Halophilic Class I Aldolase and Glyceraldehyde-3-phosphate Dehydrogenase: Some Salt-Dependent Structural Features†

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ABSTRACT: Aldolase and glyceraldehyde-3-phosphate dehydrogenase from the extremely halophilic archaebacterium *Haloarcula vallismortis* are stable only in high concentrations of KCl present within the physiological environment. Data concerning the structural changes in the two enzymes as a result of lowering of salt concentration and changes in pH were obtained by monitoring the intrinsic protein fluorescence in the presence of quenchers. When the KCl concentrations were lowered below 2 M or in the presence of 6 M guanidine hydrochloride, the emission maximum shifted to a longer wavelength, indicating enhanced exposure of tryptophyl residues to the solvent. The spectral characteristics of the two proteins in guanidine hydrochloride and 0.4 M KCl were identical. However, these denatured states appear to be different than those observed after acid denaturation. Further perturbation of fluorescence was observed due to I^-, and application of the Stern–Volmer law showed that the total fluorescence was available to the quenchers only in 0.4 M KCl solutions. The unfolding of proteins in 0.4 M KCl was a gradual process which was accompanied by a time-dependent loss in enzyme activity. The activity loss was complete within 30 min for aldolase whereas in the case of GAPDH nearly 3 h was required for the destruction of activity. For both enzymes, inactivation and protein denaturation were strongly correlated. The data on activity and thermostability measurements of the two enzymes in varying concentrations of KCl and potassium phosphate revealed that though both proteins are halophilic, the forces in the maintenance of their stability could be different. Hydrophobic interactions and hence entropy terms appeared to be dominant for the stabilization of *h*aldolase, below room temperature. For *h*GAPDH in KCl, the hydrophobicity of the core was apparently too weak to stabilize its folded structure, and the thermodynamic stability of the solution particle was mainly enthalpy-driven. However, for both enzymes in high concentrations of phosphate ions, even the hydrophobicity of the tetrameric *h*GAPDH appeared to be sufficient for its stabilization. The data suggested that the acidic *h*GAPDH may not have a strong hydrophobic core to stabilize its folded structure in KCl whereas *h*aldolase, being a larger nonacidic protein with many small subunits, depends on stronger hydrophobic interactions (core and/or between subunits) to stabilize its folded sturcture. Nevertheless, *h*aldolase, being a halophilic enzyme, the hydrophobic interactions alone are not strong enough to stabilize its folded structure.

Extremely halophilic bacteria as the name suggests are obligate halophiles which require 10–20% NaCl for optimal growth (Larsen, 1967; Kushner, 1978). They overcome the environmental osmotic stress by maintaining an internal salt concentration close to saturation in KCl (Ginsburg et al., 1970; Christian & Waltho, 1962), an environment in which most classical biochemical reactions would be inhibited (Eisenberg & Wachtel, 1987). As a consequence, the biochemical machinery of halobacteria is adapted to function at extremely high salt concentrations. Most halobacterial enzymes are also halophilic; i.e., they require high salt concentration for the maintenance of their structural integrity and activity, and at salt concentrations below 1 M KCl or NaCl, the halophilic proteins are denatured (Baxter, 1959; Holmes & Halvarson, 1965; Lanyi, 1974). Though the purification of enzymes from halobacteria is severely impaired because of the necessity to include high concentrations of salt in purification procedures, in recent years a few halobacterial proteins have been obtained in homogeneous forms (Mevarech et al., 1977; Werber & Mevarech, 1978; Leicht et al., 1978; Guinet et al., 1988). In the earlier studies, the structural changes in dilute salt solutions were seen by sedimentation analysis (Hubbard & Miller, 1970; Holmes & Halvarson, 1965) and gel permeation chromatography (Hubbard & Miller, 1969). During the last decade, structures of a few halophilic proteins, viz. ferredoxin, elongation factor Tu, and malate dehydrogenase have been characterized by techniques such as sequencing, ^1H NMR, analytical centrifugation, light scattering, neutron scattering, and circular dichroism (Rao & Argos, 1981; Guinet et al., 1988; Ebel et al., 1992; Gochin & Degani, 1985; Sussman et al., 1986; Zaccai et al., 1986a; Hecht & Jaenicke, 1989). It appears that other than these few proteins, structural aspects of other halophilic proteins have not received much attention. The structural nature of adaptation of halobacterial proteins to extreme salinity has been reviewed by Lanyi (1971), Eisenberg and Wachtel (1987), Rao and Argos (1981), and Zaccai and Eisenberg (1990). Halophilic proteins and enzymes follow the salt rank of the Hofmeister series by displaying functional and structural integrity at high salt concentrations of salting-out salts and destabilization in the presence of salting-in salts (Lanyi & Stevenson, 1970; Liberman & Lanyi, 1971; Pundak & Eisenberg, 1981). Earlier work from this laboratory (Altekar, 1975, 1977a,b; Altekar et al., 1977) detailed the interactions of salts of the Hofmeister series with several nonhalophilic proteins by the use of fluorescence spectroscopy. Since variation of salt concentrations in halobacterial protein solutions elicits changes in their emission parameters (Mevarech et al., 1977; Hecht & Jaenicke, 1989), an investigation on the solute perturbation

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of fluorescence of halophilic proteins was undertaken.

