss of tyrosine was observed for the ferric kangaroo and horse proteins treated with peroxide and subsequently reduced. It is suggested that the decreased stability of sperm whale ferryl myoglobin, as compared to horse and kangaroo ferryl myoglobins, is due, in part, to the interaction of the ferryl heme with Tyr-151, found in the sperm whale protein but not in the others.

Since 1943, pH difference spectroscopy in the ultraviolet has been used as a measurement of the extent of dissociation of tyrosyl phenolic hydroxyl groups which are components of protein structure (Crammer & Neuberger, 1943; Tanford & Roberts, 1952). Although these measurements are often made at the major absorption band for tyrosine near 295 nm, for heme proteins it is more convenient to employ shorter wavelengths, usually near 245 nm, in order to avoid complications arising from large spectral contributions of the heme Soret. Such a study has been carried out by Hermans (1962), who, from difference spectroscopy at 245 nm, was able to determine that in the CO derivative of sperm whale myoglobin two of the three tyrosyl groups could be titrated with p*K*s of 10.3 and 11.5, respectively. The third tyrosine did not titrate within the pH range employed in the study, and it was suggested that its p*K* must be greater than 12.8.

In 1964, Breslow also studied carbonmonoxy sperm whale myoglobin, as well as the cyanide derivative. For both forms of the protein, she reported that two of the three tyrosines ionized below pH 12. It remained, however, to decide which tyrosyl residues in the protein corresponded to which of the apparent p*K*s. In order to resolve this problem, we decided to study three different myoglobins in which the number of tyrosyl residues in each differed monotonically.

In the simplest case, myoglobin from *Macropus rufus*, the red kangaroo, only a single tyrosyl residue is found in the primary sequence (Air & Thompson, 1971). In myoblogin from horse, there are two while in the major chromatographic

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fraction from sperm whale, three (Garner et al., 1974). Assuming that the primary and tertiary structures are homologous in all three myoglobins, we are able to decide which of the apparent p*K*s corresponds to which tyrosyl residue in the primary structure (Uyeda & Peisach, 1975) and show by a least-squares fit of the optical difference data to a classical titration equation that the apparent p*K* of the single tyrosine at position 146 is essentially the same in the ferric form of all three proteins, but could increase in other derivatives.

Of special interest is the ferryl derivative of myoglobin, produced by the addition of H_2O_2 to the ferric protein. Although this reaction was studied by numerous investigators over a period of 50 years with ferric hemoglobin (Kobert, 1900; Haurowitz, 1931, 1935; Keilin & Hartree, 1935, 1950; George, 1952), it was not until 1952 that George & Irvine applied it to ferric myoglobin. In this classic study, these authors demonstrated that ferric myoglobin, like ferric horseradish peroxidase (Chance, 1949), could be oxidized to a higher oxidation state, which they termed the "ferryl" state.[1] They were also the first to systematically delineate the conditions for preparation and stability of this otherwise unstable derivative of myoglobin. It is this instability involving autoreduction of the protein to the ferric oxidation state that has prevented its examination in solution with various physical probes.

In this paper, we describe the formation of a ferryl myoglobin from red kangaroo that is stable for many hours in solution. From a comparison of a pH titration in the ultraviolet of this ferryl myoglobin with that obtained from sperm whale, we show that the relative instability of the latter protein is associated with the autoreduction of ferryl heme by a specific tyrosyl residue in the sperm whale protein that is not found in the kangaroo protein.

Materials and Methods

Preparation of Protein. Red kangaroo, horse, and sperm whale oxymyoglobins were prepared by the method of Yamazaki et al. (1964). Horse and red kangaroo ferric myoglobins were prepared according to the method of Hapner et al. (1968) from frozen red muscle. Purified myoglobin solutions were concentrated and dialyzed against 0.05 M Tris-HCl buffer, pH 8.2, in which the ionic strength was raised to 0.2 with KCl.

Lyophilized sperm whale ferric myoglobin, which consisted of a mixture of four chromatographically separable myoglobins, was obtained from Seravac Laboratories, Colnbrook, England. Stock solutions were prepared by dissolving the dry protein in Tris-HCl buffer, ionic strength 0.2, pH 8.2, and centrifuging at 35000*g* for 90 min to remove small amounts of insoluble material. The centrifugate was then dialyzed against the same buffer. For further purification, SP-Sephadex chromatographic fractionation was performed according to the method of Garner et al. (1974), which is a modification of the method of Nakhleh (1971). Typically, the relative yields of fractions IV, IIIB, IIIA, and II were 78, 18, 3.5, and 0.3%, respectively. Unless otherwise stated, fraction IV was used in our experiments.

The concentration of protein used in this study was based on heme content as determined from the optical properties of the pyridine hemochromogen (Keilin & Hartree, 1951) with $\epsilon_{mM} = 34.7$ at $\lambda_{max} = 556.5$ nm. The tyrosine content of each myoglobin was determined by amino acid analysis on a Hitachi Model KLA-5 amino acid analyzer equipped with a model J 211 digital integrator. Samples prepared for amino acid analysis were hydrolyzed in 6 M HCl for 24, 48, and 72 h at 110 °C in an evacuated, sealed glass tube. After hydrolysis, the sample was continuously evacuated to remove HCl (Moore & Stein, 1963). For prevention of possible oxidation of tyrosine during hydrolysis, in some experiments, 1% phenol was added to the sample. Phenol addition had essentially no effect on the results. Amino acid standards were purchased from Ajinomoto Chemical Co. Duplicate analyses were performed.

