

Isoelectric Fractionation, Analysis, and Characterization of Ampholytes in Natural pH Gradients. V. Separation of Myoglobins and Studies on their Electro-Chemical Differences

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A crude preparation of myoglobin from horse skeletal muscle containing a mixture of ferri- and ferro-myoglobin was studied by using the method for isoelectric focusing of proteins in stable pH gradients. This protein displays a heterogeneity studied in the ferric form by Theorell and Åkeson.¹⁻³ The components and sub-components of it have been resolved by this method. The most acidic components could be resolved into three sub-components, as shown now for the first time. It has been possible not only to separate but also to identify and determine the isoelectric points, pI, of the sub-components of the heterogeneity in both ferric and ferrous forms. Some of the components showing pI differences in the order of 0.02 pH units, giving additional evidence of the resolving power of this method as calculated in paper number IV of this series.⁴

The buffering capacity was determined for the main component, Mb I, after isolation by this method. It is shown that if the molecular weight, buffering capacity, and pI are known for a protein then it is possible to calculate the net charge of this protein at the pI of another protein. In the case of proteins which are closely related, *e.g.* the ferric and ferrous forms of hemoproteins or the components of protein heterogeneities, this can be valuable for conclusions on the chemical differences between such forms.

Theorell and Åkeson¹⁻³ have examined three main fractions of horse skeletal ferri-myoglobin, which they called ferri-Mb I, Mb II, and Mb III in the order of increasing acidity. They further showed that Mb II could be separated into two sub-fractions called Mb II₁ and Mb II₂ and that Mb III was also probably heterogeneous. The system of these myoglobins thus displays a spectrum of components and sub-components with slightly different electro-chemical properties. Mb II₁ and Mb II₂ are very similar. This fact was used

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in a preceding paper,⁴ in an experimental study of the resolving power of this method for isoelectric focusing and separation. It was shown that these sub-components, having a pI difference of only 0.06 pH units could be completely separated, which is in agreement with a theoretical resolving power of 0.02 pH units.

The present paper will also deal with isoelectric focusing of myoglobins. The main interest will be focused on the separation, identification, and pI determination of all myoglobin components and sub-components. The majority of the earlier studies on myoglobin heterogeneities have been carried out on ferri-Mb, probably because ferro-Mb,* although being the physiological form, is less stable than the ferric form and susceptible to oxidation to the ferric form. In this report it will be shown that the isoelectric focusing method allows separation of and measurements on the ferrous as well as the ferric forms, and that even mixtures thereof can be successfully analyzed.

MATERIALS AND METHODS

Electrolysis apparatus. An electrolysis column** of 110 ml capacity, as described in paper number IV of this series⁴ was used. Cooling water of +4°C, from a thermostated bath, was circulated through the mantle during the experiments.

Chemicals. As carrier ampholytes** fractions isoelectric between pH 6.5 and 8 of a synthetic mixture of low-molecular aliphatic polyamino-polycarboxylic acids of the same type as described in Ref. 4. Sucrose, analytical reagent grade, from Mallinckrodt Chemical Works, St Louis, USA, was used.

Myoglobins. 2 kg of skeletal muscle of horse were cooled in an icebath, 15 min after slaughter. After extraction with water, crude crystals of myoglobin were obtained by adding ammonium sulphate essentially as described by Åkeson,² yet without using ferricyanide. Moreover, by courtesy of Dr. Åkeson at the Nobel Medical Institute, two preparations "Mb II₁" and "Mb II₂" have been supplied. These had been isolated by carefully conducted ion-exchange column chromatography and were supplied in the form of crystals of ferri-Mb in a saturated ammonium sulphate solution.

Spectrophotometric analysis. A Beckman Spectrophotometer, model DU, was used for extinction measurements. Light absorption spectra were recorded in a Beckman spectrophotometer, model DK 2. Ferro- and ferri-Mb show different light-absorption spectra.¹ Quotients between the extinction values at 580 and 594 mμ were used in order to get a rough estimate of the relative amounts when both forms were present in an unresolved mixture (Shikama³).

The total amount of myoglobin was determined¹ in the ferric form, at 409 mμ, in a 0.05 M phosphate buffer of pH 7.0, and using a molar extinction coefficient of 17.1×10^4 and a molecular weight of 18 800. Conversion to the ferric form was made by addition of an equivalent quantity of a 10^{-3} M solution of $K_3Fe(CN)_6$ (Austin and Drabkin⁶).

