Redox Equilibrium of Sperm-Whale Myoglobin, *Aplysia* Myoglobin, and *Chironomus thummi* Hemoglobin*

Maurizio Brunori,† Ughetta Saggese, Giacomo C. Rotilio, Eraldo Antonini, and Jeffries Wyman

ABSTRACT: This paper reports a study of the oxidation–reduction properties of three heme proteins, *i.e.*, sperm-whale myoglobin, *Aplysia* myoglobin, and *Chironomus thummi* hemoglobin, which are characterized by a simple equilibrium behavior, the value of n in the electrode equation being one in all cases.

(1) The dependence of $E_{0.5}$ on pH has been investigated for all three proteins. The largest contribution to the oxidation Bohr effect is due to the ionization of the heme-linked water molecule, although this process by itself cannot account completely for the pH effect. Therefore the presence of a "residual" Bohr effect, already reported for hemoglobin, is substantiated. (2) *Aplysia* myoglobin and *Chironomus thummi* hemoglobin show a close similarity in the absolute value of the redox potential. For these two proteins $E_{0.5}$ is higher than that of sperm-whale myoglobin by about 75 mV at pH 7 and 30°. (3) In the case of sperm-whale myoglobin, the apparent enthalpy change, once corrected at each pH for the contribution due to the heat of ionization of the water molecule, leads to a constant value for the intrinsic enthalpy of oxidation (ΔH_i = 5–6 kcal/mole from pH 6 to pH 10). (4) Addition of imidazole to sperm-whale myoglobin decreases the value of $E_{0.5}$ by about 140 mV at pH ~8 and 30°. This allows the determination of the binding constant of imidazole to ferrous myoglobin (K_R = 1.5 M), the value of the equilibrium constant for ferric myoglobin being known ($K_O = 8 \times 10^{-3}$ M). (5) In sperm-whale myoglobin, the substitution of protoheme with either meso- or deuterohemes is associated to relatively small differences in $E_{0.5}$ indicating the dominant effect of the protein moiety in determining the redox behavior of the heme group. (6) These results are compared to those obtained by other authors on several simple heme proteins and on protoheme. Such a comparison allows one to draw significant conclusions as to the relative importance of different structural factors in controlling the free energy of electron exchange at level of the heme iron.

Previous papers in this series have been mainly concerned with the oxidation–reduction equilibrium of hemoglobin, both normal and after various chemical modifications (Antonini *et al.*, 1964; Brunori *et al.*, 1964, 1965, 1968a). The role of cooperative effects in determining the redox properties of hemoglobin has been investigated. It has been shown, among other things, that the equilibrium of hemoglobin is dominated by two opposing effects, *i.e.*, positive heme–heme interactions and intrinsic heterogeneity as between the two types of polypeptide chain (Brunori *et al.*, 1965, 1967, 1968a). At acid pH (*e.g.*, pH ~6–7) the β chains have a redox potential about 60 mV higher than that characteristic of the α chains (Banerjee and Cassoly, 1969), a fact which is not yet understood in structural terms. The difference in the Bohr effect between the two chains is largely responsible for the decrease in n with pH, the dependence of cooperativity on pH being probably small (Antonini *et al.*, 1964; Antonini and Brunori, 1970).

An interesting problem of general significance is the understanding of the structural factors which determine the free energy of electron exchange in heme protein complexes. It is known that the oxidation–reduction potential can vary by as much as several hundred millivolts between proteins containing the same prosthetic group. Thus the redox potential of protoheme may be shifted either positively or negatively as a result of interaction with a specific apo protein.

The present study represents an attempt to understand the effect of several variables on this phenomenon through a study of three simple heme proteins, namely sperm-whale myoglobin, *Aplysia* myoglobin, and *Chironomus Thummi* hemoglobin. All three behave in a simple fashion, in the sense that the redox equilibrium can be accurately described by the standard electrode equation which, at constant pH, is given by

$$E_h = E_{0.5} + \frac{RT}{nF} \ln \frac{[Fe^{3+}]}{[Fe^{2+}]} \quad (1)$$

with a value of $n = 1$. Some of the variables, such as pH, have been investigated for all three cases; this allows a direct comparison, free from pH effects, of the role of the protein moiety on the equilibrium at the level of the heme iron. In sperm-whale myoglobin the dependence of the redox equilibrium on temperature, on the chemical nature of the heme, and on the identity of the sixth (axial) ligand has also been investigated.