Recently we have isolated for the first time two native halophilic enzymes, viz., aldolase (class I, Schiff base) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)[1] from *Haloarcula vallismortis* (Krishnan & Altekar, 1990, 1991) and characterized them biochemically. Both enzymes show a number of halophilic characteristics: their activity depends on the salt concentration, and they are unstable below 1.5 M KCl. In the case of *h*GAPDH, the amino acid analysis revealed a large proportion of acidic residues, and its subunit size as seen in denaturing PAGE appeared to be slightly higher than nonhalophilic analogs. A molecular mass of 160 000 from gel filtration is compatible with a tetrameric structure. In contrast to *h*GAPDH and other halophilic proteins, the unusual class I aldolase is not a particularly acidic protein, but perhaps more hydrophobic. Besides, the data from gel filtration and PAGE under denaturing conditions revealed that *h*aldolase as compared to nonhalobacterial aldolases is made up of a large number of subunits of much smaller size, viz., 27 kDa as compared to 33–40 kDa. We suggested that while protein–solvent interactions would be important for the stability of *h*GAPDH, those between subunits would be important for the stability of *h*aldolase. These aspects will be discussed in this paper on the basis of results of the salt-dependent structural features of *h*aldolase and *h*GAPDH, obtained mainly by fluoresence spectroscopy. Additional information on the stability behavior was obtained from the study of the temperature dependence of the two proteins in KCl and K-phosphate. A preliminary report on the information obtained from the neutron-scattering studies on *h*GAPDH has appeared recently (Ebel et al. 1991).

EXPERIMENTAL PROCEDURES

Chemicals. K_2HPO_4, KH_2PO_4, diethyl barbiturate, DHAP, and acrylamide were from Sigma Chemical Co. (USA). Citric acid, sodium borate, and KI were products of Glaxo Chemicals (India). Guanidine hydrochloride was an E. Merck (Germany) product, and all salts were of analytical grade. Double-glass-distilled water was used for all experiments.

Enzymes. Homogeneous preparations of *h*aldolase and *h*GAPDH were obtained from the halobacterium *Haloarcula vallismortis* by the procedures described earlier (Krishnan & Altekar, 1990, 1991).

Protein Assay. Protein concentrations were measured by the method of Lowry et al. modified by Peterson (1983).

Enzyme Assays. (*A*) *Aldolase Assay.* Aldolase activity was measured colorimetrically by estimating the triose phosphates released, using the modified method (Krishnan & Altekar, 1991) of Sibley and Lehninger (1949).

(*B*) *GAPDH Assay.* GAPDH activity was measured spectrophotometrically (Krishnan & Altekar, 1990), following NAD reduction at 340 nm.

Effect of Anions on hAldolase Activity. The effect of anions (1–12 mM), arsenate, pyrophosphate, and phosphate on aldolase activity was determined by measuring enzyme activity in the standard assay medium in the presence of varying concentrations of the Na or K salts of the above anions.

Stability of hAldolase and hGAPDH in 0.4 M KCl. The enzymes solutions in 4 M KCl–50 mM Tris-HCl, pH 8.0, were diluted quickly with 50 mM Tris-HCl to obtain 0.4 M KCl concentration, and the stability was determined as the percent original activity remaining at the end of specified time intervals, ranging from 0, 10 min, ..., 3 h.

pH Measurements. All experiments were carried out in 50 mM Tris-HCl, pH 8.0, buffers (unless otherwise indicated), the pH being adjusted after the addition of salt. For pH titration, 500 mM Universal buffer was prepared as follows: 6.008 g of citric acid, 3.893 g of KH_2PO_4, 1.769 g of H_3BO_3, and 5.266 g of diethyl barbiturate were dissolved and diluted to 800 mL; 80 mL of this solution was titrated with different amounts of NaOH and diluted to 100 mL, for obtaining buffers with pH ranging from 4.0 to 10.0. The pHs of various buffers were measured in a Systronics digital pH meter type 335.

Stability of hAldolase and hGAPDH at Different Temperatures as a Function of Salt. The stability of *h*aldolase and *h*GAPDH was examined separately by incubating the enzyme (50–80 μg/mL) for 24 h at different temperatures (−20 to 40 °C) in salt solutions ranging from 0.2 to 2.7 M KCl or from 0.2 to 1.5 M K-phosphate. The enzyme activity was assayed in the standard assay medium and related to the enzyme activity in 4 M KCl.

Fluorescence Measurements. The ultraviolet fluorescence spectra were determined at 25 °C in a 1-cm path cell in a Hitachi fluorescence spectrophotometer (Model F-3010) as described earlier (Altekar, 1975). The tryptophyl emission spectrum was scanned from 300 to 400 nm after excitation at 280 nm. Protein concentrations of 5–10 μg/mL were used, and it was ascertained that at this concentration the absorbance at 280 nm was less than 0.1. All the enzyme solutions in the particular set were of the same absorbance.

