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# Electrostatic Effects in Myoglobin. Application of the Modified Tanford–Kirkwood Theory to Myoglobins from Horse, California Grey Whale, Harbor Seal, and California Sea Lion†

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ABSTRACT: The modified Tanford-Kirkwood electrostatic theory (Shire et al., 1974a) was applied to ferrimyoglobins from the following animal species: sperm whale (*Physeter catodon*), horse, California grey whale (*Eschrichtius gibbosus*), harbor seal (*Phoca vitulina*), and California sea lion (*Zalophus californianus*). Computations were made of the overall hydrogen ion titration curves of the proteins, and of pH and ionic strength variations of ionization equilibria for individual groups in the protein, with particular reference to the hemic acid ionization of the iron bound water molecule. Coordinates and static solvent accessibility were estimated in terms of the sperm whale myoglobin structure. Where possible, theoretical results and experimental data are compared. Some comparative features of charge and ionization properties among the various myoglobins are presented.

We have previously shown (Shire et al., 1974a,b) that the Tanford-Kirkwood electrostatic theory (Tanford and Kirkwood, 1957) can be modified to take into account the specific accessibility to solvent of individual ionizing side chains in sperm whale ferrimyoglobin by introducing a set of solvent accessibility parameters (Lee and Richards, 1971). The modified theory was shown to account adequately for the hydrogen ion titration curve of the protein as well as the titrations of individual ionizing groups. Specifically, the theory predicts the pH and ionic strength variations of the ionization p$K$ value for the iron-bound water molecule in reasonable agreement with the values determined experimentally. It also yields the ionic strength variation of the hydrogen ion titration curve of the protein, in agreement with experiment.

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Table I: Amino Acid Differences and Estimated Coordinates for Charged Group Location for Horse, Grey Whale, Harbor Seal, and Sea Lion Myoglobins.[a]

| Sperm Whale | Horse | Grey Whale | Seal | Sea Lion | $X$ | $Y$ | $Z$ | $X'$ | $Y'$ | $Z'$ |
|---|---|---|---|---|---|---|---|---|---|---|
| Glu-4 | Asp | Asp | Asp | Asp | −0.6 | 15.5 | 24.3 | 0.9 | 14.2 | 25.9 |
| Gln-8 | Asn | | His | | 7.7 | 12.2 | 27.8 | 7.8 | 10.3 | 25.1 |
| His-12 | Asn | Asn | Asn | Asn | | | | | | |
| Asp-27 | Glu | | Glu | Glu | 26.3 | 15.1 | 8.7 | 26.3 | 14.7 | 10.3 |
| Lys-34 | Thr | | | | | | | | | |
| Arg-45 | Lys | Lys | Lys | Lys | | | | | | |
| Ala-53 | | | Asp | Asp | 35.5 | 17.7 | 0.7 | 35.7 | 16.0 | 0.7 |
| Glu-54 | | | Asp | | 35.5 | 22.7 | −3.8 | 34.6 | 21.9 | −1.9 |
| Lys-56 | | | Arg | | | | | | | |
| Ala-57 | | | Arg | Arg | 35.2 | 22.7 | 4.1 | 14.5 | 26.2 | 19.7 |
| Lys-62 | | | Arg | | | | | | | |
| Val-66 | | | | Lys | 22.1 | 27.4 | 15.1 | 27.6 | 27.6 | 15.1 |
| Glu-83 | | | | Asp | −0.9 | 28.4 | 17.4 | −0.8 | 29.6 | 15.7 |
| Glu-109 | Asp | Asp | | | 11.0 | 11.1 | 4.0 | 12.1 | 11.8 | 2.4 |
| His-113 | | Asn[b] | | | | | | | | |
| His-116 | | Asn[b] | | Gln | | | | | | |
| Arg-118 | Lys | | Lys | Lys | | | | | | |
| Asn-122 | | Asp[c] | Glu[c] | Asp[c] | | | | | | |
| Gln-128 | | | | His | 12.3 | 5.2 | 10.2 | 9.8 | 7.2 | 9.1 |
| Asn-132 | | | Lys | Lys | 6.0 | 10.4 | 7.6 | 4.2 | 9.0 | 5.1 |
| Lys-140 | Asn | | Asn | Asn | | | | | | |
| Lys-147 | | | | Arg | | | | | | |
| Tyr-151 | Phe | Phe | Phe | Phe | | | | | | |
| Gln-152 | | | His | | −0.8 | 23.9 | −0.8 | 0.2 | 22.6 | −3.6 |

[a] $X$, $Y$, and $Z$ are the coordinates (in angstroms) for sperm whale myoglobin given by Watson (1969) and $X'$, $Y'$, and $Z'$ are the estimated values when the indicated substitution is allowed for. No entry is given where the sperm whale coordinates were used, or where a neutral amino acid is substituted; sources: horse, Dautrevaux et al. (1969); grey whale, R. A. Bogardt, unpublished results; seal, Bradshaw and Gurd (1969); sea lion, Vigna et al. (1974). [b] Either residue 113 or 116 is asparagine. The change was assigned to residue 113. [c] As explained in the text this residue was omitted from the computations.

