Structure–Volume Relationship. Dilatometric Study of the Acid–Base Reaction Involving Human Oxy- and Methemoglobins in Water and Denaturing Media†

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ABSTRACT: The volume changes produced by acid–base reactions with human oxy- and methemoglobins showed a strong dependence on the state of the heme, the quaternary structure, and the medium. The volume changes were determined dilatometrically at 30.0 ± 0.001°. The protonation of oxyhemoglobin produced time-dependent large negative volume changes at pH <4.5; steady-state volumes were achieved within 18 hr after mixing. A minimum of −750 ml/10^5 g of protein occurred at the point where 70 mol of H^+ were bound/10^5 g of protein. The corresponding isotherm for methemoglobin exhibited no time dependence and was qualitatively similar to the steady-state values of oxyhemoglobin. These negative volume effects can be related to the disruption of the quaternary structure and the consequent extensive structural changes in the acid environment. Similar experiments performed in 8 M urea and 6 M guanidine hydrochloride produced conventional positive isotherms; *i.e.*, the small volume increase at low levels of hydrogen ions is associated with histidyl protonation and the large volume rise at higher proton binding levels is due to the titration of carboxylate residues. These proteins proved to be more stable in alkali as indicated by a nearly linear volume increase with increasing OH^- concentration until the pH exceeded 10; at higher pH values, time-dependent volume effects which indicate structural changes were observed. The decrease of the steady-state volume at high OH^- concentration can be related to the dissociation of the hemoglobin from the tetrameric to dimeric state. Methemoglobin proved to be less stable in alkali than oxyhemoglobin, as demonstrated by the larger time-dependent effects and negative volume changes. In 8 M urea both of these proteins produced essentially identical isotherms. These isotherms exhibited a linear volume increase as a function of hydroxyl ion bound with a slope of 15 ml/mol of OH^- bound; this indicates that these hemoglobins behaved in a "normal" mode in this dissociating and denaturing medium.

The magnitude and character of the volume changes produced by the reaction of acids and bases with proteins are determined by the composition and structure of the protein and the type of medium employed (Rasper and Kauzmann, 1962; Katz and Miller, 1971a). This study proposes to investigate the influence of the quaternary structure, the state of the heme, and the denaturant on the volume isotherms produced by the protonation or neutralization of hemoglobins. Human oxy- and methemoglobin which exist as tetramers were selected for study in order to extend and supplement the results of a similar investigation of the single-chain hemoprotein, myoglobin (Katz *et al.*, 1973a).

The data obtained by this dilatometric study reveal that the quaternary structure and the state of the heme have a profound influence on the degree, character, and time dependency of the resultant volume effects. The larger magnitude of the volume decrease resulting from the protonation of hemoglobins compared to myoglobin is attributed to the structural change accompanying the disruption of the quaternary structure. The state of the incorporated heme has a decided influence on the protein stability; *e.g.*, oxyhemoglobin is more labile in acid than methemoglobin but in alkali the converse is the case.

Experimental Section

Methodology. The volume changes were determined at 30.0 ± 0.001° with Teflon-sheathed Linderstrøm-Lang-type dilatometers which could be read to 0.01 μl (Linderstrøm-Lang and Lanz, 1938; Katz and Ferris, 1966; Katz, 1972). The experimental procedure and calculations used in these dilatometric titration have been described (Rasper and Kauzmann, 1962; Katz and Miller, 1971a; Katz *et al.*, 1973a). Steady-state reading which are defined as volumes which do not vary for a 1–3-hr period generally were reached 2–3 min after mixing. When time-dependent volume effects occurred the 5 min and 18 hr values were reported; the latter readings are steady state. The protocol for the dilatometric studies involved the use of 5.00 ml of 5% protein in one arm of the dilatometer while the other arm contained 5.00 ml of 0–0.08 M acid or alkali in the same medium used to dissolve the protein. To denature the protein, the protein was exposed at 30° for 24 hr in either 8 M or 6 M guanidine hydrochloride. Spectral analysis was performed with a Cary Model 17 spectrophotometer or Beckman DU2. pH was measured with a Radiometer Model 26 pH meter using semimicro combination electrode GK2321C. Conductivity was determined with a Radiometer CDM-3 apparatus. Acrylamide gel electrophoresis was performed according to our standard procedure; Peacock's *et al.* (1965) buffer was the electrolyte used.