After separation in the column, light absorption spectra were recorded for the different hemoprotein fractions as well as for their carbon-monoxide compounds. The latter were prepared after careful reduction of the ferric forms, when necessary, with sodium dithionite, $Na_2S_2O_4$, and saturation of the solution with carbon monoxide. An absorption peak at 578 mμ for CO-myoglobin, and a corresponding peak at 570 mμ for CO-hemoglobin, were used for the identification of these hemoproteins.¹

Preparation of the column and isoelectric focusing by electrolysis. The procedure for preparing the column was described in detail in Ref. 4. In this work 1 % solutions of carrier ampholytes were used in the electrolysis experiments. Before application to the column, myoglobin crystals were collected by centrifugation, dissolved in a few ml of

* In this paper ferro-Mb is equal to MbO_2 if not especially indicated that the oxygen free form is present.

** Now available from LKB-Produkter AB, Box 76, Stockholm-Bromma, Sweden.

distilled water and dialyzed with two changes against a 0.5 % solution of carrier ampholytes, isoelectric between pH 6.5 and 7.5.

pH Determinations. The pH measurements were made with a Radiometer pH-meter, model PHM 25 SE, at +4.0°C with a relative accuracy of ± 0.01 pH. A Radiometer Standard buffer solution with pH 7.10 ± 0.02 at 4°C was used as a standard.

PROCEDURE AND RESULTS

Experiment 1 a. When filling the column with the carrier ampholyte solution, a dialyzed solution of the crude crystalline mixture, containing 16 mg of myoglobin, was treated, just before being added to the column, with a slight molar excess of $K_3Fe(CN)_6$. Spectrophotometry proved that this procedure converted all hemoproteins into their ferric forms. After isoelectric focusing with the cathode in the bottom of the column, several brown zones, but no red ones, could be seen. The zone positions were read on a ruler. The isoelectric spectrum is shown in Fig. 1. After fractionation, pH-measurements and spectrophotometric analyses were carried out. The main zone, number 1 in Fig. 1, showed a typical ferri-Mb absorption spectrum and had the highest pI of all the myoglobins; it thus corresponds to Mb I in the nomenclature of Theorell and Åkeson.¹ The next protein zone, number 2, was identified as ferri hemoglobin by spectrophotometry of its carbon monoxide form. This zone contained less than 1 % of the total protein applied to the column. For the identification of zones 3 and 4 the preparations "Mb II₁" and "Mb II₂" of Åkeson were used in the following way. After analysis of the fractions of Experiment 1a these were retransferred to the column in the order of decreasing density. At the same time 2 mg of myoglobin from the preparation "Mb II₁" was added. Renewed focusing showed that the amount in zone number 3 as seen in Fig. 1 had increased. This component could therefore be identified as Mb II₁. When the same procedure was repeated with 2 mg of the preparation "Mb II₂", this added protein focused in zone number 4. Thus this zone represents Mb II₂.

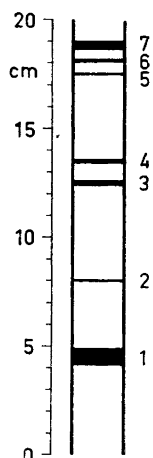


Fig. 1. A schematic diagram of the zones in Experiment 1a. Left: centimeter scale. Right: zone numbers. The zone identification revealed that; zone 1 was ferri-Mb I, zone 2 Hb, zone 3 ferri-Mb II₁, zone 4 ferri-Mb II₂, zone 5 ferri-Mb III₁, zone 6 ferri-Mb III₂, and zone 7 ferri-Mb III₃.

The myoglobin in zones 5, 6, and 7, having the lowest pI's of the components, contained together only 1 % of the total myoglobin. Theorell and Åkeson's¹ most acidic component, also present in a small amount, was called Mb III. They suspected this component to be inhomogeneous. There are thus strong reasons for regarding these three zones as subcomponents of Mb III.

Table 1. Isoelectric points (pI) of myoglobins at +4°C.