The results, combined with information now available on the three-dimensional as well as the primary structure of two of the proteins, namely sperm-whale myoglobin and *Chironomus* hemoglobin, make it possible to assess the role of the distal histidine, and they bring out the dominant influence of the protein in determining the redox properties of these molecules.

Experimental Section

Materials

Sperm-whale myoglobin was a commercial preparation from Seravac Chemical Co., recrystallized from $(NH_4)_2SO_4$ according to the procedure of Rossi-Fanelli (1949). The

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† To whom to address correspondence.

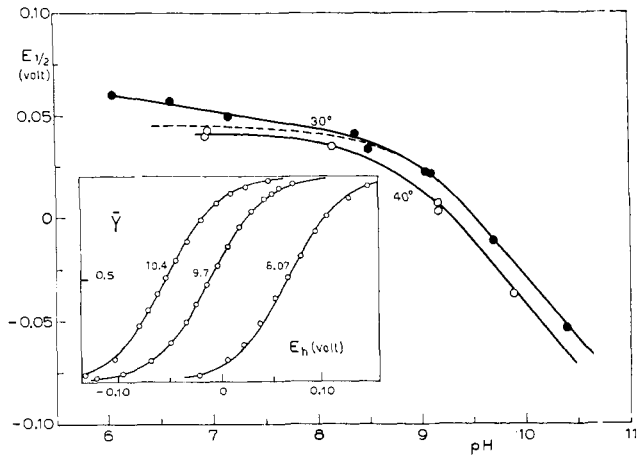

FIGURE 1: Oxidation Bohr effect of sperm-whale myoglobin at 30° (●) and 40° (○) in phosphate and borate buffers. Dashed line represents the expected dependence of $E_{0.5}$ on pH for the oxidation-linked ionization of the water molecule with a pK ~ 8.8 at 30°. The insert shows the actual equilibrium curves at various pH values; ordinate, fractional oxidation; abscissa, potential (in volts).

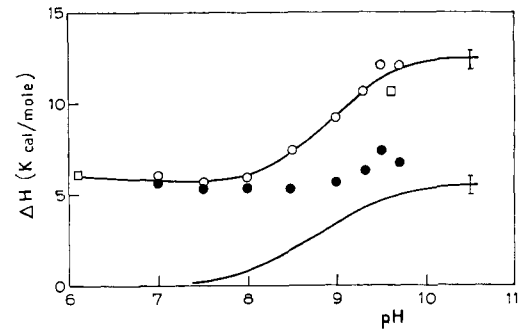

FIGURE 2: Dependence on pH of the apparent enthalpy change for sperm-whale myoglobin. Symbols as follows: ○, values obtained by (a) procedure described in the methods; temperature interval 30–40°. □, values obtained by (b) procedure described in the methods; temperature interval 20–30°. ●, values of ΔH_ϵ obtained after subtraction of the contribution due to the ionization of the water molecule (ΔH_{H^+}) indicated by the lower continuous line (see text).

material was electrophoretically heterogeneous, containing three major and two minor fractions. Isolation of homogeneous components was achieved by starch block electrophoresis accroding to Kunkel (1954). The concentration of myoglobin was determined spectrophotometrically on the basis of E_{mM} = 12 at 560 nm for deoxy myoglobin obtained with dithionite. Reconstituted sperm-whale myoglobins containing proto-, meso-, and deuterohemes were prepared by adding a stoichiometric amount of the various hemes to globin, the reconstitution procedure being performed at 4°. Globin from sperm-whale myoglobin was obtained by the method of Rossi-Fanelli *et al.* (1958).

Aplysia myoglobin was prepared from buccal muscles according to Rossi-Fanelli and Antonini (1957). Its concentration was determined spectrophotometrically in the reduced form by taking the extinction coefficient E_{mM} = 13 at 555 nm.