The concentration of protein in water was determined by drying at 100° for 24 hr *in vacuo*. Operational absorption coefficients were determined from systems which simulated dilatometric experiments in all respects; the solutions were diluted with 0.05 M acetate buffer (pH 4.8) for spectral analysis (Katz *et al.*, 1973a). The following absorptivity values are

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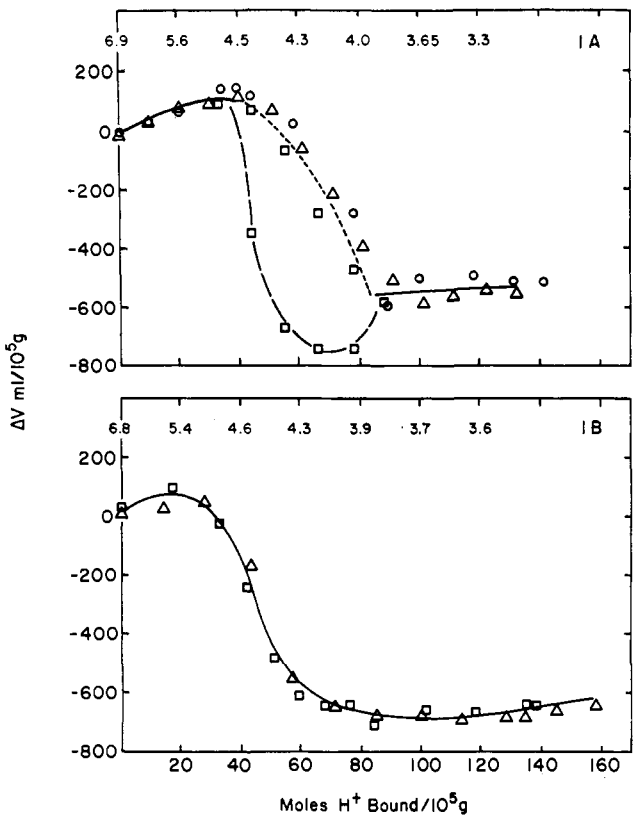

FIGURE 1: Volume changes produced by the reaction of HCl with hemoglobins of human origin. The experiments were performed at 30.0°; the data are reported in terms of 10^5 g of protein. The pH values are stated on the top portion of each graph. The top panel (1A) represents the oxyhemoglobin system: (- - - - -) the volume changes determined 5 min after mixing; (— — —) steady-state values which were determined 5–18 hr after mixing; (—) the steady-state values which occurred immediately after mixing. The bottom panel (1B) represents the methemoglobin system; steady state was achieved immediately after mixing. The different symbols represent specific experiments.

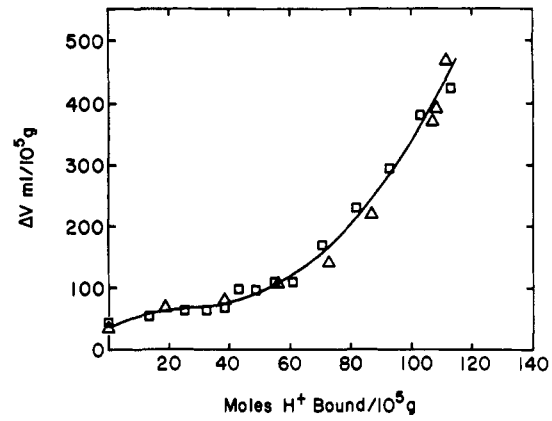

FIGURE 2: Volume changes produced by the reaction of HCl with human methemoglobin; the solvent was 8 M urea. For details refer to Figure 1.

for hypothetical 1% solutions: methemoglobin initially in 8 M urea is 18.7 ± 0.6 at 278 nm; methemoglobin initially in 6 M guanidine hydrochloride is 17.7 ± 0.8 at 275 nm. The use of water as diluent instead of acetate buffer had a profound influence on these values, *e.g.*, oxyhemoglobin diluted with water yielded a value of 22.3 ± 0.2 at 276 nm; a value of 21.5 was reported by Antonini and Brunori (1971).

Materials. Human hemoglobin was prepared by conventional procedure (Benesch *et al.*, 1972) using freshly drawn human blood. Methemoglobin was prepared by exposing hemoglobin to a fivefold excess of $K_3Fe(CN)_6$ for 10 min; this was exhaustively dialyzed until the conductivity of the diffusate and retentate was nearly the same. The hemoglobin A_1 content of these samples ranged from 96 to 98% as determined by cellulose acetate electrophoresis.

The standard acid and base solutions were Harleco products; restandardization established that maximum deviation from the stated normalities was ≤0.3%. Urea, *n*-heptane (Mallinckrodt), and guanidine hydrochloride (Sigma) were purified by our standard procedures (Katz and Miller, 1971b).

Results

The magnitude, character, and the kinetics of the volume effects produced by acid–base reactions with hemoglobins are determined not only by compositional factors and medium, but also to a large extent by the protein's quaternary organization, and the oxidation and ligand state of the heme.