Experiment Component	1 a	1 b	2 a	2 b	3 a	3 b	3 c	Average
Ferri Mb I	7.78	7.77	7.76	7.76	7.75	—	—	7.76
Ferro Mb I	—	—	7.27	—	7.25	—	—	7.26
Ferri Mb II ₁	7.32	7.34	(7.33)	7.32	(7.29)	7.30	7.31	7.32
Ferri Mb II ₂	7.26	7.28	(7.27)	7.27	(7.24)	—	7.25	7.26
Ferro Mb II ₁	—	—	6.86	—	6.84	—	—	6.85
Ferro Mb II ₂	—	—	6.80	—	6.78	—	—	6.79
Ferri Mb III ₁	6.88	6.91	—	—	—	—	—	6.89
Ferri Mb III ₂	6.84	6.86	—	—	—	—	—	6.85
Ferri Mb III ₃	6.81	6.80	—	—	—	—	—	6.80

The pH gradient and the concentration distribution of myoglobin are illustrated in Fig. 2. The pI values are given in Table 1.

Experiment 1 b. In order to examine the possible dependence of pI values on the sucrose concentration this experiment was carried out as 1a, but with the opposite current direction. The isoelectric spectrum obtained was thus inverted compared to the one in Experiment 1a.

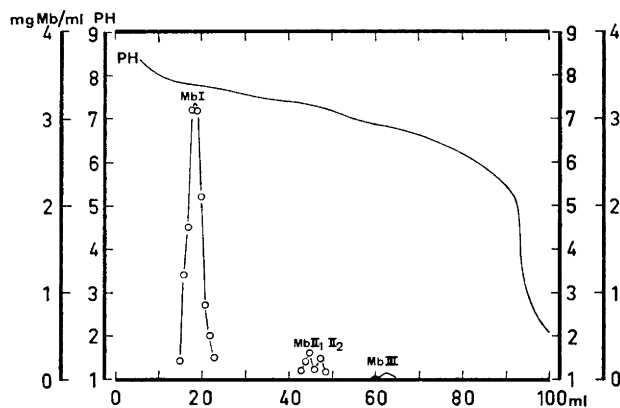


Fig. 2. pH gradient and concentration distribution of myoglobin in Experiment 1a.

Experiment 2 a. A dialyzed solution from the crude crystalline mixture, containing 40 mg of myoglobin, 60 % as ferri- and the rest as ferro-Mb as checked by spectrophotometry, was electrolyzed in the column. After isoelectric focusing, several brown and red zones were obtained (see Fig. 3). After fractionation, pH determinations and spectrophotometric analyses were carried out on the fractions. The ferri-Mb zones were identified by comparing these results with those of the preceding experiments. Zone 1 showed a typical absorption spectrum of ferri-Mb, with a pI value corresponding to ferri-Mb I. Spectrophotometric analysis revealed hemoglobin in zones 2 and 3. Zones 4 and 6 (brown) were not well separated from zone 5 (red), but it was possible to distinguish them as different zones from their different colours. The separation was of course still less clear after fractionation of the column, because of inevitable diffusion of protein and some tailing of the zones during the draining procedure.⁴ Zones 4 and 6 could nevertheless be identified as ferri-Mb II₁ and Mb II₂ by comparison with pI's obtained in the other experiments (see Table 1). The myoglobin contents in the zones, 7, 8, 9, and 10 were low, each amounting to less than 1 % of the total myoglobin. Zones 7 and 10 (brown) showed spectra typical of ferri-Mb and contained probably Mb III as judged by their pI values.

The zones, 5, 8, and 9 (red) showed spectra typical of oxygenated ferro-myoglobin. In order to identify these components, Experiment 2b was performed.

Experiment 2b. After analysis of the fractions of experiment 2a, the fractions containing zone 5 in Fig. 3, were treated with a slight molar excess of K₃Fe(CN₆) which converted the ferro-Mb in this zone into ferri-Mb. All the fractions from Experiment 2a were then put back into the column in a sequence of decreasing density. Thus a density gradient was obtained containing the blurred zones from Experiment 2a with zone 5 in the ferric form. During the isoelectric focusing it was easy to follow the migration of this oxidized zone, and its final focusing in the ferri-Mb I zone. Thus the identity of zone 5 as ferro-Mb I was proved. The small surplus of ferricyanide, not consumed for