Erythrocruorin (here called hemoglobin) from *Chironomus thummi* was generously provided by Dr. H. Formanek of the Max-Planck Institut für Eiweiss und Lederforschung in Münich. The material was one of the components isolated chromatographically and crystallized according to the method of Huber *et al.* (1964). This component has a molecular weight of 16,000 calculated by sedimentation diffusion and by X-ray analysis. Its concentration was determined spectrophotometrically in the reduced form on the basis of the extinction coefficient E_{mM} ~109 at 428 nm.

The following buffers were used: 0.1 M potassium phosphate (pH 5.7–7.8) and 0.04–0.05 M sodium borate (pH 8.2–10.1).

All reagents were commercial grade and were used without purification.

Methods

Oxidation–reduction equilibria were measured by the standard procedure already described (Antonini *et al.*, 1964). A saturated calomel electrode served as reference cell throughout the work, except for some of the experiments on the effect of temperature, in which a 0.1 M KCl cell was employed. Potentials are referred to the standard hydrogen electrode according to the convention of Clark (1960).

In the oxidation–reduction titrations the following reagents were used: anthraquinone-β-sulfonate (previously reduced by hydrogen and palladium) to reduce ferrihemoproteins, and potassium ferricyanide to oxidize ferrohemoproteins. Oxygen was removed from the system by flushing with pure argon.

In all cases, in order to facilitate attainment of equilibrium at the platinum electrode, small amounts of an electromotively active mediator (toluylene blue, methylene blue, cresyl blue, or thionine) were added. The molar concentration of these varied, in the different experiments, between 2 and 4% of that of the protein. Experiments were generally performed at 30 ± 0.1°. The apparent enthalpy change of sperm-whale myoglobin was measured by two slightly different procedures. (a) A series of titration curves performed both at 30 ± 0.1°, and at 40 ± 0.1°, the absolute redox potentials being measured against a saturated calomel electrode equilibrated to the temperature in question; (b) the redox potential of a solution containing ferro- and ferrimyoglobin in equimolar amount was measured at different temperatures from 20 to 30°. The results were the same whether the temperatures were increased or decreased successively, which provides good evidence that in each case true equilibrium was obtained.

Results and Discussion

Sperm-Whale Myoglobin. The results on sperm-whale myoglobin are comparable to earlier data on horse myoglobin obtained under similar conditions (Taylor and Morgan, 1942; Behlke and Scheler, 1961). In the presence of mediators stable potentials were reached in a reasonably short time (5–15 min for each point). The shape of the equilibrium curves conforms to a simple one-electron process at every pH, as shown by the results in Figure 1 (insert). The titrations were completely reversible, as demonstrated by the identity of the oxidative and reductive titrations.

Since sperm-whale myoglobin is electrophoretically heterogeneous, the simple behavior implies, in the absence of compensation effects, that the various electrophoretic components have the same redox potential. As a proof to this point, the redox behavior of a major component, isolated by starch block electrophoresis, was determined and found to be identical to that of the overall mixture.

EFFECT OF pH AND TEMPERATURE. The dependence of $E_{0.5}$ on pH at 30 and 40° is reported in Figure 1. In qualitative agreement with previous results, the potential decreases above

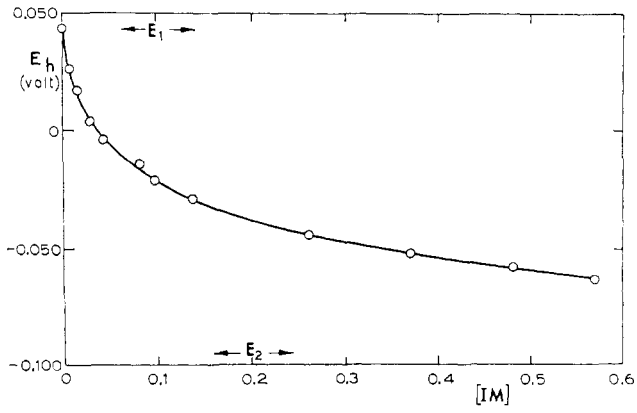

FIGURE 3: Effect of the concentration of imidazole on the redox potential of sperm-whale myoglobin at 50% of oxidation, pH ~8, 0.2 M phosphate buffer and 30°. The levels of redox potential in the absence, E_1, and in the presence of saturating amounts of imidazole, E_2, are reported.