Acid Titration. The isotherm produced by the protonation of oxyhemoglobin was characterized by regions where ΔV was reached immediately after mixing and by a zone where the volume effects were time dependent (Figure 1A). Near the origin there was a small volume rise reaching a maximum of 100 ml/10^5 g of protein at the point where 35 mol of H^+ were bound/10^5 g of protein; steady state was reached immediately after mixing. At higher binding levels, *i.e.*, where 35–85 mol of H^+ were bound/10^5 g of protein, time-dependent large volume decreases were observed. Apparent steady-state values were reached 5 hr after mixing and were constant for the ensuing 18-hr contact period. A minimum of −750 ml/10^5 g of protein was found when about 70 mol of H^+ were bound/10^5 g of protein. When the binding exceeded 85 mol of H^+/10^5 g of protein, the isotherm reached steady state immediately after mixing and exhibited little dependence on the amount of H^+ bound. The values for ΔV in these regions were about −550 ml/10^5 g of protein.

The corresponding isotherm for methemoglobin differed primarily by the absence of kinetic effects; the steady-state isotherms for these proteins were qualitatively similar (see Figure 1B). Methemoglobin exhibited a small volume increase at low binding levels, reaching a maximum of 80 ml/10^5 g of protein when 20 mol of H^+ were bound/10^5 g of protein; there was a subsequent sharp volume decrease, reaching a minimum of −700 ml/10^5 g of protein at the point where 100 mol of H^+ were bound/10^5 g of protein. At higher levels of proton binding the values for the isotherm increased slightly with binding, the slope being 1.5 ml/mol of H^+ bound.[1]

The use of denaturants as solvents altered the character of the isotherms drastically; *i.e.*, the large volume decreases observed in water were virtually eliminated (compare Figures 1–3). Data for the protonation of oxyhemoglobin in 8 M urea and 6 M guanidine hydrochloride are not presented since the oxy form is converted to methemoglobin in these media. The isotherm for methemoglobin in 8 M urea exhibited a progressive rise of slope with increasing H^+ binding, attaining a value of 425 ml/10^5 g of protein when 110 mol of H^+ were

[1] A value of 10 ml/mol is proposed for the titration of unhindered carboxylate compounds in proteins; this is based on data determined from simple amino acids and proteins (Rasper and Kauzmann, 1962; Katz and Miller, 1971b). In 8 M urea the ΔV for protonation of carboxylate groups is about 80% that in water and in 6 M guanidine hydrochloride it is about 50% that in water (Katz and Miller, 1971b, 1972).

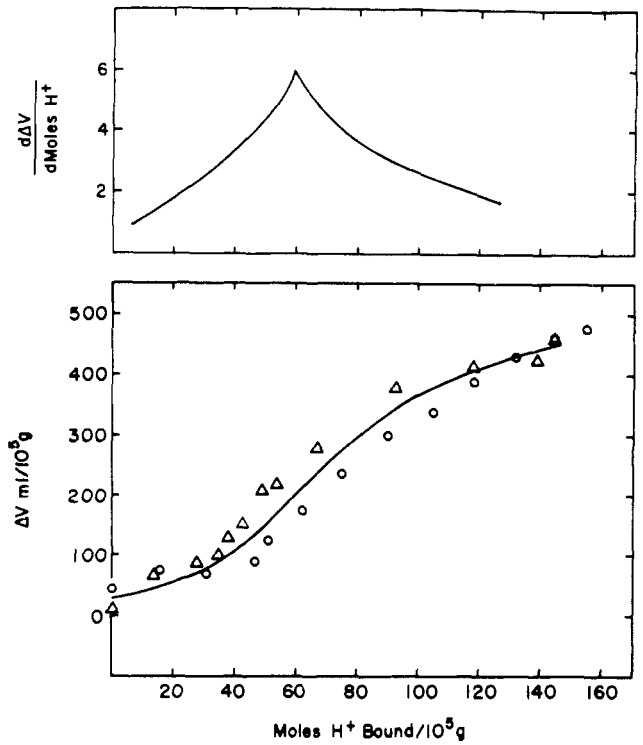

FIGURE 3: Volume changes produced by the reaction of HCl with human methemoglobin; the solvent was 6 M guanidine hydrochloride. The top panel (3A) is a plot of the derivative of the volume isotherm, as a function of the moles of H^+ bound/10^5 g of protein. The bottom panel (3B) is the conventional representation of the isotherm for this system.