In order to obtain the fluorescence spectra of the enzymes at various salt concentrations, the stock solution of each enzyme in 4 M KCl–50 mM Tris-HCl, pH 8.0, was diluted appropriately and read after 30 min unless mentioned otherwise. For pH titration, stock solutions of 4 M KCl were used for dilution with 50 mM Universal buffer, and the pH was adjusted from 4.0 to 10.0. Freshly prepared Gdn·HCl solution was used. Stock solutions of 1 M acrylamide and KI were stored in the cold and used within 1 week; 0.1 M sodium thiosulfate was added to KI to prevent free iodine formation.

The peak fluorescence of the protein in 4 M KCl was taken as 100%, and the fluorescence peak values under the given experimental conditions were calculated in relation to this value unless specified otherwise. The data on quenching were analyzed as described earlier (Altekar, 1977a,b; Altekar et al., 1977). This was done according to the equation of Stern and Volmer (1919) expressed as $F_0/F = 1 + K_Q[X]$, and its modification was developed by Lehrer (1971). In the case of proteins where two types of tryptophan fractions are present, one accessible and the other inaccessible to the quencher:

$$F_0/(F_0 - F) = F_0/\Delta F = 1/[X]f_aK_Q + 1/f_a$$

where F and F_0 are the quenched and unquenched fluorescence quantum yields or intensities, respectively, f_a is the fraction of accessible fluorescence, K_Q is the quenching constant, and [X] is the concentration of the quencher. A plot of $F_0/\Delta F$ versus $[X]^{-1}$ will be linear with an intercept of f_a^{-1} and a slope of $(f_aK_Q)^{-1}$. $(f_a)_{eff}$ is the value of the "effective" fractional accessible fluorescence from 1/intercept, and $(K_Q)_{eff}$ is the "effective" quenching constant obtained from the intercept/slope.

Estimation of Tryptophan. The tryptophan content of the two proteins was measured by the spectrophotometric method of Bencze and Schmid (1957).

[1] Abbreviations: *h*, halobacterial; Gdn·HCl, guanidine hydrochloride; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MDH, malate dehydrogenase; K-phosphate, potassium phosphate; DHAP, dihydroxyacetone phosphate; NAD, nicotinamide adenine dinucleotide; GAP, glyceraldehyde 3-phosphate; FDP, fructose 1,6-diphosphate.

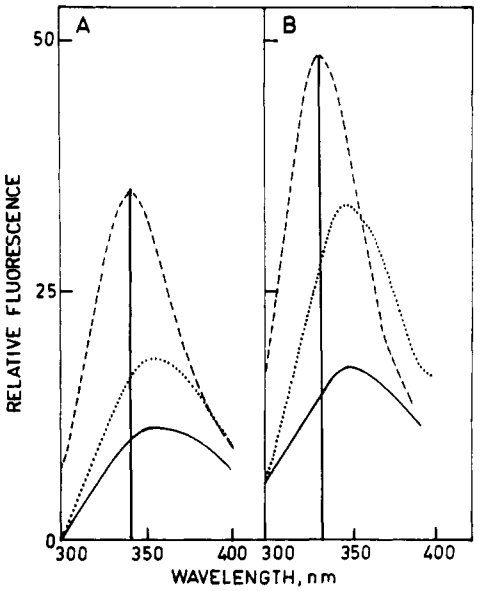

FIGURE 1: Fluorescence spectra of native and denatured (A) *h*aldolase (6 μg/mL) and (B) *h*GAPDH (7.5 μg/mL) in (- - -) 3 M KCl and in (···) 6 M Gdn·HCl–0.4 M KCl 0 min after additions and (—) after 30 min. All solutions contained 50 mM Tris-HCl, pH 8.0, and the λ_{exc} was 280 nm at 25 °C.

RESULTS

The salt-dependent properties of *h*aldolase and *h*GAPDH were examined under similar conditions, and the data are presented. Values of 50 mol/mol, decamer, for *h*aldolase, and 16 mol/mol, tetramer, for *h*GAPDH were obtained as the tryptophan content of the two proteins.

Solute Perturbation of Protein Fluorescence. (A) Effect of Protein Denaturant on the Fluorescence of hAldolase and hGAPDH. The fluorescence spectra of native *h*aldolase and *h*GAPDH showed maxima at 338 and 332 nm, respectively. Addition of Gdn·HCl to both proteins resulted in a shift of the emission maxima to a longer wavelength, 350 nm, an indication of denaturation in Gdn·HCl (Figure 1A,B). The fluorescence was quenched upon denaturation, and the change was time-dependent. The fluorescence of *h*aldolase decreased to 51% immediately after the addition of the denaturant and decreased by a further 30% of the original value by the end of 30 min. In case of *h*GAPDH, the corresponding values were 70% and 36%, respectively.

(B) Effect of Salt Concentration on the Fluorescence of hAldolase and hGAPDH. The activities of *h*aldolase and *h*GAPDH were shown to be dependent on high concentrations of salt (Krishnan & Altekar, 1990, 1991). The fluorescence intensity of *h*aldolase decreased when the salt concentration was reduced (Figure 2A) without any change in the emission maximum upto 1 M KCl. In 0.4 M KCl, however, there was a shift in the emission maximum to 350 nm.