Ferric myoglobin cyanide was prepared by adding a 10-fold molar excess of KCN to about 55 μM ferric myoglobin in 0.05 M Tris-HCl buffer, pH 8.2, ionic strength elevated to 0.2 with KCl. The solution was incubated for 15 min at room temperature before study. The fluoride derivative was prepared by adding an excess of solid KF to ferric myoglobin in the same buffer. Here too, the ionic strength was adjusted to 0.2 with KCl.

Titrations and Analysis. For the determination of pH difference spectra, a Cary 14R spectrophotometer was employed. pH measurements were made on a Radiometer PHM-52 meter. All measurements were performed at room temperature with protein solutions in which the ionic strength was adjusted to 0.2. Optical cells with a 10-mm light path were used. The pH of the reference cuvette was 8.2–8.3.

In the titration, equal volumes of KOH of various concentrations, but with ionic strength 0.2, were added with stirring to 5 mL of a 60–70 μM protein solution, and, 4 min after mixing, the pH and optical spectral difference were determined.

Optical differences at 245 nm were fit with the aid of a computer by iteration to an algorithm of the form:

$$Z = A_1 + \frac{B_1}{1 + 10^{X-C_1}} + A_2 + \frac{B_2}{1 + 10^{X-C_2}} + \ldots$$

where Z is the optical density at the measured pH, X, A is the optical density difference at the end of aitration with a p*K* of C, and B is a term related to the extinction coefficient $\Delta\epsilon_{245}$ where

$$B = \Delta\epsilon_{245}[M]$$

The subscripts in the algorithm refer to individual titratable chromophores, 1, 2, etc. The data collected in Tables I–IV are values obtained from least-squares fits of the algorithm. The reversibility of the titration was determined for the ferric sperm whale protein by a method of back-titration and analysis of the data (see below).

During the course of the titration, the optical spectrum in the visible was monitored as an indication of the ligation state of the heme. At the end of the titration, the integrity of the heme was verified from the pyridine hemochromogen procedure.

Difference Titrations of Deoxymyoglobin. For studies of deoxymyoglobin, a stock solution was prepared by repeated

[1] The oxidation state of heme in the oxidation product of peroxide with methemoglobin, metmyoglobin, or horseradish peroxidase (compound II) is termed "ferryl" by George (1952, 1955). According to this definition, a single reducing equivalent is required to convert the heme back to the met or ferric state (George & Irvine, 1951). Thus, the ferryl state is thought to be an oxidized form of heme differing from ferric heme by a single oxidizing equivalent. As H_2O_2 contains 2 oxidizing-equiv, the initial product of the reaction between ferric myoglobin and H_2O_2 should have 1 oxidizing-equiv more than ferryl heme. Although the formation of this product has been postulated (George & Irvine, 1956), its presence has never been unequivocally demonstrated, as it has been for horseradish peroxidase (compound I) (Theorell, 1941). If such a product were to form as a single addition complex of ferric myoglobin and H_2O_2, then it would have to be reduced by an unknown mechanism to the ferryl state, possibly yielding hydroxy radical as a second product (George & Irvine, 1952, 1956). On the other hand, the ferryl state may be achieved by single electron oxidation of ferric myoglobin, such as with chloroiridate (George & Irvine, 1954).

Table I: Apparent pKs of Kangaroo, Horse, and Sperm Whale Ferric Myoglobins Obtained by Difference Spectroscopic Titration at 245 nm[a]

	position									
	146		103		151					
species	pK_1	$\Delta\epsilon_1$	pK_2	$\Delta\epsilon_2$	pK_3	$\Delta\epsilon_3$	pK_4	$\Delta\epsilon_4$	pK_0	$\Delta\epsilon_0$
red kangaroo	12.8	9.0							9.0	4.6
horse	12.9	8.0	11.4	8.0					9.2	6.5
sperm whale										
commercial mixture	12.9	12.0	11.8	12.0	10.3	12.0			9.0	6.0
fraction IV	12.9	11.0	11.9	10.0	10.3	11.0			9.0	6.0
fraction IIIA	12.8	11.0	12.0	10.8	10.3	11.0	10.6	10.8	9.1	6.0

[a] The computed pKs 1, 2, and 3 refer to the dissociation of protons from tyrosyl residues at positions 146, 103, and 151, respectively (see Figure 2). The computed pK_4 is for the tyrosyl residue unique to fraction IIIA of commercial sperm whale myoglobin. $\Delta\epsilon_{1-4}$ refers to a best fit computed millimolar extinction coefficient at 245 nm for each tyrosine dissociation. $\Delta\epsilon_0$ refers to a best fit computed millimolar extinction coefficient at 245 nm for the optical transition observed for the conversion from the high-spin to the low-spin hydroxy forms of the ferric heme.

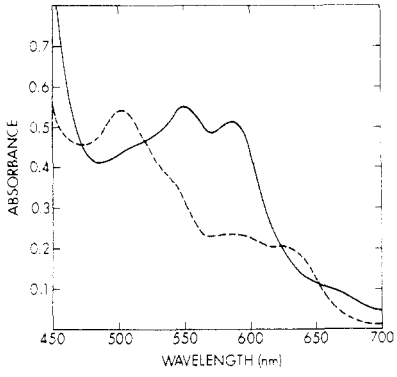

FIGURE 1: Optical spectra in the visible region of ferric (---) and ferryl (—) kangaroo myoglobin at pH 8.2. The ferryl myoglobin was prepared with a 2-fold excess of H_2O_2.

evacuation and flushing with argon of an oxymyoglobin solution with a rotary evaporator. KOH solutions of different concentration but with the ionic strength adjusted to 0.2 were purged of oxygen by bubbling with O_2-free argon. A 1-mL aliquot of deoxymyoglobin and 2 mL of deoxygenated KOH solution were anaerobically transferred with a hypodermic syringe to an argon-purged Thunberg anaerobic cuvette fitted with a rubber septum stopper and a 1-cm light-path optical cell. With gentle shaking, the samples were flushed for 5–8 min with water-saturated argon. After the visible spectrum was checked for deoxymyoglobin content, the ultraviolet difference spectrum was taken against a Thunberg anaerobic cuvette containing the same concentration of deoxymyoglobin, but to which 0.2 M KCl was added instead of KOH.