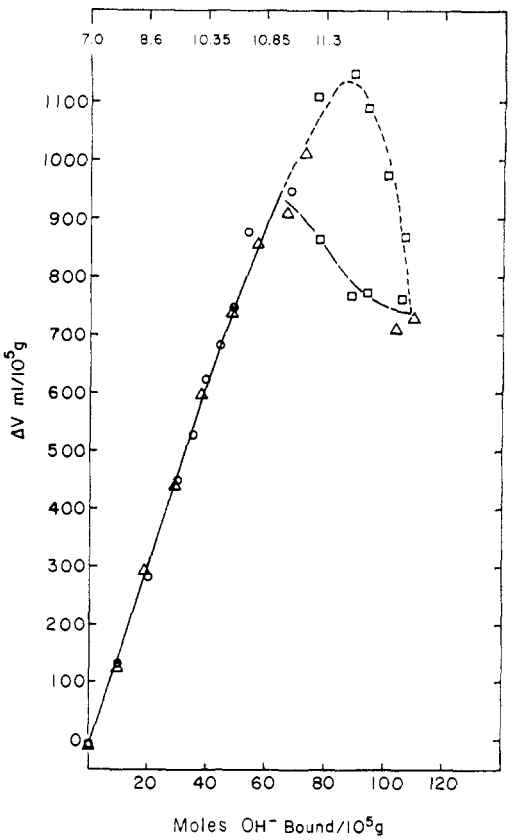

FIGURE 4: Volume changes produced by the reaction of NaOH with human oxyhemoglobin. For details refer to Figure 1.

bound/10^5 g of protein (Figure 2). Surprisingly, in 6 M guanidine hydrochloride, the isotherm was somewhat sigmoidal in nature (Figure 3B); the slope reached a maximum value of 6 ml/mol of H^+ bound when about 60 mol of H^+ were bound/10^5 g of protein and then decreased with increased H^+ binding (Figure 3A). At the point where 110 mol of H^+ were bound/10^5 g of protein, the value for ΔV was 390 ml/10^5 g of protein.

Alkaline Titration. The reaction of alkali with human oxy- and methemoglobin in water produced substantially different isotherms; however, the use of 8 M urea eliminated the difference between the two proteins. Oxyhemoglobin reacting with OH^- in water generated a linear time-independent volume increase up to the point where 50 mol of OH^- were bound/10^5 g of protein; the mean slope was 15 ml/mol of OH^- bound[2] (Figure 4). The time-independent portion of the volume isotherm continued to increase with a slightly diminished slope until a value of 925 ml/10^5 g of protein was reached, *i.e.*, where 65 mol of OH^- were bound/10^5 of protein. At higher alkalinity, time-dependent effects were observed; the 5-min and 18-hr values are depicted graphically. The ΔV determined 5 min after mixing increased with increasing pH until a maximum of 1125 ml/10^5 g of protein was obtained when 85 mol of OH^- were bound/10^5 g of protein; at higher alkalinity there was a sharp decrease of this parameter. The 18-hr values decreased in a sigmoidal manner when the binding was >65 mol of OH^-/10^5 g of protein. At the point where 110 mol of OH^- were bound/10^5 g of protein, steady-state values were achieved 5–10 min after mixing; the ΔV was 725 ml/10^5 g of protein.

The corresponding isotherm for methemoglobin differed from the above (compare Figures 4 and 5). There was a linear time-independent volume rise until 40 mol of OH^- were bound/10^5 g of protein; the slope was about 15.5 ml/mol of OH^- bound.[2] In the region encompassed by 40–100 mol of OH^- bound/10^5 g of protein, the volume effects were time dependent. The 5-min values reached a maximum of 900 ml/10^5 g of protein when 65 mol of OH^- were bound/10^5 g of protein and then decreased to a minimum of 625 ml/10^5 g of protein when 105 mol of OH^- were bound/10^5 g of protein. When the amount of OH^- binding was $\geq$105 mol of OH^-/10^5 g of protein, the ΔV isotherm achieved steady state immediately after mixing and exhibited a linear dependence on OH^- binding with a mean slope of 10 ml/OH^- bound.[2] The steady-state isotherm was similar to the 5-min values except that it was about 300–400 ml/10^5 g of protein more negative in the region where 40–100 mol of OH^- were bound/10^5 g of protein.

The exposure of these two proteins to 8 M urea at 30° for 18 hr before alkaline titration produced systems which reached steady state immediately upon reaction. The isotherms for these proteins were linear within experimental error; the values for ΔV were 1550 and 1600 ml/10^5 g of protein at the point where 110 mol of OH^- were bound for oxy- and methemoglobin, respectively.

Discussion

The contribution of the disruption of the quaternary structure to these volume effects is demonstrable by two approaches: (i) the difference between the ΔV values for acid-

[2] The value of 23 ml/mol was proposed for the volume change produced by the reaction of OH^- with "normal" imidazolium and ammonium residues in proteins (Rasper and Kauzmann, 1962). In 8 M urea there is about a 15% reduction of this volume effect. Studies in 6 M guanidine hydrochloride are not feasible since this denaturant is a weak acid and competes in the neutralization process (Katz and Miller, 1971b).

base reactions involving hemoglobins in water and in denaturing media, and (ii) the calculation of the difference between the volume effects estimated for a "normal" protein with hemoglobin's composition and the experimentally determined value.