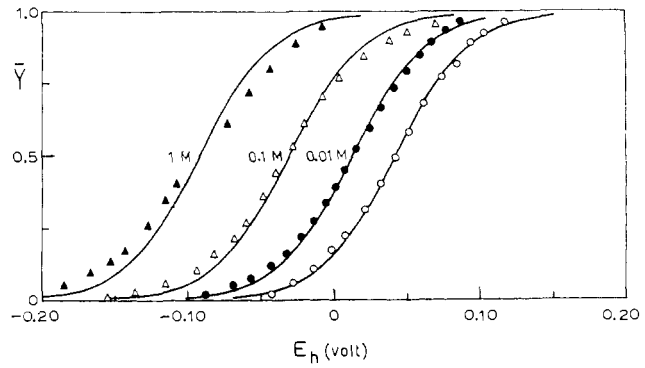

FIGURE 4: Oxidation–reduction equilibrium curves of sperm-whale myoglobin in the absence and presence of imidazole at different concentrations. ○, zero imidazole, pH 7.8; ●, 0.01 M imidazole, pH 7.8; △, 0.1 M imidazole, pH 7.94; ▲, 1 M imidazole, pH 7.90. In all cases phosphate buffer, 0.2 M and 30°; ———, theoretical curve for $n = 1$. Ordinate, fractional oxidation; abscissa, potential (in volts).

pH ~8 (Behlke and Scheler, 1961); the main cause of this is the ionization of the heme-linked water molecule, which has a pK of ~8.8 at 30° as determined directly by spectroscopy (Brunori *et al.*, 1968b). However, this ionization process by itself does not completely account for the oxidation Bohr effect, as will be seen from a comparison of the experimental data at 30° with the curve calculated on the basis of the ionization of the water molecule. The difference between the contribution due to the water molecule and the total pH effect is a feature common to all heme proteins, both normal and modified, so far examined (Antonini *et al.*, 1964; Brunori *et al.*, 1964, 1969).

The data in Figure 1 show that the effect of temperature on $E_{0.5}$ is relatively small. The apparent enthalpy change (ΔH_{app}) at different pH values is reported in Figure 2. It can be seen that the results (open squares and circles) obtained by the two different methods, a and b, described in the Experimental Section, are in good agreement; this is a very strong indication of the attainment of a true equilibrium. The dependence of ΔH_{app} on pH arises from the enthalpy changes accompanying oxidation-linked ionizations. The net enthalpy change, obtained by subtracting, at each pH, the heat of ionization of the water molecule ($\Delta H \sim 5.5$ kcal/mole) from the total heat, is identified in Figure 2 by the black circles. It was calculated from eq 2 where ΔH_ϵ = intrinsic enthalpy change for the

$$\Delta H_{app} = \Delta H_\epsilon + \nu \Delta H_{H^+} \qquad (2)$$

oxidation process, ΔH_{H^+} = heat of ionization of the water molecule, and ν is the number of protons dissociated at the given pH as a result of oxidation. The essential constancy of ΔH_ϵ (= 5–6 kcal) over the whole pH range is evidence that the only significant contribution to the overall heat is that due to the water molecule. The additional Bohr effect acid to pH 7, which is evident in Figure 1, does not show up, whether because the number of protons involved is too small or because their heat of ionization is negligible. These results show that any attempt to estimate the enthalpy of oxidation on the basis of experiments obtained at one pH value can be very misleading.

The simple behavior of sperm-whale myoglobin stands in marked contrast to that of hemoglobin, where the value of ΔH_ϵ obtained in exactly the same way by subtracting ΔH_{H^+} from ΔH_{app}, instead of being constant, is about 8 kcal greater at pH 6.1 than at pH 9, where it is about 3–4 kcal (Antonini *et al.*, 1964). This obviously implies some other pH-dependent process; since in the absence of any large oxidation Bohr effect at acid pH there can be no considerable net liberation of proton, it would seem that this 8 kcal must represent an internal transfer of proton (Brunori *et al.*, 1965).