Ferryl Myoglobin.[1] A 30% H_2O_2 solution was purchased from Mallinckrodt or Mitsubishi Gas Chemical Co. The hydrogen peroxide concentration was determined by titration with $KMnO_4$ that had been assayed with primary standard sodium oxalate (Kolthoff & Belcher, 1957).

A 2-fold molar excess of H_2O_2, which is the minimal concentration of H_2O_2 required for maximal formation of ferryl myoglobin (Fox et al., 1974), was added to a metmyoglobin solution (60–70 μM) in 0.05 M Tris-HCl buffer, pH 8.2, at room temperature. After the solution was mixed, it was maintained at 5 °C.

Aliquots were taken at various time intervals, and the percent conversion to ferryl myoglobin was determined from the 577-, 580-, and 587-nm absorptions for sperm whale, horse, and kangaroo myoglobins, respectively (Figure 1). Here, the difference between these wavelength absorptions for the ferric protein and the largest absorbance obtained in this region after mixing with H_2O_2 was taken as an indication of both yield and purity. The millimolar absorption difference between the ferric and ferryl proteins at pH 8.2 was 7.1 for both sperm whale and horse myoglobins and 7.0 for kangaroo myoglobin. Difference spectroscopic studies at 245 nm were carried out as for the ferric protein.

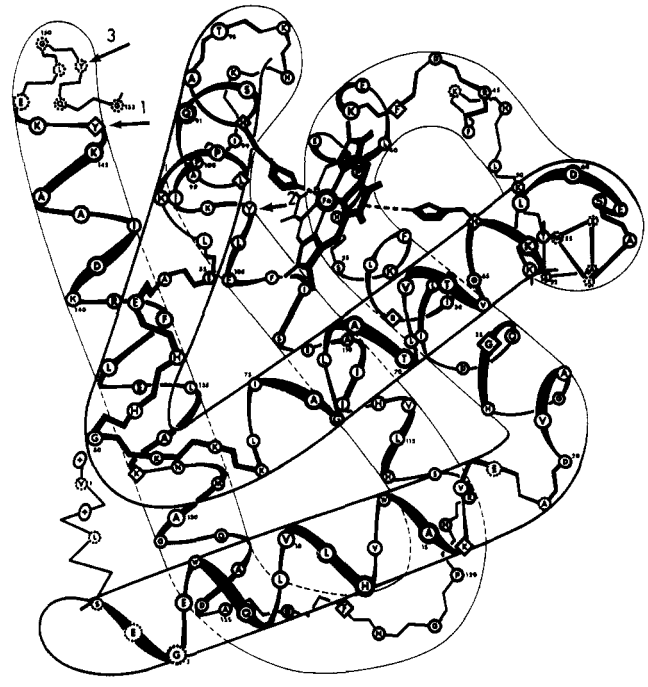

FIGURE 2: Position of tyrosyl residues in a model of the sperm whale myoglobin molecule obtained from 2-Å X-ray crystallographic analysis. The tyrosyl residues are indicated by arrows 1, 2, and 3. The positions from the N-terminal residue are 146, 103, and 151, respectively (Takano, 1977). Reproduced from Dayhoff et al. (1976), which is an adaptation from Dickerson (1964).

Results

pK of Tyrosyl Groups. For the simplest case, ferric myoglobin from red kangaroo, the single tyrosyl residue at position 146 (Figure 2) is half-titrated at a pH of 12.8 (Figure 3 and Table I). In addition, one observes an inflection in the titration curve with a computed apparent pK of 9.0, far below pH 10.02, the pK of phenolic proton dissociation of tyrosine in aqueous solution at 25 °C, ionic strength 0.2, calculated by the Debye–Hückel theory from the data of Tanford & Roberts (1952). This low pK (pK_0 in Table I) is ascribed to the spectral difference at 245 nm between high-spin ferric myoglobin and its low-spin hydroxy form (see below).

The data for the ferric horse protein can be fit with three pKs, two ascribable to phenolic dissociations of tyrosyl residues, and a third with a pK near 9 which is ascribed to the effect

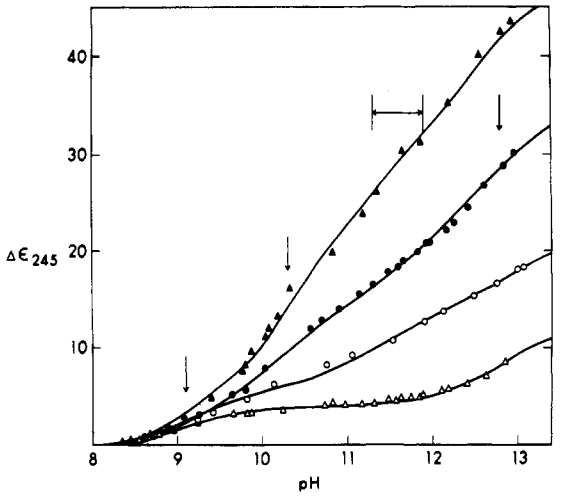

FIGURE 3: Difference spectrophotometric titrations at 245 nm of ferric myoglobins from kangaroo (△), horse (○), and sperm whale, fraction IV (●) and fraction IIIA (▲). Curves are iterated computer fits to the data. Vertical arrows indicate computed pK values given in Table I. The horizontal arrow indicates the span of computed pK values for Tyr-103 found in the horse and sperm whale proteins but not in the kangaroo protein.