As a further test of the theory, it was applied to two closely related proteins, the major and minor components of sperm whale ferrimyoglobin, assuming identical model parameters including the same crystal structure and solvent accessibility parameters for both proteins, the only difference being in the charge of two amino acid sites (Shire et al., 1974a). Again it was shown that the computed and experimental values were in agreement. The question arises as to whether the theory would apply to other myoglobins where there are greater differences from sperm whale myoglobin in amino acid composition and sequence, such as myoglobins from other animal species. In this paper, we report on the application of the modified theory to the interpretation of hydrogen ion equilibria in ferrimyoglobins from horse, California grey whale, harbor seal, and California sea lion.

## Materials and Methods

The main components of the various myoglobins were obtained in lyophilized or crystalline form, and were redissolved in $CO_2$-free glass-distilled water prior to the titration work.

Horse ferrimyoglobin was purchased from Pierce Chemical Co., lot no. 05201-7, crystallized and salt free. It was further purified on a CM-Sephadex C-50 column with an eluting phosphate buffer of pH 6.9 and ionic strength 0.05 $M$. A preparation of California grey whale ferrimyoglobin was made following the procedure for sperm whale myoglobin (Hartzell et al., 1968). The major fraction was separated on a CM-Sephadex C-50 column eluted with phosphate buffer at pH 6.4 and ionic strength 0.1 $M$. A preparation from harbor seal muscle was made as for sperm whale myoglobin (Hartzell et al., 1968). It was purified on CM-Sephadex C-50, the eluting buffer being phosphate of pH 6.8 and ionic strength 0.1 $M$. A sample of purified major fraction of California sea lion ferrimyoglobin was kindly provided by Dr. R. Vigna. Details of its preparation and purification are given elsewhere (Vigna, 1973; Vigna et al., 1974).

The apparatus for potentiometric titration of the protein, and the apparatus for spectrophotometric determination of ionization constants, as well as details of procedures, have been described (Shire et al., 1974a). Titration curves were determined in 0.01 $M$ KCl, at 25°.

## Computations

Computations and treatment of data followed the methods of the previous papers, and involve a number of parameters that have been discussed (Shire et al., 1974a). The parameters include crystallographic coordinates (Watson, 1969) and intrinsic p$K$ values for the ionization of titratable sites. These intrinsic p$K$ values are defined as p$K$ values for the residues in a hypothetically discharged protein molecule, and the choice of these values is based on a number of model compound studies (Cohn and Edsall, 1943; Tanford, 1962; Nozaki and Tanford, 1967; Gurd et al., 1971). All computations also included static solvent accessibility factors which are based on a comparison of myoglobin with model peptides (Lee and Richards, 1971). The computations involve the use of an algorithm first introduced by Tanford and Roxby (1972) in their calculations on lysozyme. Other details are as given previously (Shire et al., 1974a).

## Results and Discussion

The results of the measurements and computations are

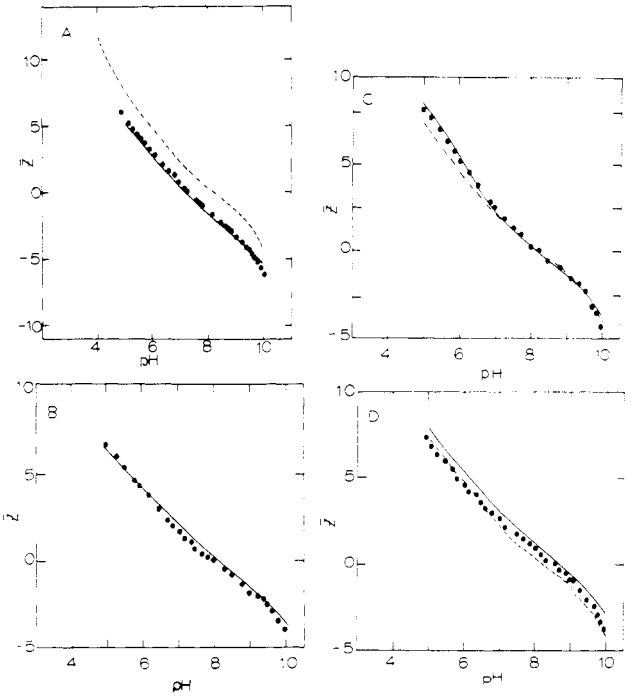


FIGURE 1: Various titration curves expressing $\bar{Z}$ as a function of pH: (A) comparison of horse and sperm whale ferrimyoglobins at 25°, $I$ = 0.01 $M$; theoretical curve for horse protein (—); theoretical curve for sperm whale protein (- - -); experimental points for horse protein at 0.1 m$M$ (●); (B) computed curve for California grey whale ferrimyoglobin at 25° in 0.01 $M$ KCl, with experimental results shown by filled circles; (C) computed curves for seal (—) and sperm whale (- - -) ferrimyoglobins at 25° in 0.01 $M$ KCl, with experimental results for the seal protein shown by filled circles; (D) computed curves for California sea lion (—) and sperm whale (- - -) ferrimyoglobins at 25° in 0.01 $M$ KCl, with experimental results for the sea lion protein shown by filled circles.

first presented for each of the myoglobins, and then some of the comparative features are discussed.