EFFECT OF IMIDAZOLE. Imidazole is known to coordinate with the iron atom in the oxidized state and the equilibrium constant for the process can be measured spectrophotometrically. Replacement of the water molecule by an imidazole is accompanied by a change in spin state of the system from high to low (Russell and Pauling, 1939). Imidazole is known to interact also with ferrous myoglobin, although with much lower affinity (Keilin, 1966). There is thus a linkage between imidazole binding and the oxidation process. This is borne out by the fact that addition of imidazole to sperm-whale myoglobin at 50% oxidation results in a drop in potential, which, at pH 8, is ~140 mV, as shown in Figure 3. This decrease in potential, which is to be expected in view of the higher affinity of imidazole for oxidized myoglobin, is also proton linked. Knowledge of the redox potential in the absence of imidazole, E_1, and under saturating conditions, E_2, permits the calculation of the ratio, K_O/K_R, between the equilibrium constant of the oxidized and reduced forms, by means of the simple approximate relation

$$E_2 = E_1 + \frac{RT}{nF} \ln \frac{K_O}{K_R} \qquad (3)$$

Given the value of $K_O = 8 \times 10^{-3}$ M at pH 8 and 30°, K_R can be estimated to be about 1.5 M under the same conditions. The results presented in Figure 4 on the effect of different concentrations of imidazole on the redox equilibrium curves reveal the same phenomenon from a different point of view. Comparison of the $E_{0.5}$ values from Figure 4 with those plotted in Figure 3 shows that the two sets of experiments are in good agreement.

It may be noticed that the titration curve at 1 M imidazole in Figure 4 shows a certain amount of distortion from the simple case where $n = 1$. The reason for a value of $n < 1$ is not clear; certainly it is not related to the electrophoretic

TABLE I: Oxidation–Reduction Potentials of Different Hemes and of Reconstituted Myoglobins.[a]

	Hemes		Myoglobins	
	$E_{0.5}$ (V)	pH	$E_{0.5}$ (V)	pH
Proto-	−0.24	9	+0.047	7.06
Meso-	−0.278	9	+0.041	7.10
Deutero-	−0.19	9	+0.025	7.08

[a] Temperature 30°, except for deutero, 25°. The vinyls, which occupy positions 2 and 4 of the porphyrin ring in protoheme, are substituted: (a) in mesoheme by ethyl groups and (b) in deuteroheme by hydrogens. Results for proto-, meso-, and deuterohemes from Clark (1960).

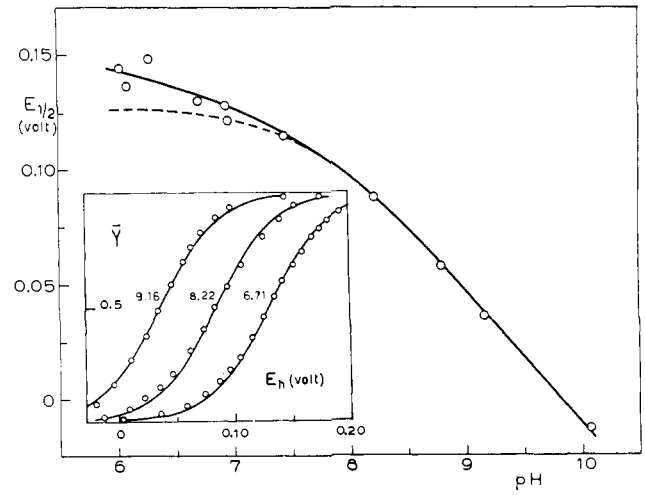

FIGURE 5: Oxidation Bohr effect of *Aplysia* myoglobin at 30°, in phosphate and borate buffers. Dashed line represents the expected dependence of $E_{0.5}$ on pH for the oxidation-linked ionization of the water molecule with a pK ~ 7.5 at 30°. The insert shows the actual equilibrium curves at various pH values; ordinate: fractional oxidation; abscissa: potential (in volts).

heterogeneity of sperm-whale myoglobin, since an electrophoretically homogeneous component shows the same effect.