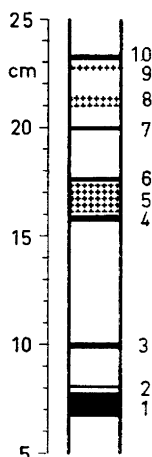


Fig. 3. A schematic diagram of the zones in Experiment 2a. +++ ferrous forms of Mb. ■ ferric forms of Mb. Left: centimeter scale. Right: zone numbers. The zone identification revealed that; zone 1 was ferri-Mb I, zones 2 and 3 Hb, zone 4 ferri-Mb II₁, zone 5 ferro-Mb I, zone 6 ferri-Mb II₂, zone 7 ferri-Mb III₁, zone 8 ferro-Mb II₁, zone 9 ferro-Mb II₂, and zone 10 ferri-Mb III₃. Above zone number 10 another three red zones were discernible, having pI values at about 6.5. They contained such small amounts of Mb that analysis of them was difficult. Most probably these zones represented ferrous forms of Mb III.

the oxidation of the ferro-Mb component, migrated to the anode during electrolysis. During this transport, it also passed the components more acid than that in zone 5. The myoglobins in the originally red zones 8 and 9 were thus also oxidized. Due to the small amount of myoglobin in these zones, estimated to about a few tenths of a mg, it was not easy to follow their migration, but apparently they focused together with the Mb II zones. The zones 8 and 9, therefore, probably represent ferro-Mb II₁ and ferro-Mb II₂.

Experiment 3a. After several months of storage, the amount of ferri-myoglobin had increased, and only about 10 % of ferro-myoglobin in the crude crystalline mixture were left. The ferri-Mb was converted into ferro-Mb by addition of Na₂S₂O₄. Some precautionary measures were necessary in order to avoid a reoxidation to the ferric form. The myoglobin solution was kept in a test-tube in which the oxygen was removed by applying vacuum for 10 min, after which the test-tube was filled with argon before adding the necessary quantity of dithionite. The required amount of dithionite was checked by spectrophotometry. After reduction, the spectrum of oxygen-free ferro-Mb was first obtained. By shaking the test-tube gently under access of air, the spectrum of MbO₂ appeared. The solution of MbO₂ was then put into the column while preparing the density gradient. The current was switched on as soon as the column was filled.

After isoelectric focusing, a picture containing red and brown zones was obtained as in Experiment 2a. Measurements of pI values and of the light absorption spectra of the zones proved their identity. The MbO₂ components showed a remarkable stability. After two weeks of storage a refrigerator, only a few per cent of ferri-Mb had been formed. In order to check the identity of the most acidic ferro-Mb zones, Experiments 3b and 3c were carried out.

Experiment 3b. The fraction from Experiment 3a with a pI of 6.84, which was supposed to contain the ferrous form of Mb II₁, was treated with a slight molar excess of K₃Fe(CN₆), in order to convert it into the ferric form. This myoglobin solution was then subjected to isoelectric focusing in a new experiment. After focusing, only one zone could be observed in the column. pH-measurements revealed this zone as ferri-Mb II₁. Thus it was found that the ferro-Mb zone, with a pI value of 6.84, was actually ferro-Mb II₁.

Experiment 3c. The fraction from Experiment 3a with a pI of 6.78, containing ferro-Mb was treated with K₃Fe(CN₆) and put into a column together with the fractions from Experiment 3b. After focusing, another zone could be observed in the column. Its pI corresponded to ferri-Mb II₂. The second ferro-Mb zone with a pI of 6.78 must thus be identified as ferro-Mb II₂.

Titration experiments. 5 ml of a solution of ferri-Mb I, isolated by isoelectric focusing, was dialyzed first overnight against a 0.2 M sodium chloride solution, after that against distilled and deionized water for 24 h with 3 changes. The water used in the dialysate was boiled in order to remove carbon dioxide, and special care was taken to keep carbon dioxide away. The myoglobin solution was then transferred to a titration vessel belonging to a Radiometer Titrigraph TTT 1 with cooling mantle, through which water of +4°C from a thermostated bath was circulated. The myoglobin solution was protected from contact with air by a slow stream of argon. The pH of the solution was adjusted to 5.0 with 0.1 M HCl. Titration was then carried out automatically,