The experimental hydrogen ion titration curves were compared with the computed titration curves. Computed ionization p$K$ values for individual histidine residues and for the N-terminal amino acid are also reported for each of the myoglobins, and where possible these are compared with experimentally determined values. The computed p$K$ values are reported as p$K_{1/2}$ which is the value of p$K$ for a given group at the pH where 50% of the amino acid is dissociated. This definition is needed to establish a reference pH for each individual group, since p$K$ values vary with pH for all groups in the protein. In the special case of the iron-bound water molecule, the theoretical ionic strength variation of the ionization p$K_{1/2}$ is compared with experimental data, and the p$K_{1/2}$ value corresponding to ionic strength zero is referred to as p$K^0$ for each species of myoglobin. The choice of the ionic strength function $I^{1/2}/(1 + I^{1/2})$ has been discussed (Shire et al., 1974b; Hanania and Nakhleh, 1975).

All computations were performed using the same set of parameters including crystallographic coordinates, model and solvent accessibility factors, and intrinsic p$K$ values, as was used in the sperm whale ferrimyoglobin computations (Shire et al., 1974a). The only changes are those given in Table I involving minor alterations in site coordinates, for instance in the change from glutamic acid to aspartic acid, as well as the important changes in charge. The choice of site coordinates was made with reference to the Kendrew model for sperm whale ferrimyoglobin, by replacing the appropriate residue and estimating the best position which appears to be free of steric hindrance. In cases such as replacement of arginine by lysine, the coordinates of the charged site could be left unaltered since judicious bending of the side chain resulted in the same placement of the positive charge. Furthermore, the appropriate intrinsic p$K$ value was used, for instance that of lysine where arginine is replaced by lysine, and so on.

As discussed below, the results for the horse ferrimyoglobin can be dealt with quite closely in terms of the previous treatment for the sperm whale protein. The results for the grey whale, seal, and sea lion proteins can be made to fit by abandoning the assumption of strong ion binding near residue 45, an assumption that took the form of omitting this residue from the computation in the case of the sperm whale protein (Shire et al., 1974a,b). This change would offset a negative charge at position 122, relative to the asparagine residue in the sperm whale protein, since this residue is aspartic acid in the grey whale[1] and sea lion sequences (Vigna et al., 1974) and glutamic acid in the seal sequence (Bradshaw and Gurd, 1969). However, this argument does not respond to the fact that the horse protein also contains lysine at position 45, but like the sperm whale case is well fit with the omission of that residue. Rather than change the assumption of anion binding at residue 45 in an arbitrary way we have chosen to retain residue 122 as uncharged. Despite the finding of aspartic acid at this position in the sequence determinations, there is evidence that the amide form at this position is particularly labile[2] and deamidation may occur during removal of heme, an extreme condition that is avoided in the present work. The assumption of near identity of tertiary structure for the different myoglobin species is itself unproven. If it turns out that residue 122 is indeed asparagine only in the sperm whale and horse myoglobins it would still be possible to reconcile the observed results on the basis of currently unrecognized small structural differences.[3]

*Horse Ferrimyoglobin.* As Table I shows, three positive groups in the sperm whale protein are replaced here by neutral groups and there is no need to introduce new solvent accessibility factors. As described above, the masking of residue 45 assumed for sperm whale myoglobin (Shire et al., 1974a) was also assumed in this and the other cases dealt with here. The resulting agreement between the theoretical and experimental hydrogen ion titration curves is shown in Figure 1A. Included for comparative purposes is the dashed curve corresponding to the major component of sperm whale ferrimyoglobin, illustrating the charge difference mentioned above.

Table II gives the p$K_{1/2}$ values for the N-terminal amino group, as well as for the six titratable histidine residues computed at zero, 0.01 $M$, and 0.1 $M$ ionic strengths. Included in this table are the intrinsic p$K$ values, p$K_{int}$, used previously in the sperm whale myoglobin computation. As

[1] R. A. Bogardt, unpublished results.