The fluorescence of *h*GAPDH also decreased with decreasing KCl concentrations and was accompanied by a gradual shift in the emission maximum to longer wavelength (Figure 2A). This shift which was apparent in 2 M KCl (334 nm) reached 350 nm in 0.4 M KCl. The foregoing data thus indicated that as a result of lowering the KCl concentration, the unfolding of two enzymes with concomitant exposure of tryptophan residues took place (Chen, 1967).

The changes in the fluoresence emission of the two enzymes were time-dependent. The changes in 0.4 M KCl concentrations were monitored and compared with the loss in the enzyme activity, at similar KCl concentrations (Figure 2A, inset, *h*aldolase; Figure 2B, inset, *h*GAPDH). The fluorescence of *h*aldolase was quenched immediately to 42% upon lowering the KCl concentration to 0.4 M. However, there was no shift in the emission maximum at this time, which appeared after 10 min. At the end of 75 min, when the total enzyme activity was lost, the spectrum resembled that of *h*aldolase in 6 M Gdn·HCl.

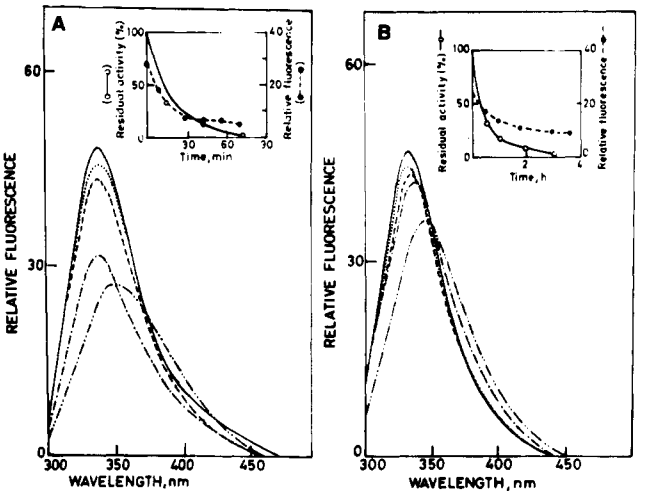

FIGURE 2: Fluorescence of (A) *h*aldolase (6 μg/mL) and (B) *h*GAPDH (7 μg/mL) in varying KCl concentrations: (—) 4 M; (···) 3 M; (- - -) 2 M; (– · –) 1 M; (– ·· –) 0.4 M. Insets shows the time-dependent loss of enzyme activity and the fluoresecence loss in 0.4 M KCl. Other conditions as in Figure 1.

Under similar conditions, the fluorescence behavior of *h*GAPDH was different. Quenching accompanied by a 12-nm shift of the fluorescence maximum to the longer wavelength was evident immediately upon lowering the KCl concentration to 0.4 M. However, the change in the microenvironment of tryptophan did not affect enzyme activity immediately. The quenching was only 18% of the original value, and a further time-dependent decrease in the fluorescence yield was quite gradual as compared to the changes seen for *h*aldolase (30 min). At the end of 3 h, when the total enzyme activity was lost, the spectrum resembled that of *h*GAPDH in 6 M Gdn·HCl. For both proteins, the kinetics of the loss of fluorescence in 0.4 M KCl followed the pattern for the loss of enzyme activity under similar conditions (see inset).

(C) KI Quenching of hAldolase and hGAPDH Fluorescence in Varying KCl Concentrations. The effects of I^- ions (0.1–0.5 M) on the emission of the enzymes were tested at different concentrations of salt. In these experiments, 3 M KCl was the highest concentration of salt used to obviate the volume changes resulting from other additions. Fluorescence spectra in 3 and 4 M KCl were identical. The fluorescence which was already decreased as a result of lowering the KCl concentration was further quenched due to the addition of KI.

In the presence of 0.2 M KI in 3 M KCl, the *h*aldolase fluorescence was quenched by 16%, accompanied by a shift of the emission maximum by 4 nm to the shorter wavelength (data not shown). No shifts in the emission maxima were seen when the enzyme was in 0.4 M KCl, though the fluorescence at these salt concentrations was further quenched by 38% and 23%, respectively. The quenching of aldolase by I^- in 0.4 M KCl appears to be quite low, since in the control itself the fluorescence yield was decreased. The Stern–Volmer relationships shown in Figure 3A for quenching of *h*aldolase by I^- in 3 and 1 M KCl were nonlinear. The modified Stern–Volmer plot gave values of 25% and 75%, respectively, for the tryptophan residues accessible to the quencher. In 0.4 M KCl, however, a linear relationship for KI quenching was