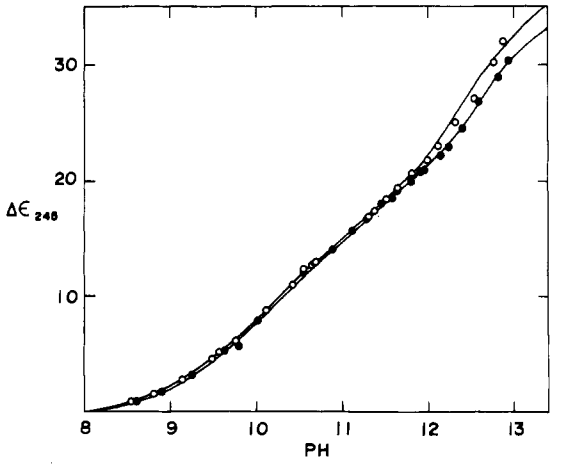

FIGURE 4: Reversibility of optical difference titration at 245 nm of sperm whale ferric myoglobin. The solid circles are the data for the forward titration, and the curve is an iterated least-squares fit of the data to a standard titration equation described under Materials and Methods. No difference is observed in the fit for protein brought up to pH 12.9 and subsequently back-titrated. The open circles and the accompanying fit are for ferric protein brought to pH 12.9 for 22 h and subsequently back-titrated. As can be seen, the apparent pK for Tyr-146 is moderately lower for the long-term base-treated protein.

of spin-state change on the optical spectral contribution of the heme (Table I). It is noteworthy that one of the pKs is essentially the same as the single apparent pK for the kangaroo protein and is thus assigned to the homologous tyrosine at position 146 (Figure 2). The second pK near 11.4 is thus assigned to tyrosine-103.

For the sperm whale protein, either the commercially prepared product or the chromatographically purified fraction IV from the commercial material, a third apparent pK is determined, now at 10.3, and is thus assigned to tyrosine-151 (Figure 2).

For fraction IIIA from sperm whale, yet another tyrosyl residue can be titrated (Table I), and its apparent pK, 10.6, is close to that of the tyrosyl group found in fraction IV of sperm whale ferric myoglobin but not in either the horse or the kangaroo proteins.

Reversibility of Reactions. Attempts to reversibly titrate phenolic groups of tyrosyl residues of heme proteins have not always proven successful. For example, raising the pH of ferric hemoglobin irreversibly exposes tyrosyl residues which otherwise would titrate at a higher pH (Nagel et al., 1966). With ferric myoglobin, this is not the case. All the tyrosyl residues are reversibly titratable.

In order to test the reversibility of dissociation of the phenolic groups of ferric myoglobin, a solution of sperm whale myoglobin was raised to pH 12.9 and was then back-titrated with HCl. The time-dependent changes of tyrosine ionization are neglibible after exposure of the protein to high pH from 90 s to 1 h. There was essentially no change in the difference spectrum of the protein that was first brought to a pH of 12.9 and reversibly titrated and the protein not raised to high pH at all. In fact, over a wide range of pHs (up to 12), the protein is stable for at least 22 h as monitored by the difference absorption at 245 nm.

On the other hand, after exposure of the protein to a pH above 12 for long time periods, there is a drift in the measured pH to lower values, suggesting a consumption of hydroxyl groups by protons from the protein structure. When sperm whale myoglobin is subjected to pH 12.9 treatment for 22 h and then back-titrated, the apparent pKs for tyrosines-146 and -103 were 12.7 and 12.1, respectively, while the apparent pK of tyrosine-151 did not perceptively change (Figure 4). Thus,

Table II: Apparent pKs for the Ionization of Tyrosine-146 in Various Derivatives of Kangaroo Myoglobin (See Table I for a Description of Legends)

derivative	pK_1	$\Delta\epsilon_1$	pK_0	$\Delta\epsilon_0$	calculated[a] pK_0
met fluoride					
0 M F^-	12.8	9.0	9.0	4.6	8.90
0.057 M F^-	12.8	11.0	9.4	3.2	9.45
0.311 M F^-	12.7	10.0	9.9	2.5	10.06
met CN	13.5	8.6	*b*		
deoxy	12.9	9.0	*b*		
oxy	13.4	8.0	*b*		
ferryl	13.3	9.0	*b*		
met reduced from ferryl	12.9	9.0	9.0	4.6	

[a] By the method of Beetlestone & Irvine (1968). See text for a description. [b] Not observed.

an alteration of protein structure had taken place in which the local environment of the tyrosyl residues had been slightly changed.

Transition to Low-Spin Forms. Ferric myoglobin has a transition from the acidic high-spin form to the hydroxy low-spin form (Keilin & Hartree, 1951). Titrating the ferric kangaroo protein and fitting the optical absorption at 583 nm as a function of pH to an algorithm similar to that given above yield a pK of 8.83, which is in excellent agreement with published values of 8.93 (George & Hanania, 1952) and 8.99 (Brunori et al., 1968) for horse and sperm whale proteins, respectively. In all of the difference spectral titrations at 245 nm shown in Figure 3, an inflection is observed which is ascribed to this same transition (Table I). The midpoint of this inflection is referred to as "pK_0".

Addition of Fluoride and Cyanide. The pKs for tyrosine dissociations in the various ferric myoglobin fluorides are essentially the same as for the ferric myoglobins (Tables II–IV). The pK_0, however, is altered since fluoride competes with hydroxide for the heme.