RECONSTITUTED MYOGLOBINS. The influence of the nature of the heme upon the oxidation–reduction equilibrium of sperm-whale myoglobin was examined at pH 7 and 30°. Reconstituted protomyoglobin is identical with native myoglobin. Exchanging proto- with either meso- or deuteroheme results in the changes in $E_{0.5}$ shown in Table I. The differences in $E_{0.5}$, although certainly significant, are small in comparison with those between the corresponding free hemes, for which even the trend is different. This shows the extent to which the behavior of the system is dictated by the protein moiety.

Aplysia Myoglobin. Redox curves of *Aplysia* myoglobin at different pH values and 30° are shown in Figure 5 (insert). Under all conditions the shape of the curves corresponds to a one-electron process and the reaction is completely reversible. Comparison of these results with those on sperm-whale myoglobin shows a considerable difference, the value of $E_{0.5}$ for *Aplysia* myoglobin being about 75 mV higher than that for sperm-whale myoglobin at neutral pH. The pH dependence of $E_{0.5}$ is compared in Figure 5 with that expected on the basis of oxidation-linked ionization of the water molecule, which, in *Aplysia* myoglobin, has a pK of ~7.5 (Brunori *et al.*, 1968b). It is clear that, as reported above for sperm-whale myoglobin the oxidation Bohr effect cannot be accounted for solely on the basis of the ionization of the water molecule. The difference, which represents what has been called "residual oxidation Bohr effect" (Brunori *et al.*, 1969), amounts to about 10 mV at pH 6.5.

Chironomus Hemoglobin. The pH dependence of $E_{0.5}$ for the monomeric component of the hemoglobin of *Chironomus thummi* for which X-ray crystal analysis at 2.8-Å resolution is available (Huber *et al.*, 1969) is compared in Figure 6 to that expected on the basis of the dissociation of the water molecule, which in this case has a pK of ~7.4 (Brunori *et al.*, 1968b). It will be seen that the discrepancy, though of the same type, is much larger than that observed in both sperm-whale and *Aplysia* myoglobins. The similarity both as regards values of $E_{0.5}$ and the oxidation Bohr effect between *Aplysia* myoglobin and *Chironomus* hemoglobin is striking and probably not casual in view of the structural similarities of the two proteins.

Concluding Remarks

Systems containing ferroporphyrin have been extensively investigated both on account of their biological importance and the possibility of comparing their behavior to that of model systems. For several of these, such as the cytochromes, the reversible change in the valence state of the iron represents the physiological function; for others, such as the proteins investigated here, oxidation of the iron, although it represents a true reversible equilibrium and throws light on the physical properties of the molecule, does not enter into their biological role.

In this paper we have confined our attention to ferroporphyrin–protein complexes which are characterized by a simple equilibrium behavior. Figure 7 shows the redox potential, $E_{0.5}$, as a function of pH for several such systems, all of which possess the same prosthetic group (protoheme IX); $E_{0.5}$ for ferroprotoporphyrin (IX) itself is included for comparison. The change in $E_{0.5}$ in passing from cytochrome *c* to horseradish peroxidase amounts to ~0.52 V at pH 7.0; the other proteins lie in between. It is natural to investigate the

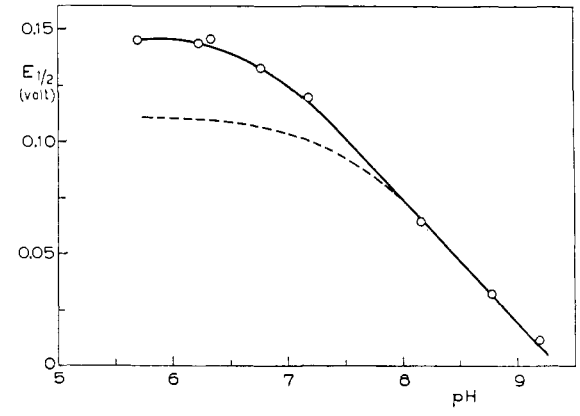

FIGURE 6: Oxidation Bohr effect of *Chironomus thummi* hemoglobin at 30° in phosphate and borate buffers. Dashed line represents the expected dependence of $E_{0.5}$ on pH for the oxidation-linked ionization of the water molecule with a pK ~ 7.4 at 30°. The titration at pH acid to 7 show a certain degree of heterogeneity; therefore the value of n in these experiments is subject to a larger uncertainty, around an average of 0.9. At all other pH values n = 1.