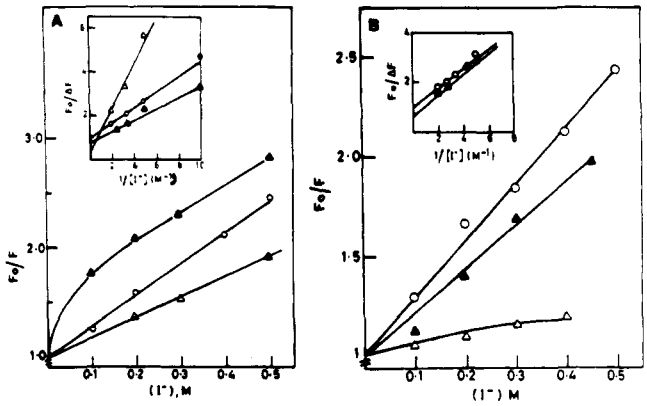

FIGURE 3: Stern–Volmer plots of the quenching (A) *h*aldolase (6 μg/mL) and (B) *h*GAPDH (7.5 μg/mL) by I^- from values obtained in the presence and absence of I^- in varying concentrations of KCl: (△) 3 M KCl; (▲) 1 M KCl; (○) 0.4 M KCl. Insets show the modified Stern–Volmer plots of the same data. Other conditions as in Figure 1.

Table I: Quenching Constants and $(f_a)_{eff}$ Values of *h*Aldolase and *h*GAPDH

quencher	enzyme and M KCl	$(K_Q)_{eff}$	K_Q	$(f_a)_{eff}$
KI	*h*aldolase			
	3	0.18		0.25
	1	2.14		0.75
	0.4	2.77	6.1	1.0
KI	*h*GAPDH			
	3	0.05		0.1
	1	0.66		0.6
	0.4	1.33	5.3	1.0
acrylamide	*h*GAPDH			
	3		20	

obtained with $K_Q = 6.1\ M^{-1}$ (Figure 3A). From the modified Stern–Volmer plot (Figure 3A, inset), it was evident that all tryptophyls in *h*aldolase were accessible to the quencher.

The quenching of *h*GAPDH fluorescence by I^- in 3, 1, and 0.4 M KCl is shown in Figure 3B. The Stern–Volmer plot of KI quenching in 3 M KCl was nonlinear. Comparatively, little quenching of *h*GAPDH due to I^- was observed in 3 M KCl, and the modified Stern–Volmer relationship gave a value of 10% for the fraction of fluorescence accessible to the quencher (Figure 3B, inset). The fraction of accessible tryptophyl residues in 1 M KCl was found to be 60%. The linear Stern–Volmer for KI quenching in 0.4 M KCl indicated that the total fluorescence was accessible to the quencher (Figure 3B, inset). The K_Q of the enzyme for KI under these conditions was determined as $5.3\ M^{-1}$.

Table I shows the various quenching constants for *h*aldolase and *h*GAPDH at different concentrations of KCl.

(D) Acrylamide Quenching of hAldolase and hGAPDH Fluorescence. The fluorescence of *h*aldolase and *hGAPDH* was also quenched in the presence of acrylamide. However, no shifts in the emission maxima were observed in either case. The Stern–Volmer relationships for the two proteins are plotted in Figure 4.

For *h*aldolase, the relationship was linear up to 0.08 M quencher concentration and nonlinear beyond this concentration. The K_Q value for the linear portion was $15.6\ M^{-1}$. For *h*GAPDH, the Stern–Volmer relationship was linear with $K_Q = 20\ M^{-1}$.

pH Titration. The pH titrations of the fluorescence of *h*aldolase and *h*GAPDH were carried out in the presence of 3 and 0.4 M KCl, and the values for the peak fluorescence are plotted in panels A and B, respectively, of Figure 5.

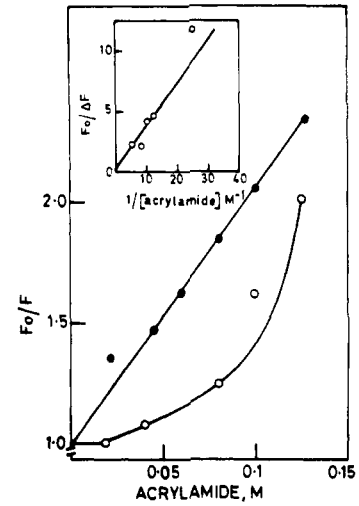

FIGURE 4: Stern–Volmer plot for acrylamide quenching of *h*aldolase (6 μg/mL) (○) and *h*GAPDH (7.5 μg/mL) (●) in 3 M KCl. K_Q for linear portion = $15.6\ M^{-1}$ for *h*aldolase and $20\ M^{-1}$ for *h*GAPDH. The inset shows the modified Stern–Volmer plot for *h*aldolase. Other conditions as in Figure 1.