From the equilibrium constant, K_f, for fluoride binding to the high-spin ferric protein, Beetlestone & Irvine (1968) have shown that the hydrogen ion concentration at the apparent pK_0 in the presence of fluoride is given by $K_f/[(K_f + F^-)(1.26 \times 10^{-9})]$ where $K_f = 0.023$ and F^- is in mol/L.

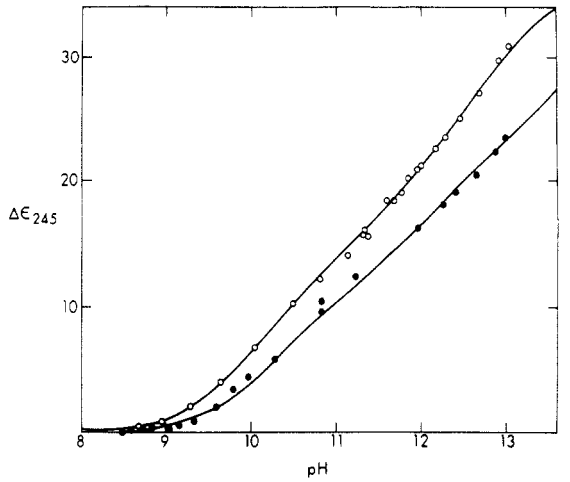

FIGURE 5: Difference spectrophotometric titration of sperm whale (O) ferric myoglobin fluoride ([F^-] = 0.057 M) and (●) ferric myoglobin cyanide at 245 nm. The curves are least-squares fits to the difference titration data, assuming three individual pKs arising from tyrosyl phenolic dissociation. For the fluoride derivative, an additional inflection is observed near pH 9.4 which arises from a spin-state change of the heme. The data are collected in Tables II and III.

Table III: Apparent pKs for Tyrosine Ionization in Various Derivatives of Horse Myoglobin (See Table I for an Explanation of Legends)

	position					
	146		103			
derivative	pK_1	$\Delta\epsilon_1$	pK_2	$\Delta\epsilon_2$	pK_0	$\Delta\epsilon_0$
met F^- (0.057 M)	13.0	10.0	11.8	8.0	9.5	6.7
met CN^-	13.1	8.7	11.4	7.6	*a*	
oxy	13.1	8.7	11.6	8.0	*a*	
ferryl	13.2	9.0	11.0	8.0	*a*	
met reduced from ferryl	12.9	8.0	11.0	8.0	9.2	6.5

[a] Not observed.

We have added fluoride to kangaroo metmyoglobin and determined the apparent pK_0 for the high- to low-spin transition by examining the difference spectrum at 245 nm. Our results (Table II) are in good agreement with those predicted by Beetlestone & Irvine (1968). For instance, in the presence of 0.31 M fluoride, the calculated apparent pK_0 is 10.1 (Figure 5). From the optical difference titration, at 245 nm, we obtained a value of 9.9. Thus, at pH 8.2 and with this fluoride

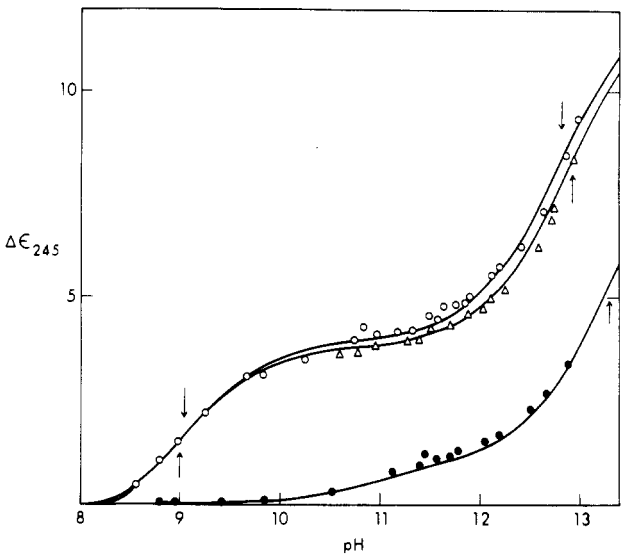

FIGURE 6: Difference spectrophotometric titration at 245 nm of (O) ferric kangaroo myoglobin, (●) ferryl kangaroo myoglobin formed with a 2-fold molar excess of H_2O_2 at pH 8.2, and (Δ) ferric kangaroo myoglobin reduced from ferryl myoglobin with ferrocyanide after a 24-h incubation at 5 °C. The pH of the reference sample is 8.2. In the ferryl protein, only a single inflection attributed to the pK of tyrosine-146 is seen. For the ferric protein, a second inflection is seen. This is ascribed to the pK of the ferric heme. The arrows are the computed pKs (Tables I and II) based on a least-squares fit of the optical data to standard titration equations.

concentration, the protein is 99% in the high-spin form. If this same titration is carried out in the absence of fluoride but in the presence of 3 mM cyanide, only the optical changes ascribed to tyrosine dissociation are observed.

For the kangaroo and sperm whale proteins, cyanide ligation to the heme shifts the pK ascribed to tyrosine-146 to higher values (Figure 5 and Tables II–IV). A comparison of the optical absorption at 245 nm for a metmyoglobin solution at pH 8.2 containing cyanide, where the protein is essentially all low spin, with a solution of protein at pH 8.2 containing 0.057 M fluoride leads to a $\Delta\epsilon_{mM}$ at 245 nm of 6.5, 7.6, and 7.9 for kangaroo, horse, and sperm whale proteins, respectively. As the pH of the reference samples is near 8.2, they contain roughly 20% low-spin protein, and the apparent extinction coefficients given for the pK_0 in Table I ($\Delta\epsilon_0$) are significantly smaller.