Acid Titration. Prior to analysis of the data produced by the protonation of oxy- and methemoglobins, a review of the structural changes generated in hemoglobins by acidification is useful. Exposure of hemoglobins to systems more acid than pH 6 causes the dissociation of hemoglobins from the tetrameric to dimeric form (Fanelli *et al.*, 1964; Sharonova *et al.*, 1972). At pH values <4, structural changes occur which normalize "masked" prototropic groups (Steinhardt and Zaiser, 1953). Even though question exists regarding the number and types of ionic groups released, there is agreement that most of these are imidazoles; titration studies indicate that about 18 of the 38 imidazoles in human hemoglobin molecule are masked in the native state (Geddes and Steinhardt, 1968; Janssen *et al.*, 1972). Three other processes involving heme become significant at pH <4 (Allis and Steinhardt, 1969). There is a fast reaction causing a shift of the Soret band to the red which is followed by the expulsion of heme from the protein and the concomitant disruption of the protein's tertiary structure. The third step is the dimerization of the heme in the solution. The rates of these processes are strongly pH dependent (Allis and Steinhardt, 1969).

At present, one cannot designate the specific process responsible for a given volume effect unequivocally for these systems; however, there is substantial evidence that the disruption of the quaternary structure of hemoglobin is the factor primarily responsible for the large negative ΔV changes observed by these acid–base reactions. The negative volume effects resulting from the protonation of hemoglobins (see Figure 1) are in marked contrast to the volume rises produced by the protonation of globular proteins (Rasper and Kauzmann, 1962; Katz and Miller, 1971a). The small volume rise which occurs at the initial stages of protonation of hemoglobins is readily explicable in terms of the simultaneous protonation of imidazoles[3] and carboxylates.[1] However, at pH < 5 where positive ΔV is predicted on the basis of the titration of carboxylates, the hemoglobins exhibit a sharp volume decrease. This volume decrease of -700 ml/10^5 g of protein is substantially larger than the ΔV of -100 ml/10^5 g of protein observed for myoglobin under similar conditions (Katz *et al.*, 1973a). It should be recalled that myoglobin's transition is attributed to the structural transition which exposed 34 masked histidyl residues/10^5 g of protein. However, for hemoglobins it is proposed that this initial large volume decrease occurs at the pH where hemoglobin is converted from the tetrameric to dimeric form and this process exposes shielded polar groups to water, thus causing a volume decrease in accordance with the electrostriction concept (Gurney, 1953). The absence of a volume rise at higher protonation levels, *i.e.*, at pH values < 4, is indicative that continuing progressive structural changes are occurring which expose additional masked ionic groups. This phenomenon is probably related to the structural changes involving heme which have been referred to previously (Allis and Steinhardt, 1969).

The magnitude of the volume effect attributable to a structural transition of a protein can be determined by taking the difference between the ΔV calculated for this protein assuming

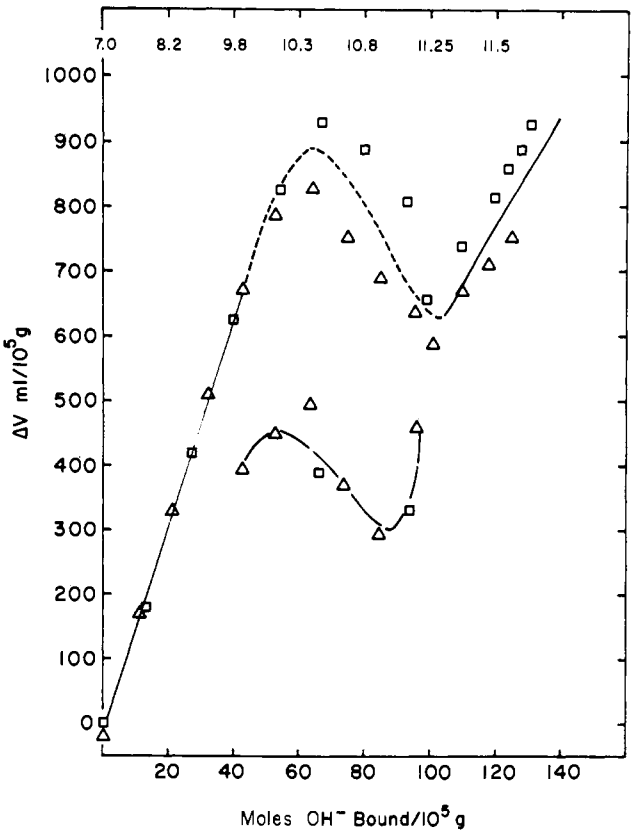

FIGURE 5: Volume changes produced by the reaction of NaOH with human methemoglobin. For details refer to Figure 1.