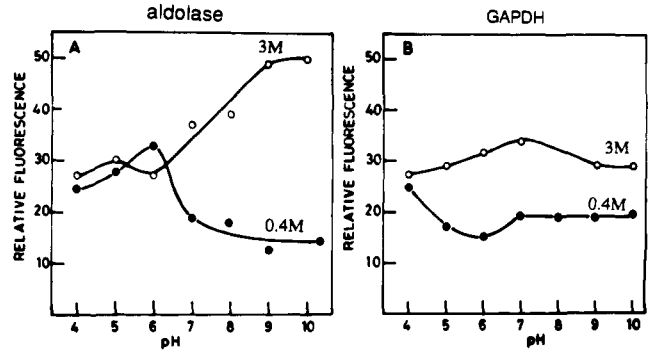

FIGURE 5: Fluorescence pH titration of (A) *h*aldolase (6 μg/mL) and (B) *h*GAPDH (7.5 μg/mL) in Universal buffers of varying pHs. (○) 3 M KCl; (●) 0.4 M KCl. Except for the buffer, other conditions were as in Figure 1.

The fluorescence intensities of both proteins either in concentrated or in dilute solutions of KCl were affected considerably by the changes in pH. In the case of *h*aldolase in 3 M KCl, a high yield of fluorescence was obtained between pH 7.0 and 10.0 while low intensities were seen between pH 4.0 and 7.0 with no change in the emission maximum. In 0.4 M KCl between pH 4.0 and 7.0, the fluorescence pattern was more or less similar to that in 3.0 M KCl. However, at pHs above 7.0, the emission maximum of 338 nm was shifted to the longer wavelength of 350 nm, and the fluorescence yield was less than that seen in 3 M KCl.

The fluorescence of *h*GAPDH in 3 M KCl increased with increases in pH though the changes were not as pronounced as that for *h*aldolase. No change in the emission maximum was seen. In 0.4 M KCl, at pH 4.0, the fluorescence yield was the same as that in 3 M KCl, but decreased above pH 5.0. While there was no shift in the emission maximum at pH 4.0, a shift to longer wavelength was evident at pH 5.0, which shifted further at pH 6.0 and remained unaltered above neutral pH.

pH Titration of Iodide and Acrylamide Quenching. The variation of peak fluorescence intensity as a function of pH in the presence of 0.2 M KI or 0.05 M acrylamide is shown in Figure 6A for *h*aldolase and in Figure 6B for *h*GAPDH. The results were obtained in 3 M KCl, and are compared with the values obtained in the absence of the quenchers. The changes in fluorescence yields in the presence of the two

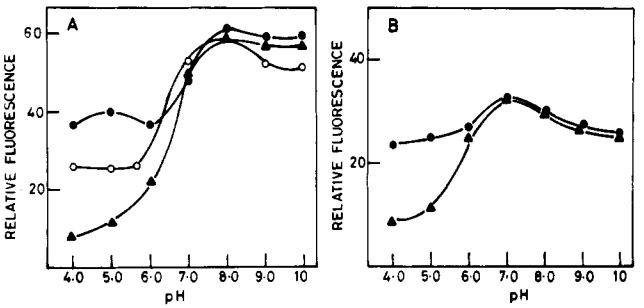

FIGURE 6: Fluorescence pH titration of (A) *h*aldolase (6 μg/mL) and (B) *h*GAPDH (7.5 μg/mL) in Universal buffers in the presence of quenchers. (●) Control; (○) in the presence of 0.05 M acrylamide; (▲) in the presence of 0.2 M KI. Except for the buffer, other conditions were as in Figure 1.

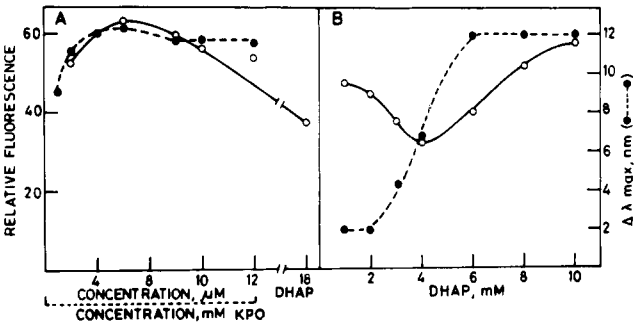

FIGURE 7: Fluorescence of *h*aldolase (7 μg/mL) in 3 M KCl. (A) Effect of micromolar DHAP and millimolar potassium phosphate (read lower scale). (B) Effect of millimolar DHAP. Other conditions as in Figure 1.

quenchers were qualitatively similar to those observed in their absence. Thus, the fluorescence at acidic pHs was less than that seen above pH 7.0, and the highest value was seen between pH 8.0 and 10.0. However, the reduction of the fluoresence of *h*aldolase in the presence of KI was more pronounced. The fluorescence at alkaline pHs did not change much due to the quenchers. Qualitatively similar results were obtained for *h*GAPDH in the presence of KI.

Additional Observations with hAldolase. (A) Effect of DHAP and Phosphate. Figure 7 shows the effect of DHAP and phosphate on *h*aldolase fluorescence. The fluorescence intensity was enhanced in the presence of 2–12 μM DHAP and 1–12 mM phosphate concentrations. At higher concentrations (1–10 mM) of DHAP, a decrease in fluorescence was seen in the presence of 1–4 mM DHAP, but the yield increased above this concentration. The increase in fluorescence was accompanied by a gradual shift in the emission maximum to longer wavelength which reached 350 nm at 10 mM DHAP.