Deoxy- and Oxymyoglobin. Difference spectrophotometric titrations of oxy- and deoxymyoglobins were also carried out (Tables II–IV). The computed apparent pK for the dissoci-

Table IV: Apparent pKs for Tyrosine Ionizations in Various Derivatives of Sperm Whale Myoglobin, Fraction IV (See Table I for Explanation of Legends)

	position							
	146		103		151			
derivative	pK_1	$\Delta\epsilon_1$	pK_2	$\Delta\epsilon_2$	pK_3	$\Delta\epsilon_3$	pK_0	$\Delta\epsilon_0$
met F (0.057 M F^-)	12.9	11.0	11.9	10.0	10.3	11.0	9.4	6.0
met CN	13.4	11.0	11.9	10.0	10.3	11.0	*a*	
deoxy	13.0	11.0	12.2	10.0	10.4	11.0	*a*	
oxy	13.4	11.0	11.8	10.0	10.4	11.0	*a*	
ferryl	13.0	9.9	10.9	9.5	*a*		*a*	
met reduced from ferryl 10 min after formation followed by ferrocyanide	12.6	12.0	11.0	12.0	10.3	2.8	9.0	6.0
24 h after formation followed by ferrocyanide	12.9	12.0	11.3	10.8	10.3	1.8	9.0	6.8
spontaneously reduced after 7 days	12.8	12.4	11.3	9.7	10.3[b]	<1	9.1	6.4

[a] Not observed. [b] Assumed pK required to fit the titration data.

Table V: Number and Position of Tyrosine and Phenylalanine Residues in the Myoglobin from Various Species[a]

species	Tyr (position)	Phe (position)
sperm whale		
fraction IV	3 (103, 146, 151)	6 (33, 43, 106, 123, 138)
fraction IIIB	3	6
fraction II	3	6
fraction IIIA	4	6
human	2 (103, 146)	7 (33, 43, 46, 106, 123, 138, 151)
horse	2 (103, 146)	7 (33, 43, 46, 106, 123, 138, 151)
bovine	2 (103, 146)	7 (33, 43, 46, 106, 123, 138, 151)
harbor porpoise	2 (103, 146)	7 (33, 43, 46, 106, 123, 138, 151)
Black Sea dolphin	2 (103, 146)	7 (33, 43, 46, 106, 123, 138, 151)
harbor seal	2 (103, 146)	7 (33, 43, 46, 106, 123, 138, 151)
red kangaroo	1 (146)	9 (33, 43, 46, 103, 106, 123, 138, 149, 151)

[a] Data were obtained from Garner et al. (1974) and Dayhoff et al. (1976).

ation of tyrosine-146 is significantly higher for the oxyprotein than for the deoxyprotein.

Titration of Tyrosyl Residues of Ferryl Myoglobins. In Figure 6, we show the difference spectral titration of kangaroo ferryl myoglobin (prepared with a 2-fold molar excess of peroxide) which shows an inflection ascribed to the apparent pK of the dissociation of the single tyrosyl residue at position 146 in the primary structure (Air & Thompson, 1971). This pK (Table II) is 0.5 pH unit higher in the ferryl protein as compared to that observed in the ferric protein (Table I). This same shift of pK was observed for the horse ferryl protein as well (Table III). In addition, the pK ascribed to tyrosine-103 in the horse protein is shifted to a slightly lower value (Table V). For ferryl myoglobins from both species the inflection in the titration curve near pH 9 observed for the high-spin ferric protein is absent, as no titratable chromophore other than tyrosine is seen at 245 nm.

After the addition of a 2-fold molar excess of peroxide to both horse and kangaroo ferric myoglobins, the product was incubated for 24 h at 5 °C. Reduction was then effected with excess ferrocyanide, leading to the re-formation of the respective ferric myoglobins. After excess reagents were removed with Sephadex G-25, the apparent pKs of the tyrosyl residues were determined again by difference spectroscopic titration (Figure 6). For residue 146, the pK was restored to nearly the original value. Also, the infection at pH 9 ascribed to a spin-state change was once more observed. In addition, the intensity of the chromophore as evidenced from its extinction coefficient did not change. In the cycled horse protein, however, the pK ascribed to tyrosine-103 was permanently shifted to a lower value.

For the sperm whale protein, a different course of events takes place. Adding a 2-fold molar excess of peroxide does not alter the pK of Tyr-146 by as much as was observed for the horse and kangaroo proteins. However, the pK of Tyr-103 does shift to a lower value, as was noted for the horse protein. Lastly, the tyrosine at position 151 with an apparent pK of 10.3 in the ferric protein could not be titrated, even up to pH 13.

If sperm whale ferryl myoglobin, incubated from 10 min to 24 h with peroxide at 5 °C, is reduced to the ferric protein with ferrocyanide, and excess reagents are subsequently removed by gel filtration, the pK of Tyr-103 is permanently

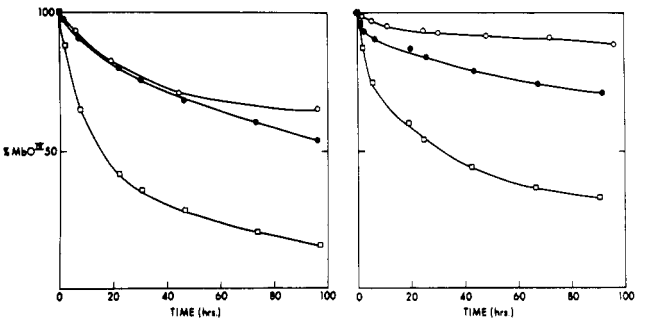

FIGURE 7: Relative stability of ferryl myoglobin from kangaroo (○), horse (●), and sperm whale (□) formed with a 2-fold molar excess of H_2O_2, pH 8.2. The ferryl proteins were stored at 5 °C either before (left) or after (right) gel filtration. The integrity of the ferryl protein was determined from the optical spectrum.

shifted to a lower value as compared to the uncycled protein, much in the same way as was observed for horse myoglobin. The pK ascribed to Tyr-151, however, is barely titratable. Its millimolar extinction coefficient drops from 11.0 for the uncycled protein to 2.8 for the protein incubated with peroxide for 10 min, or to 1.8 for the protein treated with peroxide for a longer period of time. Ferryl sperm whale myoglobin spontaneously autoreduced for 7 days and then treated with ferrocyanide still only shows a small absorption ascribable to the third tyrosyl residue. In all cases, however, recycled ferric protein exhibits an apparent pK near 9 in the difference titration which is due to the change of the spin state of the heme iron. The extinction coefficients for the first two titratable tyrosines, however, remain unchanged, even though the apparent pKs are altered.