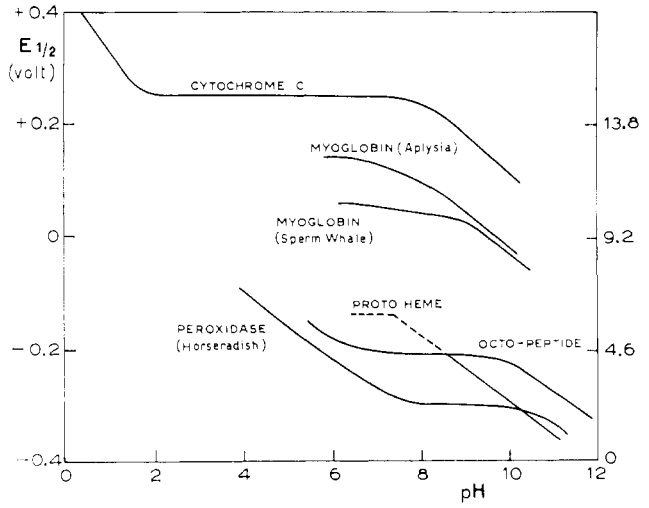

FIGURE 7: Relationship between $E_{0.5}$ and pH for protoheme and various simple heme proteins, at 30°. The figures on the right-hand scale indicate relative free-energy change (in kilocalories per mole). References: Clark (1960), for protoheme; Rodkey and Ball (1950), for cytochrome *c;* Harbury and Loach (1960), for octopeptide of the cytochrome *c* and Harbury (1957), for horseradish peroxidase.

TABLE II: Oxidation–Reduction Potentials of Various Simple Heme Proteins.[a]

	$E_{0.5}$ (V) (pH 7, 30°)	State of Aggregation	Distal Imidazole
Sperm-whale myoglobin	+0.050	Monomer	Present
α chain	+0.050	Monomer	Present
Aplysia myoglobin	+0.125	Monomer	Absent
Chironomus hemoglobin	+0.125	Monomer	Absent
β chain	+0.110	Tetramer	Present

[a] Results for α and β chains from Banerjee and Cassoly (1969).

reasons for the large differences between the various members of the series and to find their structural basis.

It will be seen that depending on the nature of the conjugated protein the potential of the complex may be shifted either up or down in respect to free heme. From a formal point of view, the shift can be considered in terms of a difference in the free energy of binding of the two axial ligands to the oxidized and reduced heme. This subject has been extensively investigated for complexes of metalloporphyrins with various simple ligands, such as pyridine, cyanide, imidazole, and α-picoline (Clark, 1960). Along the same lines the shift observed upon binding of heme to a specific site in the protein might be visualized as a difference in the affinity of the apoprotein for ferrous and ferric heme. However this type of approach, valuable as it is, gives no information on the structural factors involved, which must obviously include the chemical nature and the mutual interactions of the components.

The experiments on sperm-whale myoglobin reconstituted with proto-, meso-, and deuterohemes clearly show that modification of the side chains in positions 2 and 4 of the porphyrin ring has only a minor effect. This, small as it is, might be taken to represent the effect that the porphyrin side chains exert on the electron density at the level of the iron atom. Larger effects of a similar type are evident in the case of the free hemes (see Table I). It would appear that the inherent differences between the hemes tend to vanish upon combination with the protein and be replaced by other, more important effects. These probably depend on the structural constraints and on the special hydrophobic environment provided by the polypeptide chain folded around the heme.