"normal" behavior and the experimentally observed ΔV (Katz *et al.*, 1973b).[4] A value of 850 ml/10^5 g of protein was calculated for "normal" hemoglobin based on the ΔV determined in 6 M guanidine hydrochloride at the point where 130 mol of H^+ were bound/10^5 g of protein. This compares favorably with the quantity 950 ml/10^5 g of protein which was based on the protonation of 30 imidazoles and 100 carboxylates. Thus we obtain a value of -1600 ml/10^5 g of protein which represents the volume decrease resulting from the disruption of hemoglobin structure at the point where 130 mol of H^+ are bound/10^5 g of methemoglobin.

The exposure of hemoglobins to 8 M urea and 6 M guanidine hydrochloride disrupts their structure substantially; this is graphically demonstrated by the contrast of the volume effects in these denaturants compared to that observed in water (see Figures 1–3). Inspection of methemoglobin's isotherms in these dissociating and denaturing media reveal that most of the histidines are titrated and that some degree of organized structure is still retained. The low volume rise near the origin indicates that both imidazoles[2] and carboxylates[1] are being jointly titrated (Janssen *et al.*, 1970). The residual structure of methemoglobin in 8 M urea is progressively lost with increasing acid concentration as the progressive increase of slope with

[3] The protonation of imidazole in water, 8 M urea, and 6 M guanidine hydrochloride produces the following ΔV values: -1.7, -2.75, and -3.3 ml/mol, respectively (Katz and Miller, 1971b).

[4] The "normal" volume effect can be calculated by two approaches: (i) using ΔV values for prototropic groups determined from simple organic compounds (Kauzmann *et al.*, 1962) and then multiplying by the number of contributing ionic groups in the protein as established by composition, titration, and pK values; (ii) by extrapolation from the volume isotherm determined in 6 M guanidine hydrochloride. The second approach introduces the errors associated with the assumption that the protein is normalized in this denaturant and the use of the conversion factor, 2, which is required to convert this value to that in water.[1-3]

acid concentration indicates (Figure 2). The value of the slope, 10 ml/mol of H^+ bound, near the terminus of the isotherm is larger than that predicted from model carboxylate studies; this is indicative of a structural contribution.[1] In 6 M guanidine hydrochloride the volume effects differ from that observed in urea; the slope reaches a maximum when 60 mol of H are bound/10^5 g of protein and then decreases with increasing H concentration (Figure 3A). Obviously, the differing character of the isotherms in the two denaturants is proof that different structural changes are generated as a function of the medium and acidity. The change of slope with pH may be interpreted as the operation of progressive structural change and/or that different categories of carboxylates are titrated as a function of pH.

The protonation behavior of these hemoglobins differs markedly from sperm-whale metmyoglobin when water and 8 M urea are employed as solvents but all the isotherms were qualitatively similar in 6 M guanidine hydrochloride. Myoglobin in water produced a quasisinusoidal isotherm with a maximum of 125 ml/10^5 g of protein and a minimum of -100 ml/10^5 g of protein where 85 mol of H were bound/10^5 g of protein (Katz *et al.*, 1973b). It should be recalled that myoglobin, a single-chain hemoprotein, upon exposure to acid undergoes an unmasking phenomenon similar to hemoglobins, *i.e.*, an acid transition which results in the unmasking of 34 "buried" histidyl residues/10^5 g of protein at pH > 4 (Hartzell *et al.*, 1968). Since the difference of composition and sequence of these hemoproteins is not sufficient to account for the disparity in the volume isotherms this lends support to the hypothesis that the disruption of the quaternary structure is the primary factor. Interestingly, while the isotherms for these hemoproteins in 6 M guanidine hydrochloride are similar the volume effects in 8 M urea are in marked contrast. Methemoglobin in 8 M urea (Figure 2) exhibits a positive volume rise with increasing acid; but metmyoglobin in the same medium exhibits kinetic effects and produces a steady-state U-shaped isotherm with a minimum of -275 ml/10^5 g of protein when 70 mol of H^+ are bound/10 g of protein. The basis for this contrasting behavior in 8 M urea, a dissociating and denaturing medium, is not apparent.

Inspection of the volume changes produced by the protonation of oxyhemoglobin in water reveals a kinetic effect in the region where 35–85 mol of H^+ are bound/10^5 g of protein (Figure 1A). This time-dependent effect is a manifestation of the molecular events associated with the conversion of oxyhemoglobin to methemoglobin. The small differences between the steady-state isotherms of oxy- and methemoglobin may reflect the contribution of the intermediate forms involved in this transition.