(B) Enhancement of hAldolase Activity by Anions. An enhancement in the activity of *h*aldolase was observed in the presence of > 1 mM concentrations of Na or K salts of arsenate, pyrophosphate, and phosphate. The order of enhancement of activation was arsenate > pyrophosphate > phosphate (Figure 8). The enzyme activity was unaffected when tested in the standard assay buffer in the presence of 0.5 and 1 mM concentrations of the chaotropic ions, viz., SCN^-, Br^-, I^-, ClO_4^-, and NO_3^- (Krishnan, 1990). *h*Aldolase activity was retained in 50 mM Gdn·HCl, but was totally lost beyond this concentration.

Stability of hAldolase and hGAPDH as a Function of Temperature and Salt Concentration. Stability measurements for the two halophilic enzymes as a function of incubation temperature and salt concentrations were studied, and the results are shown in Figures 9 and 10. The measurements

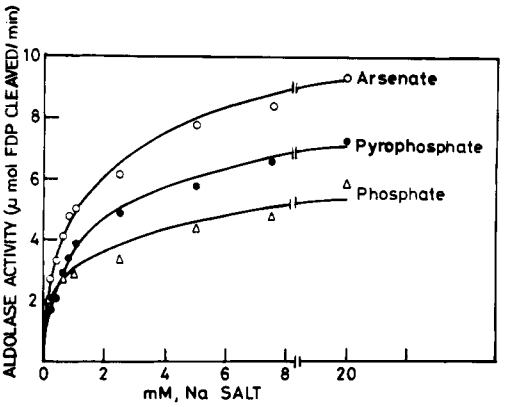

FIGURE 8: Activation of *h*aldolase by the presence of arsenate pyrophosphate and phosphate in the standard assay medium. Na salts of anions were used. Protein in assay, 0.5 μg.

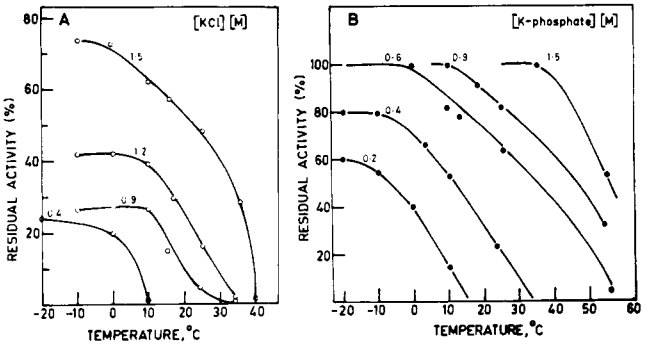

FIGURE 9: Enzymatic activity of *h*aldolase in standard buffer after each incubation for 24 h at a given salt concentration at various temperatures. (A) KCl; (B) potassium phosphate. For details, see Experimental Procedures.

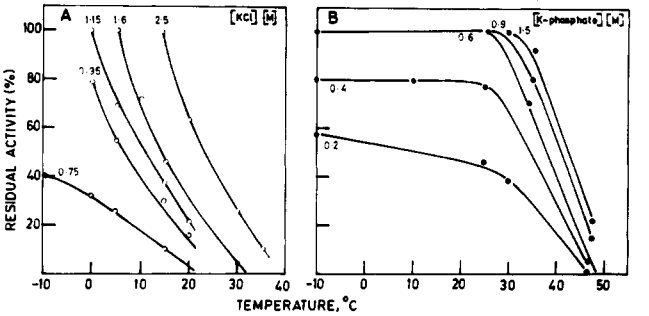

FIGURE 10: Enzymatic activity of *h*GAPDH in standard buffer after each incubation for 24 h at a given salt concentration at various temperatures. (A) KCl; (B) K-phosphate. For details, see Experimental Procedures.

reflect the proportion of the active protein after incubation in the stated conditions.

The stability of the *h*GAPDH in KCl increases with decreasing temperature down to −10 °C (Figure 10). The stability of enzyme in decreasing KCl concentration and decreasing temperature showed a linear relationship, and the original activity was retained in 1.15 M KCl and incubation at 0 °C.

In contrast, for *h*aldolase in KCl, the relationship was not linear. The complete original activity was not retained under the conditions used; moreover, it reached a limiting value as the incubation temperature decrased (Figure 9). The limiting value was also dependent on salt concentration.

In the case of both enzymes, however, for all phosphate concentrations the stability reached a limiting value at a certain temperature depending on the salt concentration, with a higher temperature for higher concentrations. K-phosphate was a

better stabilizer than KCl, and there was no loss in the activities of *h*aldolase as well as *h*GAPDH in 0.6 M K-phosphate concentration at 25 °C.

DISCUSSION

The compactness of folding of polypeptide chain(s) in solvents is an individual property of proteins (Privalov, 1979). The nonhalophilic proteins that function in the absence of salt are precipitated out and cease to function in concentrated salt solutions. In the absence of salt, halophilic proteins from halobacteria undergo structural changes that are reflected by changes in various parameters, e.g., sedimentation coefficient (Hubbard & Miller, 1970; Holmes & Halvarson, 1965), mainly due to the loss of unbound water (Zaccai et al., 1989) measured by various methods. It is known that some of the interactions that are ordinarily involved in the stabilization of proteins structure are weaker in halophilic systems whereas others are stronger because of the presence of salt (Lanyi, 1971).