[2] Deamidation in a similar sequence has been observed (Robinson, 1974). Romero Herrera and Lehmann (1974) find an acid residue at position 122 in 12 mammalian myoglobins, including aspartic acid in both the sperm whale and horse proteins. The original finding of asparagine at position 122 in sperm whale myoglobin (Edmundson, 1965) has been confirmed recently in this laboratory (F. E. Dwulet and R. A. Bogardt, unpublished results).

[3] In the case of the harbor seal protein, for example, there is evidence that histidine residue 36 is less fully masked than in the sperm whale protein (Nigen and Gurd, 1973).

Table II: Computed $pK_{1/2}$ Value for Valine and Comparison of Computed $pK_{1/2}$ Values with Experimental p$K$ Values for Histidine in Horse Ferrimyoglobin, Major Component.

| Residue | $pK_{1/2}$ at Ionic Strength ($M$) | | | | | |
|---|---|---|---|---|---|---|
| | $pK_{int}$ | 0 | 0.01 | 0.1 | $pK_{exp}$ | |
| Val-1 | 7.7 | 7.8 | 7.8 | 7.8 | *a* | *b* |
| His-48 | 6.8 | 6.7 | 6.8 | 6.9 | 7.0 | 6.9 |
| His-64 | 7.8 | 7.8 | 7.8 | 7.8 | 7.6 | 7.6 |
| His-81 | 6.3 | 6.1 | 6.2 | 6.3 | 6.9 | 6.8 |
| His-113 | 6.3 | 5.4 | 5.6 | 5.8 | 5.7 | 5.5 |
| His-116 | 6.3 | 5.9 | 6.0 | 6.1 | 6.6 | 6.5 |
| His-119 | 6.3 | 5.6 | 5.7 | 5.9 | 6.0 | 5.8 |

[a] Cohen et al. (1972). [b] L. Botelho, G. I. H. Hanania, and F. R. N. Gurd, unpublished results.

already mentioned these same values are used throughout all the computations and are presented in all the tables for the other myoglobins for comparison to the computed $pK_{1/2}$ values. Experimental p$K$ values for the histidine residues as determined with proton nuclear magnetic resonance (NMR) titrations (Cohen et al., 1972)[4] are listed for comparative purposes, but not meant as definite assignments. The computed results are seen to be in fair agreement with the experimental data of Botelho et al.[4] which are somewhat lower than the data of Cohen et al. (1972) that apply to slightly different experimental conditions.

The results for the ionization of the iron-bound water molecule are shown in Figure 2A. The filled circles represent the experimental points, and the dashed curve is the computed ionic strength variation. As can be seen, although the magnitude of the p$K$ value is somewhat different, the computations predict the correct ionic strength variation. Indeed, if the intrinsic p$K$ value is changed from 8.32, its original value in sperm whale myoglobin, to 8.30, the resulting full curve shown in Figure 2A is in good agreement with experiment. The dashed-dotted curve is for the computation based on the smeared charge Linderstrøm-Lang model (Linderstrøm-Lang, 1924).

*California Grey Whale.* Figure 1B presents the theoretical and experimental titration curves for this protein at 0.01 $M$ ionic strength and 25°. The filled circles are experimental values and the curve is computed. The corresponding titration curve for sperm whale ferrimyoglobin is not included because of its similarity to the curve of grey whale ferrimyoglobin. The computations involve an additional uncertainty at amino acid positions 113 and 116 as indicated in Table I. It is known that either position 113 or 116, both of which are occupied by hisitidine in sperm whale myoglobin, is a side-chain amide in grey whale myoglobin.[1] In the present computations the amide replacement was assigned to position 113. Similar results are obtained if the change was assigned to position 116. No attempt was made to modify the original sperm whale parameters in order to improve the fit. The differences between the computed and experimental curves in the pH range of 7 to 8 may well be due to the fact that some of the groups in grey whale myoglobin have slightly different spatial relationships and hence different solvent accessibilities from sperm whale ferrimyoglobin.

[4] L. H. Botelho, G. I. H. Hanania, and F. R. N. Gurd, manuscript in preparation.