with carbon dioxide-free 0.020 M NaOH. The titration curve of the same volume of water was then recorded. The myoglobin solution was then dried at 105°C to a constant weight. The total amount of myoglobin was 36.0 mg after correction for the added chloride and sodium. A molecular weight of 18 800 for myoglobin was adopted.¹ Third order polynomials were adapted to the two titration curves between pH 5.5 and 9.0 by using a simplified calculation method described by Svensson.⁷ By taking the difference between the two polynomials, the proton binding of ferri-Mb I could be represented by the equation:

$$m = a + 2.209 (\text{pH} - 7) - 0.1745 (\text{pH} - 7)^2 - 0.014 (\text{pH} - 7)^3 \quad (1)$$

9.0 > pH > 5.5 (4°C)

where m is the amount of alkali in equivalents per mole of protein, and a is a constant.

DISCUSSION

The present report deals with the complete isoelectric spectra of both ferri- and ferro-myoglobins of horse skeletal muscle. Preparations obtained without using ferricyanide contain both ferro- and ferri-myoglobins in proportions that have been shown (Shikama⁵) to depend on the method of preparation and on the storage conditions. This phenomenon was confirmed in the author's investigations. Although it was noted that the yield of ferro-myoglobins was higher in one preparation, the main intention of the present investigation was not to establish optimum conditions for getting good yields of either ferro- or ferri-myoglobins. Instead, the author's efforts have been concentrated on exact measurements of the isoelectric points of the various ferro- and ferri-Mb fractions, on their identification, and on possible chemical and physiological interpretations of the pI differences thus found.

The main components Mb I, Mb II and Mb III have fairly large differences in electrophoretic mobility and in isoelectric points and are easily resolvable by the isoelectric as well as by classical methods.

Theorell and Åkeson^{1,2} have shown the existence of the sub-components ferri-Mb II₁ and ferri-Mb II₂ and indicated a heterogeneity in ferri-Mb III. These findings have also been confirmed here. The pI differences between the sub-components are very small (a few hundredths of a pH unit). The sub-components of Mb III have not been separated earlier. All sub-components are, however, well resolvable by the isoelectric method. The complete separation of ferri-Mb II₁ and ferri-Mb II₂ was described in the previous communication.⁴ In the present report, ferri-Mb III is shown to contain three sub-components. Following the nomenclature principles adopted by Theorell and Åkeson, the notation Mb III₁, Mb III₂, and Mb III₃ is herewith suggested for these sub-components, the Roman as well as the Arabic numbers increasing with an increasing acidity of the myoglobins.

The identification of the various ferro- and ferri-myoglobins was facilitated by the convertibility between ferrous and ferric forms by chemical agents. The complete conversion of ferro- to ferri-Mb by the action of potassium ferricyanide,⁶ K₃Fe(CN)₆, involves no problems, and an isoelectric spectrum

containing only the ferric forms is therefore easily obtained. Such a spectrum is shown in Fig. 1.

The complete conversion of ferri- to ferro-Mb is much more difficult. Sodium dithionite, $\text{Na}_2\text{S}_2\text{O}_4$, is generally used for this purpose, but it is known that an excess of this reagent often causes a rather fast re-oxidation to the ferric form, probably due to the hydrogen peroxide formed from oxygen and an excess of dithionite. By working with the precautions described in Experiment 3a, it has been possible to obtain an almost pure isoelectric spectrum of ferro-Mb. However, in Experiments 2a and 3a, preparations containing both ferro- and ferri-Mb were run, and accordingly mixed isoelectric spectra of ferro- and ferri-Mb were obtained. Experiment 2a was run with a fresh preparation of myoglobin, whereas in Experiment 3a, an originally low ferro-Mb content was increased by reduction with dithionite. The mixed spectra of ferro- and ferri-myoglobins in Experiment 3a were useful for the comparison between the native and the regenerated ferrous forms. The chemical agents used did not appear to cause any other change in the myoglobin molecule than the desired shift between 3 and 2 for the valency of the iron atom. Artificially produced ferri- and ferro-myoglobins gave the same isoelectric points and the same degree of focusing as the native forms found in fresh preparations from muscle extracts. The isoelectric points of the various myoglobins found here are presented in Table 1. When the ferri-myoglobins are concerned it is possible to make some comparison with pI values determined by moving boundary experiments. Åkeson³ found for Mb I a pI = 7.08 and for both components of Mb II a pI = 6.58. The pI difference between these forms is thus 0.50 pH units. Exactly the same value is found when taking the difference between the average pI values for ferri-Mb I and ferri-Mb II₂ in Table 1. The discrepancy of the absolute pI values as determined by electrophoresis and the pI values ascertained by the isoelectric methods is discussed in paper number IV of this series.⁴