Amino Acid Analysis. To elucidate the reason why the tyrosine residue at position 151 in sperm whale ferryl myoglobin, or ferric myoglobin which was reduced from the ferryl protein, is not titratable, we performed an amino acid analysis. We found that uncycled sperm whale ferric myoglobin has 3.1 tyrosyl residues per molecule while ferryl myoglobin or cycled ferric myoglobin which was either autoreduced or chemically reduced with ferrocyanide has 2.1. Thus, the conversion of sperm whale ferric myoglobin to the ferryl protein is accompanied by the destruction of tyrosine-151. No such loss of tyrosine was observed for either the horse or the kangaroo proteins.

Stability of Ferryl Myoglobin. Although in theory one should be able to convert metmyoglobin to the ferryl form with a single stoichiometric equivalent of H_2O_2 [one of the oxidizing equivalents being presumably converted to the hydroxy radical (George & Irvine, 1956)], one requires, as noted by Fox et al. (1974), about a 2-fold molar excess to completely convert the protein to the ferryl form at pH 8.2. Even in the presence of excess oxidant, ferryl myoglobin reverts to the ferric form. However, both kangaroo and horse proteins are about 5 times more stable than the sperm whale protein (Figure 7, left panel). If after 5 min of reaction with peroxide the excess oxidant is removed by gel filtration, the stability of all ferryl proteins is increased, but the one from kangaroo is still more stable than the others (Figure 7, right panel). Even after 24 h, ferryl kangaroo myoglobin is more than 90% pure while the horse and sperm whale ferryl myoglobins are less than 85 and 55% pure, respectively, based on their optical properties in the visible range.

Discussion

Assignment of pKs. The single tyrosine found at position 146 (Figure 1) in kangaroo myoglobin has an apparent pK of 12.8. As the homologous position of this tyrosine is invariant

in all the myoglobins studied and also has the same pK in all of them, it is suggested that its local environment is virtually the same in each case.

X-ray crystallographic analysis of sperm whale myoglobins shows that this tyrosine at position H22 in the Dickerson (1964) notation (Figure 1) is buried within the protein structure with its hydroxyl group hydrogen bonded to a main-chain carbonyl residue at isoleucine-99 (FG5), cross linking helices G and H (Kendrew, 1962; Takano, 1977). Thus, it is not surprising that the apparent pK for proton dissociation from this buried tyrosyl residue is unusually high. It is this tyrosine in sperm whale myoglobin that cannot be iodinated at near neutral pH, while the other two can (Kretzinger, 1968). Furthermore, as tyrosine-146 is invariant in all myoglobins, it probably plays an important role in the stability of the protein.

The second tyrosine residue found in the horse protein at position 103, but not in the kangaroo protein, has an apparent pK lower than that at position 146 (Table I). For the sperm whale protein, X-ray crystallographic analysis demonstrates that this tyrosine is more exposed to the surface of the molecule but still within a helical region, and a lower pK than that for the tyrosine at position 146 is to be expected. It is noteworthy that the apparent pK for this tyrosyl residue in the sperm whale myoglobin, at position G4, has a slightly higher value, suggesting a species-dependent lack of congruence in the local environment of this residue.

The last tyrosine, exclusive to the sperm whale protein at position 151 (position HC3), resides near the C-terminal helix and is more exposed to solvent than the others. Thus, its apparent pK is the closest to that of free tyrosine in aqueous medium (Tanford & Roberts, 1952).

In 1976, Wilbur & Allerhand studied the pKs of the tyrosines in the cyanide derivatives of the three ferric proteins reported here by using NMR spectroscopy. For Tyr-151 in the sperm whale protein, they report a value of 10.6 while the values they report for Tyr-103 in both the sperm whale protein and the horse protein vary from 11.5 to 11.7, in good agreement with the values we obtain from the optical titrations[2] (Tables III, IV). The pK of Tyr-146 found in all three proteins could not be accurately titrated by Wilbur & Allerhand, probably because they did not carry out their experiments to a high enough pH. They did suggest, though, that the pK is above 12.5.

Among the myoglobin molecules that have been investigated, there appears to be a near constancy in the sum of tyrosyl and phenylalanyl residues (Table V). Of the nine such residues in most proteins studied (red kangaroo and fraction IIIA of sperm whale being the exception with ten), an increase in the number of tyrosines is equal to the decrease in the number of phenylalanines. A minority chromatographic fraction of sperm whale myoglobin, fraction IIIA, described by Garner et al. (1974), has been shown to contain four tyrosyl residues. We have titrated this material and have assigned an apparent pK of 10.6 to the fourth tyrosyl residue (Table I), suggesting that like tyrosine-151 it must reside near the surface of the molecule. As the apparent pK of this fourth tyrosine is rather low, it is suggested that it is homologous with one of the phenylalanines that is exposed near the surface in the kangaroo protein. In kangaroo myoglobin, the phenylalanyl residue at position 149 is not found in any of the species examined (Table V). From perusal of the Dickerson model, however, this position is not exposed to the surface of the molecule, and it is doubtful that the fourth tyrosyl residue in fraction IIIA of sperm whale myoglobin is found there.