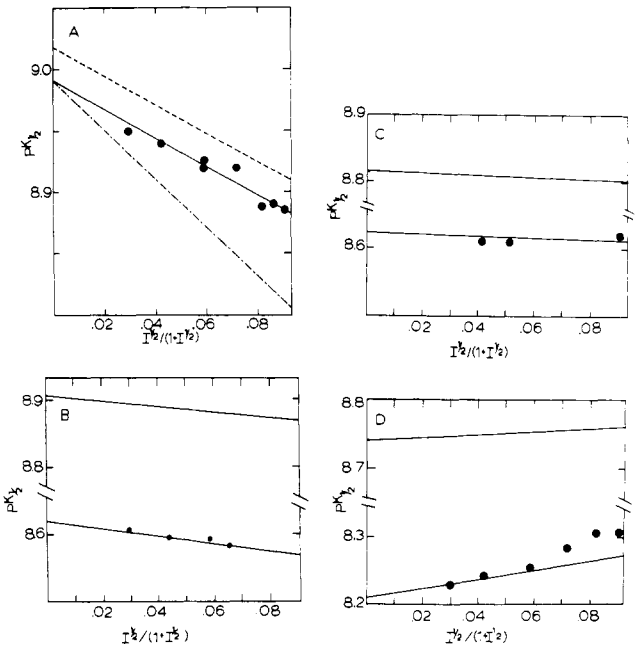


FIGURE 2: Plots of the p$K$ value for half-titration, $pK_{1/2}$, against the ionic strength function $I^{1/2}/(1 + I^{1/2})$, for the ionization of the iron-bound water molecule in various ferrimyoglobins at 25°. In each case experimental values are given by filled circles. (A) Plot for the horse protein computed with intrinsic p$K$ = 8.32 (- - -), with intrinsic p$K$ = 8.30 (—), and according to the Linderstrøm-Lang treatment (- —). The intrinsic p$K$ value of 8.32 corresponds to that for sperm whale ferrimyoglobin (Shire et al., 1974b). (B) Plot for California grey whale ferrimyoglobin. The upper curve is computed with intrinsic p$K$ = 8.32 (see above), and the lower curve with intrinsic p$K$ = 8.12. (C) Plot for harbor seal ferrimyoglobin. The upper curve is computed with intrinsic p$K$ = 8.32, the lower with intrinsic p$K$ = 8.22. (D) Plot for California sea lion ferrimyoglobin. The upper curve is computed with intrinsic p$K$ = 8.32, the lower with intrinsic p$K$ = 8.00.

Table III: Comparison of Computed $pK_{1/2}$ Values with Experimental p$K$ Values for Valine and Histidine in Grey Whale Ferrimyoglobin, Major Component.

| Residue | $pK_{1/2}$ at Ionic Strength ($M$) | | | | |
|---|---|---|---|---|---|
| | $pK_{int}$ | 0 | 0.01 | 0.1 | $pK_{exp}$ |
| Val-1 | 7.7 | 7.74 | 7.79 | 7.80 | 7.74[a] |
| His-48 | 6.8 | 6.6 | 6.8 | 6.8 | 6.8[b] |
| His-64 | 7.8 | 7.7 | 7.8 | 7.8 | 8.0[b] |
| His-81 | 6.3 | 6.1 | 6.2 | 6.3 | 6.6[b] |
| His-116 | 6.3 | 5.9 | 6.0 | 6.2 | 6.2[b] |
| His-119 | 6.3 | 5.5 | 5.7 | 5.9 | 5.7[b] |

[a] Garner et al. (1973). [b] L. Botelho, G. I. H. Hanania, and F. R. N. Gurd, unpublished results.

Figure 2B gives the ionic strength variation of the ionization p$K$ value for the iron-bound water molecule. The experimental points are shown as solid circles. The upper curve is the computed ionic strength variation. The computed magnitudes of the p$K$ values are approximately 0.3 p$K$ unit higher than the experimental values, but this can be easily corrected as was done in the case of horse myoglobin by introducing a change in the intrinsic p$K$ value for the water molecule, in this case from 8.32 to 8.12. As can be seen the slope is in very good agreement with the experimental data.

Table III presents the computed $pK_{1/2}$ values for the N-

Table IV: Computed $pK_{1/2}$ Values for Histidine and Comparison of Computed $pK_{1/2}$ Value with the Experimental p$K$ Value for Glycine in Harbor Seal Ferrimyoglobin, Major Component.

| Residue | $pK_{1/2}$ at Ionic Strength ($M$) | | | |
|---|---|---|---|---|
| | $pK_{int}$ | 0.0 | 0.01 | $pK_{exp}$ |
| Gly-1 | 7.7 | 7.67 | 7.76 | 7.76[a] |
| His-8 | 6.3 | 5.8 | 6.0 | |
| His-48 | 6.8 | 6.6 | 6.7 | |
| His-64 | 7.8 | 7.6 | 7.7 | |
| His-81 | 6.3 | 6.0 | 6.2 | |
| His-113 | 6.3 | 5.0 | 5.4 | |
| His-116 | 6.3 | 5.6 | 5.9 | |
| His-119 | 6.3 | 5.3 | 5.6 | |
| His-152 | 6.3 | 5.9 | 6.1 | |

[a] Garner et al. (1973).

terminal valine and the histidine residues. Experimental values are also presented for the N-terminal residue determined from the kinetics of cyanate addition (Garner et al., 1973). The proton NMR p$K$ values as determined by Botelho, Hanania, and Gurd[4] are matched up with the computed values to illustrate the compatibility of the spread of these values with experiment, but these listings are not meant to be firm assignments of resonance peaks to p$K$ values. As can be seen the overall agreement is very satisfactory.