Alkaline Titrations. The volume effects produced by the reaction of hemoglobins with OH^- are in accord with the maxim that these proteins are more resistant to alkali than to acid (Sharonova *et al.*, 1972) and that oxyhemoglobin is more stable in alkali than methemoglobin (compare Figures 4 and 5). Oxyhemoglobin, neutralized by OH^-, produces a nearly monotonic volume increase until the point where 60 mol of OH^- are bound/10^5 g of protein; the mean volume rise of 15 ml/mol of OH^- bound is substantially less than the 22 ml/mol of OH^- bound predicted for the neutralization of imidazolium and lysyl ϵ-ammonium groups.[2] The alkaline isotherms for hemoglobins are radically dissimilar to that produced by metmyoglobin (Katz *et al.*, 1973a); the reaction of OH^- with myoglobin generates a linear volume rise which reaches steady state immediately after mixing. This suggests that the kinetic effect and subsequent volume decrease which occurs when OH^- binding to hemoglobin exceeds 65 mol of OH^- bound/10^5 g of protein is caused by tetramer to dimer conversion process and the consequent structural reorganization. Magnetooptical rotatory dispersion studies by Sharonova *et al.* (1972) reveal that human deoxyhemoglobin dissociates to dimers when the pH exceeds 10 and that denaturation becomes significant at pH >11. Interestingly, the difference between the initial maximum volume reading and the steady-state values of -375 and -450 ml/10^5 g of protein found for oxy- and methemoglobin compare favorably to the ΔV values of -300 and -400 ml/10^5 g of protein proposed for the disruption of the quaternary structure of these hemoglobins by sodium dodecyl sulfate (Katz *et al.*, 1973b). The contrasting character of the isotherms for these proteins (compare Figures 4 with 5) is due to the different rates of alkali-facilitated reactions such as the translocation of heme, the change of the ionization state of several prototropic groups (Sharonova *et al.*, 1972), the neutralization of tyrosyls,[5] and the alkaline hydrolysis of disulfide bonds (Donovan and White, 1971; Katz *et al.*, 1973a).

In 8 M urea the isotherms for oxy- and methemoglobin are superimposable straight line functions of OH^- neutralization; this indicates the conversion of oxyhemoglobin to the met form by the prior 18-hr contact with 8 M urea. The linear isotherm is characteristic of a normal protein; however, the value for the slope, 14 ml/mol of OH^- bound, is about 25% lower than that anticipated for the neutralization of protonated nitrogen bases in this medium[2] (Katz and Miller, 1971b). This suggests that either these proteins' prototropic groups are less hydrated than the model compounds selected as reference (Kauzmann *et al.*, 1962; Katz and Miller, 1971b), thereby producing reduced electrostriction effects (Gurney, 1953), or else that the ΔV values proposed should be reevaluated (Rasper and Kauzmann, 1962; Katz and Miller, 1971a).

The volume changes which hemoglobins exhibit upon reaction with acid and bases in water are unique and specific for the individual hemoproteins and are in marked contrast to that produced by simple globular proteins (Rasper and Kauzmann, 1962; Katz and Miller, 1971a). The extremely large negative volume effects observed in water but not in denaturing media are due primarily to the disruption of the quaternary structure by the action of acids or bases. The specific character of the individual isotherms is determined by the spatial organization of the protein, the prosthetic groups, composition, and the primary structure of the protein.

References

Allis, J. W., and Steinhardt, J. (1969), *Biochemistry 8*, 5075.

Antonini, E., and Brunori, M. (1971), Hemoglobin and Myoglobins in Their Reactions with Ligands, New York, N. Y., Elsevier, p 19.

Benesch, R. E., Benesch, R., Renthal, R. D., and Maeda, M. (1972), *Biochemistry 11*, 3576.

Donovan, J. W., and White, T. M. (1971), *Biochemistry 10*, 32.

Fanelli, R. A., Antonini, E., and Caputo, A. (1964), *Advan. Protein Chem. 19*, 74.

Geddes, R., and Steinhardt, J. (1968), *J. Biol. Chem. 243*, 6056.

Gurney, R. W. (1953), Ionic Processes in Solution, New York,

[5] Krausz (1970) stated that the ΔV of the reaction of OH^- with phenolic hydroxyl of tyrosyl should be about 3 ml/mol of OH^-. This value should not be altered by more than 1–3 ml/mol of OH^- in 8 M urea.