Recently, Lee & Richards (1971) and Shire et al. (1974) have suggested that only one of the phenylalanyl residues, at position 106, of the major fraction of sperm whale myoglobin is accessible to solvent, the other five being buried within the protein structure. If in fraction IIIA of sperm whale myoglobin this phenylalanyl residue were substituted by a tyrosyl group, then its pK would more than likely be close to that of free tyrosine, and a pK of 10.6 would not be unreasonable.

Stability of Ferryl Myoglobin. Because of spectral similarities of compound II of various peroxidases with ferryl myoglobin, the reaction of H_2O_2 with metmyoglobin is considered as a model for the peroxidase system. Unlike the peroxidases, especially cytochrome *c* peroxidase (Coulson et al., 1971), the reaction of H_2O_2 with ferric myoglobin has been found not to be stoichiometric with heme since the extent of reaction is dependent, in part, on the molar ratio of H_2O_2 to protein, but not on the absolute concentration (George & Irvine, 1952; Fox et al., 1974). In addition, for maximal formation of the ferryl protein, more than equimolar amounts of H_2O_2 are required. Even if one assumes a molar equivalence of peroxide and ferric heme in the reaction, one of the two oxidizing equivalents from H_2O_2 is retained by the heme, and the second is destroyed by an as yet obscure process.

George & Irvine (1952) suggested that the extra oxidizing equivalent from H_2O_2 which is not taken up by the heme is dissipated as a hydroxyl radical, $OH^{\cdot}$, and it is this species that can undergo further reactions in protein solution. Indeed, King & Winfield (1963) demonstrated that free-radical intermediates are generated from the reaction of ferric myoglobin and H_2O_2. However, no implications concerning stoichiometry or mechanism can be derived from these studies as these authors were unaware of the relationships between EPR line shape and quantitation of unpaired spins. Although the formation of free radicals of various types can be demonstrated after the addition of H_2O_2 to ferric myoglobin (Peisach et al., 1968), the yield of radical at any time after the onset of reaction is far less than either the total heme content or the concentration of ferryl heme formed. Thus, if free-radical species are produced from the remaining oxidizing equivalent of peroxide or from a breakdown product of an addition compound of ferric myoglobin and H_2O_2 where both oxidizing equivalents are retained by the protein, then this radical is short-lived and must participate in subsequent chemical reactions. Since ferryl myoglobin, either in the presence or in the absence of excess peroxide, is an unstable species, its autoreduction to the ferric protein must involve the formation of a free-radical species. Since ferryl myoglobin is less stable in the presence of excess peroxide (Figure 7, left panel), this radical probably participates in some sort of peroxide-dependent reaction.

The role of a specific, species-dependent protein moiety interacting with an oxidant was first observed with sperm whale but not with horse myoblobin. King & Winfield (1966) and King et al. (1967) titrated both proteins with chloroiridate at pH 6.5. For the horse protein, the ferryl state was achieved. For the sperm whale protein, the ferryl state did not form, yet chloroiridate was consumed. These findings suggest that when ferric myoglobin is treated with an oxidant not only the heme

[2] In order to fit their NMR titration data for Tyr-103 in both sperm whale and horse myoglobins, Wilbur & Allerhand (1976) required a second pK, at 9.8. These authors suggested that this pK represents a localized, pH-dependent, conformational change of the protein, brought about from the titration of a neighboring group near residue 103. The higher pK near 11.5 that we observe optically is that of the deprotonation of the phenolic group of the tyrosine.

reacts, leading to the formation of the ferryl state, but also the protein moiety reacts. If this altered protein is a free radical, then it must be unstable since its measured concentration is far from stoichiometric with heme (J. Peisach, unpublished observations). In comparison, cytochrome *c* peroxidase reacts stoichiometrically with H_2O_2 to produce ferryl heme and a stable free radical associated with the protein moiety (Coulson et al., 1971). This protein moiety must contain a local environment which, unlike that in myoglobin, stabilizes the radical product (Poulos et al., 1980).

From the study presented here, we suggest that the instability of sperm whale myoglobin can, in part, be explained by the interaction of the ferryl heme with that tyrosine closest to the surface of the proteins, at position 151. Of the tyrosine residues found in sperm whale myoglobin, the reactive one at position 151 is farther away from the heme than the unreactive one at position 103 (Figure 2). Although one cannot rule out either a direct or an indirect intramolecular reduction of ferryl heme by Tyr-151, it seems more reasonable that this reaction takes place as an intermolecular process.

Other evidence for participation of the protein moiety with the autoreduction process of the ferryl heme comes from studies which show that ferric myoglobin that had been cycled to the ferryl form and then reduced back to the ferric state is oxidized more slowly by H_2O_2 and reverts back more quickly to the ferric state by the autoreduction process (King & Winfield, 1963). The alteration of the apparent p*K*s of tyrosines in cycled myoglobin also bears evidence for a conformational change of the protein.

In summary, we should like to iterate the conclusions drawn by others (George, 1952; King & Winfield, 1963) that the reaction of ferric myoglobin and H_2O_2 is a very complicated one. What we have been able to show from this study is that in the presence of excess peroxide the absence of tyrosine-151 in ferryl kangaroo myoglobin endows this protein with greater stability than the ferryl protein from sperm whale where this tyrosine residue is present. In addition, if excess peroxide is removed from the reaction mixture, ferryl myoglobin is decidedly more stable. Yet, the autoreduction process is never completely inhibited under the best of circumstances.

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