The role of the axial ligands has been emphasized several times, and may well be the most important effect of all. In all hemeproteins studied in this work, and most probably also in others, the fifth ligand is likely, if not certain, to be the imidazole group of a hystidyl residue of the protein. This is true for sperm-whale myoglobin (Kendrew, 1962), *Chironomus thummi* hemoglobin (Huber *et al.*, 1969), for the α and β chains of human hemoglobin (Perutz *et al.*, 1968), as well as for mammalian cytochrome *c* (Dickerson *et al.*, 1967), and is highly probable also in *Aplysia* myoglobin and horseradish peroxidase. The bond involved represents a direct interaction of the metal atom with the protein and constitutes a handle by which the protein controls the reactivity of the iron atom.

The role of the so-called "distal" imidazole is clearly assessed by the results reported here. The chemical composition and three-dimensional structure of sperm-whale myoglobin and *Chironomus thummi* hemoglobin show that the distal position is occupied by the imidazole of histidine E7 in sperm-whale myoglobin (Kendrew, 1962), and by the side chains of isoleucine E11 in *Chironomus thummi* hemoglobin (Huber *et al.*, 1970). Therefore a distal histidine is absent in *Chironomus* hemoglobin, and the same is likely to be true for *Aplysia* myoglobin which contains only one histidine per molecule (Tentori *et al.*, 1968). The redox potential of these two heme proteins is higher (by about 75 mV) than that of sperm whale myoglobin or that of the isolated α chains, both of which are very similar. However, the redox potential of the isolated β chains, which also have a distal histidine, is very similar to that of the two proteins lacking the distal imidazole. These results, which are summarized in Table II, show that no unique role can be attributed to the distal imidazole, either in the redox equilibria or in the formation of a stable O_2 complex with the ferrous form. Evidently it is the character of the protein as a whole, rather than the identity of the groups specifically interacting with the porphyrin, which determines the behavior of the molecule.

Acknowledgments

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Dimerization of α-Chymotrypsin. I. pH Dependence in the Acid Region*

Kirk C. Aune† and Serge N. Timasheff‡

ABSTRACT: The dimerization equilibrium constant of α-chymotrypsin was determined by the method of sedimentation equilibrium as a function of pH. The pH dependence was interpreted in terms of short-range electrostatic interactions between two pairs of identical ionizable groups in the enzyme. From a consideration of the known crystal structure, these groups were identified as the imidazole ring of histidine 57 and the α-carboxyl of tyrosine 146.

In recent years, it has become evident that the association between protein molecules plays an important role in the control of enzyme activity. Such associations affect catalytic rates by altering binding constants for substrates and products. The allosteric models of Koshland *et al.* (1966) and of Monod *et al.* (1963) discuss the linkage of subunit interactions with enzyme activity. The specific interactions which would supply the free energy needed to affect the activity can be expected to differ greatly for various systems. Therefore, it is highly desirable to specify the types of interactions which might be operative in any given system, rather than to generalize. The present study on the dimerization of α-chymotrypsin is directed toward that goal.

The self-association of α-chymotrypsin has been the subject of a number of studies. About 20 years ago, Schwert (1949) and Schwert and Kaufman (1951) observed that the sedimentation coefficient of the protease, bovine pancreatic α-chymotrypsin was concentration and pH dependent, even though the sedimentation pattern was characterized by only a single symmetrical schlieren peak. They suggested that this was the result of a monomer–dimer equilibrium, but they considered that the reaction was complex, because the concentration dependence of $s_{20,w}$ could not be described in terms of a simple monomer–dimer equilibrium constant. Later, Steiner (1954) investigated the aggregation reaction by means of light scattering, showing that the reaction was in fact a monomer–dimer equilibrium and that the association was enhanced at pH 4.4 by an increase in ionic strength. Egan *et al.* (1957) reported that the sedimentation coefficient of α-chymotrypsin

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† Recipient of American Cancer Society Postdoctoral Fellowship No. PF-484; present address: Department of Biochemistry and Molecular Biology, The Ohio State University, Columbus, Ohio 43210.

‡ To whom correspondence should be addressed at the Graduate Department of Biochemistry, Brandeis University, Waltham, Mass. 02154.