N. Y., McGraw-Hill, pp 187–196.
Hartzell, C. R., Bradshaw, R. A., Hapner, K., and Gurd, F. R. N. (1968), *J. Biol. Chem. 243*, 690.
Janssen, L. H. M., DeBruin, S. H., and Van Os, G. A. J. (1970), *Biochim. Biophys. Acta 221*, 214.
Janssen, L. H. M., DeBruin, S. H., and Van Os, G. A. J. (1972), *J. Biol. Chem. 247*, 1743.
Katz, S. (1972), *Methods Enzymol. Part C 26*, 395.
Katz, S., Crissman, J. K., Jr., and Beall, J. A. (1973a), *J. Biol. Chem. 248*, 4840.
Katz, S., and Ferris, T. G. (1966), *Biochemistry 5*, 3246.
Katz, S., and Miller, J. E. (1971a), *Biochemistry 10*, 3569.
Katz, S., and Miller, J. E. (1971b), *J. Phys. Chem. 75*, 1120.
Katz, S., and Miller, J. E. (1972), *J. Phys. Chem. 76*, 2778.
Katz, S., Miller, J. E., and Beall, J. A. (1973b), *Biochemistry 12*, 710.
Kauzmann, W., Bodanszky, A., and Rasper, J. (1962), *J. Amer. Chem. Soc. 84*, 1777.
Krausz, L. M. (1970), *J. Amer. Chem. Soc. 92*, 3168.
Linderstrøm-Lang, K., and Lanz, H. (1938), *C. R. Trav. Lab. Carlsberg 21*, 315.
Peacock, A. C., Bunting, S. L., and Queen, K. G. (1965), *Science 147*, 1451.
Rasper, J., and Kauzmann, W. (1962), *J. Amer. Chem. Soc. 84*, 1771.
Sharonova, N. A., Sharonova, Y. A., and Volkenstein, M. V. (1972), *Biochim. Biophys. Acta 271*, 65.
Steinhardt, J., and Zaiser, E. M. (1953), *J. Amer. Chem. Soc. 75*, 1599.

Variability in the Tertiary Structure of α-Chymotrypsin at 2.8-Å Resolution†

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ABSTRACT: The structures of the independent monomeric units of dimeric native α-chymotrypsin have been compared at 2.8-Å resolution *via* a difference electron density map between the two molecules. Approximately 16% of the density (one-sixth of a molecule) showed differences >0.7 eÅ^{-3} or three times the expected standard deviation of the difference density. Representative differences in the electron density have been related to variability in the tertiary structure in the two molecules. The variability also manifests itself in a number of other ways: in heavy atom derivatives, in the behavior of localized sulfate ions in crystallographic sulfate-selenate exchange experiments, and in derivatives of α-chymotrypsin (inhibitors, substrate-like molecules, transition state analogs, changes in structure with change in pH). The largest number of differences resides in the dimer interface region and in a 5–6-Å shell around the surface of the dimer; the smallest number occurs in the interior of the monomeric molecules. The differences in structure are most often associated with the orientation and configuration of side chains and less so with the main chains. The surface variability probably reflects the relatively large degree of adaptability in tertiary structure in this region of the enzyme and might be a fairly general phenomenon also common to functioning molecules in solution and in biological systems. From the nature of the variability in structure in the dimer interface, the formation of dimer must be accompanied by structurally asymmetrical and dynamical changes in this region of the enzyme. This could conceivably be a characteristic of most oligomeric structures. Aromatic residues tend to aggregate within the molecular structure suggesting a source of stabilizing interactions involving the delocalized electrons of the aromatic side chains. Two sulfate ions participate in a hydrogen bonding scheme which appears to be an important interaction in maintaining the dimeric structure. The ions form bridges, in a reciprocating manner between the independent molecules, which involve the catalytically crucial Ser-195 of one molecule and the phenolic hydroxyl group of terminal Tyr-146 of the other molecule.

At about pH 4.0, α-chymotrypsin crystallizes from approximately half-saturated ammonium sulfate solutions and the crystals are usually stored at a similar pH but at higher concentrations of ammonium sulfate (65–75% saturated) (Sigler *et al.*, 1966; Tulinsky *et al.*, 1973). The crystals belong to the monoclinic crystal system, space group $P2_1$, with four molecules (mol wt ~25,300) per unit cell or two molecules of enzyme per asymmetric unit. Consequently, to describe the space arrangement in the crystal precisely and completely, the molecular structures of both crystallographically independent molecules in the asymmetric unit (mol wt ~51,000) must be determined. The two molecules have been shown to be related to each other by an approximate noncrystallographic twofold rotation axis (local twofold axis) in a manner that interfaces the independent molecules with one another to yield a dimeric unit (Blow *et al.*, 1964; Sigler *et al.*, 1968; Cohen *et al.*, 1970; Tulinsky *et al.*, 1973). Such an interaction is con-

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