*Harbor Seal.* Figure 1C presents the theoretical and experimental titration curves of harbor seal at 0.01 $M$ ionic strength and 25°. The solid circles are experimental values and the solid curve is computed. Included in the figure for comparative purposes as a dashed curve is the sperm whale myoglobin titration curve. The agreement of theory with the experimental values is very good throughout the titration range of pH 5–10. Table IV presents the $pK_{1/2}$ values at zero and 0.01 $M$ ionic strength for the N-terminal residue as well as for the histidine residues. At the present time there are no experimental histidine p$K$ values for comparison, but it should be noted that the computed spread of histidine values is very large particularly at low ionic strengths. It is interesting that the overall charge of the seal myoglobin both experimentally and theoretically is higher than for sperm whale myoglobin in the pH range of 5–7, attaining a maximum difference of about 1 unit charge. This net increase in charge is distributed at loci which are close to some of the histidine residues, thereby lowering the p$K$ values of these groups.

The p$K$ of the N-terminal glycine residue has been determined by Garner et al. (1973), with reasonable agreement with the computations. The computed value at zero ionic strength is lower than for both grey whale and sperm whale and this is observed experimentally, reflecting in part the choice of the same intrinsic p$K$ value for the N-terminal glycine in harbor seal myoglobin as for the N-terminal valine in the grey whale and sperm whale myoglobins. A different intrinsic p$K$ will of course lead to a different value, but it is still significant that in the harbor seal case the computed p$K$ value decreased at zero ionic strength whereas in the grey whale and sperm whale cases it increased in value.

In Figure 2C the experimental ionic strength variations of the p$K$ values of Ysern (1973) for the iron-bound water molecule are shown as solid circles. The uppermost line is that computed with the intrinsic p$K$ value of 8.32 used for sperm whale myoglobin. The bottom line is computed with a lower intrinsic p$K$ value of 8.22 and shows how well the computed ionic strength variation agrees with that for the limited experimental data. It is interesting that the computed slope is almost negligible, in very good agreement with that obtained experimentally.

Table V: Computed $pK_{1/2}$ Values for Glycine and Histidine in Sea Lion Ferrimyoglobin, Major Component.

| Residue | $pK_{int}$ | $pK_{1/2}$ at Ionic Strength ($M$) | |
|---|---|---|---|
| | | 0.00 | 0.01 |
| Gly-1 | 7.7 | 7.63 | 7.75 |
| His-48 | 6.8 | 6.5 | 6.7 |
| His-64 | 7.8 | 7.4 | 7.6 |
| His-81 | 6.3 | 5.9 | 6.1 |
| His-113 | 6.3 | 5.1 | 5.4 |
| His-119 | 6.3 | 5.3 | 5.6 |
| His-128 | 6.3 | 5.7 | 5.9 |

*California Sea Lion.* Figure 1D gives the experimental values as solid circles and the theoretical titration as a solid curve for California sea lion myoglobin at 0.01 $M$ ionic strength and 25°. Again the corresponding curve for sperm whale myoglobin is indicated with the dashed curve. As can be seen the agreement is not as good as for the other myoglobins reported here, the computed results being in fair agreement in the pH range of 6 to 9 but deviating below pH 6 and above pH 9. The reasons for these deviations may involve slight structural differences between the sperm whale and sea lion myoglobins. This ferrimyoglobin has the highest isoionic point of any measured to date, about 8.6 at an ionic strength of 0.01 $M$ for 0.1 m$M$ protein at 25°.

The $pK_{1/2}$ values for the corresponding N-terminal and histidine residues are given in Table V. There are no available experimental data at this time for comparative purposes but the computed results are presented for future reference, particularly for comparisons with proton NMR titration work which is currently in progress.[4] It is noteworthy that on the whole the computed $pK_{1/2}$ values for the histidine residues in sea lion myoglobin are slightly lower than those computed for sperm whale myoglobin, especially that of histidine residue 64.

The ionic strength variation of the iron-bound water molecule dissociation p$K$ value is shown in Figure 2D. The experimental values are shown as solid circles. There are two interesting features. First, the p$K$ values, between 8.2 and 8.3, are lower than for any myoglobin whose hemic acid p$K$ value has been determined in this laboratory. As already mentioned this protein has a high isoionic point (pH ~8.7 of a deionized solution, 0.1 m$M$ in protein) and therefore a deionized sample of sea lion myoglobin is mainly in the alkaline form and is reddish in color. The second feature is the ionic strength variation itself. In all the other ferrimyoglobins studied the p$K$ value for the water molecule decreases with increased ionic strength, but the reverse is found in sea lion myoglobin. The upper curve in Figure 2D is the computed variation and is based on an intrinsic p$K$ value of 8.32 as used in the sperm whale myoglobin computations. The figure shows that a slight increase is expected as a consequence of the electrostatics. A lower intrinsic p$K$ value of 8.00 yields the lower line which is in good agreement with the limiting slope. The data indicate an upward curvature at an ionic strength of about 0.005 $M$ but more