The isoelectric spectra of mixed ferro- and ferri-myoglobins contained components with very small pI differences. Ferro-Mb I was found to focus between ferri-Mb II₁ and ferri-Mb II₂. Ferro-Mb I and ferri-Mb II₂ could not be resolved but due to their different colours a partial separation could never the less be observed (*cf.* Fig. 3 and Table 1). This partial separation gives additional evidence of the resolving power claimed in the previous report,⁴ $\Delta\text{pI} = 0.02$ pH units.

It has thus been possible to identify and to measure the isoelectric points of the ferrous forms of Mb I and Mb II. As far as known to the author, isoelectric points for the ferro-Mb forms have not been determined by moving boundary electrophoresis.

When ferro-myoglobins are concerned one also has to distinguish between its oxygenated and its oxygen-free forms. Due to the very high affinity of ferro-myoglobin for oxygen, it is ordinarily oxygenated unless special precautions are taken to keep oxygen away; this was also confirmed by measuring absorption spectra. The data presented for ferro-Mb fractions refer to their MbO₂ forms. In a preliminary experiment more rigid precautions were taken in order to keep the oxygen away. Before filling the column, a vacuum was applied for 10 min on the test-tubes containing the fractions for the preparation of the

density gradient. The test-tubes and the column were then filled with argon for protection from contact with oxygen. The contents of the test-tubes were then transferred into the column in the order of decreasing density, using a syringe and a capillary tube. It was then possible to focus the oxygen-free ferro-Mb isoelectrically, but it proved to be less stable than other forms of myoglobin and some of it was denaturated and precipitated. This is in agreement with what has been observed on other hemoproteins.⁸ The binding of oxygen to ferro-Mb seems to have no influence on its isoelectric point. This may be compared with Theorell and Ehrenberg's⁹ result that the titration curves for ferro-Mb and CO-ferro-Mb are identical.

Experiments 1a and 1b were designed so as to focus the myoglobin components on different levels in the column. As is shown in Table 1, no significant differences in isoelectric points were found. Consequently, the influence of the sucrose concentration on the pI values can be neglected in the case of myoglobins. The reproducibility in the pI determinations is on the whole very good; it is of the same order of magnitude as that of the pH measurements.

The separability of proteins with different pI values by this isoelectric method depends on sharp focusing at their respective isoelectric points, where their net charges are zero. For isoenzymes and other closely related proteins, it is, however, more interesting to know something of the charge difference at a constant pH than to know the pI difference, because a charge difference may be interpreted in terms of difference in molecular structure.

In cases where the charge difference between two closely related protein species at a certain pH is found to be an integer number of electronic charges, this is a strong indication of a difference of an integer number of protolytic groups with pK values distant from the actual pH. On the other hand, if a charge difference of a fraction of an electronic unit is found, this is probably due to protolytic groups dissociating in the vicinity of the actual pH. Two interpretations are then possible. One of the protein species may have a protolytic group in the actual pH region, and the other not. The two proteins may also have identical protolytic groups but there is a difference in one or more of the pK values of corresponding protolytic groups dissociating in the actual pH region. It is well known that a pK value may be more or less influenced by electronic or electrostatic interaction with neighbouring groups in the molecule.

A warning must, however, be expressed against uncritical conclusions regarding differences in molecular structure from charge differences alone. For example, pK shifts in two or more protolytic groups may well add up to a unit charge difference. A fractional charge difference may also be localized in one or in more protolytic groups. We must keep in mind that a change of a charge in a protein molecule can result into another conformation, which in turn may influence more than one of the pK values. However, no such phenomenon were observed in the myoglobins. Risks for misinterpretations increase of course with the number of protolytic groups in the molecule. Safe conclusions regarding molecular structure differences can only be gained by comparing the results of other methods such as titrations, amino acid analyses, and convertibility investigations.