Table VI: Comparative Ionization and Charge Properties of Ferrimyoglobins.

| | Isoionic pH | Hemic Acid Ionization | | | |
|---|---|---|---|---|---|
| | | $pK^0$ | $pK_{1/2}$ | $q^a$ | $\bar{Z}$ |
| Sperm whale IV | 8.3 | 8.9 | 8.87 (0.01) | −0.1 | −1.0 |
| Sperm whale II | 7.3 | 9.0 | 8.92 (0.02) | −0.9 | −3.0 |
| Horse | 7.3 | 9.0 | 8.89 (0.02) | −1.0 | −3.0 |
| Grey whale | 8.0 | 8.6 | 8.62 (0.02) | 0 | −1.0 |
| Harbor seal | 8.3 | 8.6 | 8.62 (0.02) | +0.4 | −0.6 |
| Sea lion | 8.7 | 8.2 | 8.31 (0.01) | +1.2 | +0.4 |

[a] $q$ = effective (Debye—Hückel) charge in the vicinity of the iron atom as obtained from the ionic strength variation of p$K$ values; see text.

data would be needed in this region to verify this observation.

## Comparative Features

For ready reference to the differences in the hydrogen ion equilibria of the six proteins studied, a compilation of pertinent data is presented in Table VI. The p$I$ values for the isoionic pH value for deionized $10^{-4}$ $M$ ferrimyoglobin at 25° were carefully measured and were reproducible to within 0.1 pH unit in all cases. As can be seen, the isoionic points vary from a low value of 7.3 for horse ferrimyoglobin and for minor component II of sperm whale ferrimyoglobin up to a value of 8.7 for California sea lion ferrimyoglobin. The second column of Table VI lists under the heading $pK^0$ the theoretical ionization p$K$ values for the iron bound water dissociation obtained by computation at zero ionic strength. Large variation is seen, ranging from 8.2 for California sea lion ferrimyoglobin up to 9.0 for horse and for the minor component II of sperm whale ferrimyoglobins. Also listed are experimental $pK_{1/2}$ values for the ionization determined at ionic strength $I$ = 0.01 $M$ (with the mean deviation given in parentheses). The value for the major component of sperm whale ferrimyoglobin is taken from Nakhleh (1971).

The observed ionic strength variation of this hemic acid ionization p$K$ can be used to obtain values for the effective charge in the vicinity of the iron atom. Thus, if $q$ is the effective charge on the iron atom in ferrimyoglobin, $Fe(H_2O)$, and $(q - 1)$ is that of its conjugate base Fe-OH, and if a Debye-Hückel ionic strength function $f(I) = I^{1/2}/(1 + I^{1/2})$ is assumed to apply, then it follows that for a dilute aqueous solution of myoglobin at 25°

$$pK = pK^0 + (q - 0.51)I^{1/2}/(1 + I^{1/2})$$

(Hanania and Irvine, 1970), where p$K$ is the experimentally determined value and $pK^0$ that obtained by extrapolation to zero ionic strength. Hence, $q$ = 0.51 + limiting Debye-Hückel slope; the resulting $q$ values are listed in Table VI. For comparison, Table VI also gives the corresponding $\bar{Z}$ values as obtained from the experimental hydrogen ion titration curves. These are the net protein charges at a pH equal to the p$K$ value at ionic strength $I$ = 0.01 $M$ and 25°. As can be seen, the effective charge $q$ is about 1 or 2 units less negative, or more positive, with respect to the net protein charge $\bar{Z}$. Closer agreement between $q$ and $\bar{Z}$ can be achieved by using a steeper ionic strength function, and in fact this was the criterion used by Beetlestone and Irvine (1968) in choosing the ionic strength function $f(I) = I^{1/2}/(1 + 12I^{1/2})$ for hemoglobin, and the function $I^{1/2}/(1 + 8I^{1/2})$ for myoglobin. However, there is no theoretical reason for adopting such functions. Furthermore, whereas this function may improve the apparent agreement in the case of sperm whale and horse myoglobins, it widens the discrepancy for harbor seal and sea lion myoglobins. It would seem more reasonable to assume that the different effective charges simply reflect the electrostatic microenvironment of the site and do not necessarily have to be equivalent to the overall net protein charge. Indeed, even in the case of sperm whale ferrimyoglobin where the apparent agreement between $\bar{Z}$ and $q$ for the iron site is improved by the use of steep functions, there is a widening of the difference between $\bar{Z}$ and $q$ for some sites other than the iron atom, for example the terminal valine, Lys-42, and His-64.