Table 2 Calculated net charge of Ferri Mb I and Ferri Mb II₂ at the pI of the other sub-components of myoglobin

Component	pI	Net charge of	
		Ferri Mb I	Ferri Mb II ₂
Ferri Mb I	7.76	0.00	-1.01
Ferri Mb II ₁	7.32	0.88	-0.13
Ferro Mb I	7.26	1.01	0.00
Ferri Mb II ₂	7.26	1.01	0.00
Ferri Mb III ₁	6.89	1.82	0.81
Ferro Mb II ₁	6.85	1.91	0.90
Ferri Mb III ₂	6.85	1.91	0.90
Ferri Mb III ₃	6.80	2.02	1.01
Ferro Mb II ₂	6.79	2.04	1.03

If two proteins have different isoelectric points, and if the titration curve and the molecular weight of one protein is known, then it is possible to calculate the net charge which the latter protein would carry at the pI value of the other protein. It is especially convenient to make such calculations if the titration data are available in the form of a polynomial like that in eqn. (1). The charge difference is obtained by inserting the two pI values into eqn. (1) and by taking the difference between the two m values thus obtained. Such data are given in Table 2.

No titration experiments on the sub-components have been carried out so far because sufficient amounts thereof have not been available. Nevertheless, the net charges of ferri-Mb II₂ at the various pI values have also been calculated and included in Table 2. This is feasible because the mobility curves of ferri-Mb I and ferri-Mb II₂ between pH 5 and 10 run exactly parallel (Åkeson³). There is thus reason to believe that ferri-Mb II₂ also obeys eqn. (1), yet with another value of the constant a .

To begin with, let us focus our attention on the net charges of ferri-Mb I and ferri-Mb II₂ at the pI's of the corresponding ferrous forms. One finds in both cases a charge difference of one electronic unit within the experimental error. One thus finds exactly the charge difference which is to be expected from the valence change of the iron atom. From this it may be concluded that this change in valency is not accompanied by any detectable shift in pK's in the vicinity of the actual pI's. According to current opinions on the pK of one of the ironbound imidazole groups, this pK is abnormally low, about 5 (Goldsack *et al.*¹⁰). A possible small shift in such a low pK value cannot influence the pI values of these myoglobins, because it is too distant from the pI value.

The transformation from ferrous to ferric form is accompanied not only by a valence change, but also by a replacement of an oxygen molecule by a water molecule. The latter is likely to undergo protolysis just as hydration water of free ferri ions is known to do. This protolysis has been studied by George and Hanania,¹¹ who found it to be strongly dependent on ionic strength and temperature. From their data this pK can be estimated to be about 9.2 at conditions prevailing in these electrolysis experiments. Thus, at a pH of 7.8, where ferri-Mb I is isoelectric, the water ligand of the iron atom can be regarded

as almost completely non-ionized. The water ligand, consequently, cannot either be suspected to cause any marked deviation from a unit charge difference between ferric and ferrous forms.

Let us then consider the charges of ferri-MbI and ferri-Mb II₂ at the pI's of the other ferric forms. Åkeson³ believed Mb I to be about one electronic charge more electropositive than Mb II₂. As is evident from Table 2, this hypothesis has now been verified with good precision. In addition, the table shows that there is also a unit charge difference between ferri-Mb II₂ and ferri-Mb III₃.

Under the assumption that these integer charge differences are attributable to single protolytic groups, it may be concluded that these groups are most probably carboxyl groups. This conclusion, again, is based on Åkeson's mobility measurements. As already pointed out, the mobility curves³ of Mb I and Mb II₂ run exactly parallel between pH 5 and 10. This presumably excludes differences in imidazole, phenolic, and terminal as well as epsilon amino groups. A difference in arginine content is excluded by his amino acid analyses.²

The separable sub-components of sperm whale myoglobin^{12,13} have been proposed to be due to different numbers of amide groups. In recent investigations on other hemoproteins, cytochrome *c*¹⁴ and lactoperoxidase,^{15,16} the heterogeneities found are attributable to successive deamidation of less stable amide groups. Work is now in progress to test a similar hypothesis regarding the relationships between the main components Mb I, Mb II, and Mb III.

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