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# Amino Acid Sequence of Dogfish Trypsin†

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ABSTRACT: The amino acid sequence of pancreatic trypsin from the spiny Pacific dogfish (*Squalus acanthias*) has been determined and compared with the sequences of bovine and porcine trypsin. Dogfish trypsin contains one less amino acid residue (222) than the other two enzymes. Two-thirds of the residues in corresponding positions in dogfish and bovine trypsin are identical and the sequences of all three enzymes are homologous. Of the 223 amino acid residues of bovine trypsin, 77 are replaced without significant changes in function. Seven replacements, all conservative, occur in the interior of the protein; the remainder are on the surface. All residues known to be components of the active site of bovine trypsin are present in corresponding positions in dogfish trypsin. Comparison of the three enzymes suggests calcium binding sites in dogfish trypsin. A corrected sequence of bovine trypsin identifies residue 67 as Asn and residues 84–87 as Ser-Asn-Thr-Leu.

The recognition that the homologous serine proteases, bovine chymotrypsin (Matthews et al., 1967), elastase (Shotton and Watson, 1970), and trypsin (Stroud et al., 1971), have similar three-dimensional conformations has provided an experimental basis for relating the details of their structure both to the specificity of their function and to their evolution (de Haen et al., 1975). Although no crystallographic data are yet available for pancreatic serine proteases of other species, knowledge of their amino acid sequences in conjunction with the model of the analogous bovine enzymes can yield useful information about phylogenetic evolution and about the tolerance of function to structural change.

We have previously reported a partial amino acid sequence of trypsin from the Spiny Pacific Dogfish *Squalus acanthias* (Bradshaw et al., 1970) and the complete amino acid sequence of porcine trypsin (Hermodson et al., 1973). Both enzymes are homologous with the bovine enzyme but a detailed comparison had to await the completion of the amino acid sequence of dogfish trypsin which is presented herein. The functional analogy of bovine and dogfish trypsins was previously demonstrated by Tye (1971).

## Materials and Methods

Sephadex of various grades was obtained from Pharmacia Fine Chemicals. 4-Vinylpyridine (Baker) was distilled under reduced pressure and stored at −20°. Pepsin and α-chymotrypsin were products of Worthington Biochemical Corp. Before use, chymotrypsin was treated with α-*N*-tosyllysine chloromethyl ketone to inactivate trypsin.

*Dogfish trypsin* was isolated from frozen pancreas glands which had been freshly excised from dogfish caught in Puget Sound. Within 3 days the frozen glands (400 g containing approximately 250 mg of trypsinogen) were minced and blended in a Waring Blendor for 40 sec with 2 vol of cold water. The slurry was agitated with paddles for 1 hr at 4° in an electric ice cream freezer to complete the extraction and then centrifuged at 12,500 *g* for 1 hr. After filtration through a pad of glass wool, the supernatant was pumped at a rate of 1800 ml/hr onto a 9 × 75 cm column of DEAE-cellulose at 4°, previously equilibrated with 0.01 *M* NaCl–0.005 *M* Tris-HCl (pH 8.0). The column was washed overnight with the same buffer and then with 3 l. of 0.25 *M* NaCl–0.005 *M* Tris-HCl (pH 8.0). At this point the salt concentration was raised to 0.50 *M* NaCl (in 0.005 *M* Tris, pH 7.0) and 400 fractions of 25 ml each were collected. Fractions containing trypsinogen were pooled (yielding 160 mg of trypsinogen) and acidified to pH 2.7 with concentrated HCl and a precipitate was discarded after centrifugation at 15,800*g*. Solid ammonium sulfate was added (313.5 g/l.) to 50% saturation and the trypsinogen collected as a precipitate by centrifuging at 15,800*g* for 30 min. The precipitate (110 mg of trypsinogen) was dissolved in 100 ml of 1 m*M* HCl, dialyzed overnight against 1 m*M* HCl, and lyophilized. The dry powder was dissolved in 50 ml of 0.01 *M* Tris-HCl–0.05 *M* $CaCl_2$–0.5 *M* KCl (pH 8), and the solution readjusted to pH 8. The trypsinogen was converted to trypsin by treatment with 3 mg of dogfish trypsin for 1–2 hr at 36°, pH 8.0. A small precipitate was removed by centrifugation at 4° and the supernatant dialyzed for 24 hr against 1 m*M* HCl to remove salts.

The lyophilized product (114 mg of trypsin) was dissolved in 60 ml of 0.1 *M* Tris–0.05 *M* $CaCl_2$–0.5 *M* KCl (pH 8), the pH readjusted to pH 8, and a small precipitate discarded after centrifugation. A 1.5 × 84 cm column of chicken ovomucoid immobilized on Sepharose (Robinson et al., 1971) was equilibrated with 0.02 *M* Mes buffer[1] (pH

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[1] Abbreviations used are: PE-, *S*-pyridylethyl-; Mes, 2-(*N*-morpholino)ethanesulfonic acid; BzArgOEt, benzyl-L-arginine methyl ester; Pth derivative, phenylthiohydantoin derivative.