The deleterious effects of lowering the KCl concentration on the structures of *h*aldolase and *h*GAPDH were seen in our experiments, and these could be correlated to the loss of enzyme activity under similar conditions. The quenching of fluorescence accompanied by a shift of the emission maximum to longer wavelength was indicative of unfolding of the protein molecule (Chen, 1967; Altekar, 1977a,b). The degree of quenching for *h*aldolase was more pronounced as compared to *h*GAPDH. Though fluorescence shifts (from 338 or 332 nm to 350 nm) in both cases were indicative of exposure of tryptophan to the solvent (Van Duuren, 1961), those for *h*GAPDH appeared as soon as the KCl concentration was reduced below 3 M, while those for *h*aldolase were apparent only after the concentration of 0.4 M was reached. The unfolding of *h*aldolase was not gradual, and the exposure of tryptophan to the solvent was rather abrupt as indicated by the sudden shift of the emission maximum to 350 nm in 0.4 M KCl. These results perhaps show weak and strong effects on protein structure in dilute salt solutions. These differences in the shift of the emission maximum and hence unfolding between the two proteins may be a reflection of their subunit associations. The interactions between the subunits of *h*aldolase appear to be stronger than those of *h*GAPDH. Proteins assume random-coil configurations in concentrated Gdn·HCl solution (Tanford, 1988), and Kronman and Holmes (1971) have shown that denaturation of proteins in Gdn·HCl resulted in an increase in fluorescence accompanied by a shift in the fluorescence maximum to 350 nm. However, in the case of halophilic MDH in Gdn·HCl though a shift of the emission maximum to 350 nm indicated protein denaturation, the fluorescence was quenched (Hecht & Jaenicke, 1989). Our results also indicated that in the case of halophilic proteins in Gdn·HCl the fluorescence was quenched upon denaturation. The shift of the emission maximum to 350 nm was a clear indication of the denaturation of halophilic proteins. The fluorescence spectra of *h*aldolase in Gdn·HCl and 0.4 M KCl were similar, and the changes were over in 30 min. Those of *h*GAPDH in 0.4 M KCl showed a similar fluorescence yield as in Gdn·HCl only after 3 h. The fluorescence quenching and inactivation of *h*aldolase are more rapid in 0.4 M KCl as compared to those of *h*GAPDH. This confirms that *h*GAPDH retains its stability and structure in dilute KCl for a much longer time as compared to the other enzyme. We are studying the neutron scattering and hydrodynamic properties of *h*GAPDH at different KCl concentrations. A constant value of 32 Å was obtained as the radius of gyration for *h*GAPDH, in the range of KCl concentrations above 2 M. The preliminary results from these studies have clearly indicated that *h*GAPDH contains loosely packed subunits and the protein begins unfolding when KCl concentrations are lowered (Ebel et al., 1991; unpublished data).

Two quenchers, iodide and acrylamide, were used to probe the degree of exposure and the nature of the microenvironment of tryptophyl residues. Further proof that the inactive forms of enzymes after incubation with low salt concentrations were extensively unfolded was obtained by the use of these quenchers.

The effect of I^- quenching on *h*GAPDH is less pronounced (in comparison to *h*aldolase) in 3 M KCl. Also, the blue emission maximum of the former protein (332 nm) would suggest that the tryptophyl residues of *h*GAPDH are more buried in 3 M salt and inaccessible to the quencher.

In 1 M KCl solution (though the counterion concentration is still high), the unfolding of the protein has taken place as indicated by the greater percentages of fluorophore available to the quencher in both cases. In 0.4 M KCl, however, all the fluorophores are available to the quencher. While the tryptophyl fluorescence was only partially available to the quencher in 3 and 1 M KCl, all of it was available in 0.4 KCl, confirming greater unfolding of the proteins in dilute salt solutions.

Fluorescence quenching of the two proteins was also observed in the presence of the polar uncharged quencher acrylamide which quenches predominantly by collisional quenching (Eftink & Ghiron, 1976). The straight-line relationship of the Stern–Volmer plot in the case of *h*GAPDH indicated that the heterogeneously emitting population of tryptophyls was accessible to this quencher. The efficiency of this quencher is apparent from the quenching constants indicated in Table I. In contrast, the nonlinear and upward-curving relationship for *h*aldolase of the acrylamide quenching suggested that the quencher cannot reach all the fluorophores in this protein (Eftink & Ghiron, 1976). This could probably be due to the compact nature of *h*aldolase as suggested here.

Some influence of the local charge around tryptophan was obtained from the results of pH variation on the peak fluorescence intensity of *h*aldolase and *h*GAPDH in 3 and 0.4 M KCl, in the presence and absence of quenchers. In the case of *h*aldolase in 3 M KCl, a drop in fluorescence around pH 6.0 may be due to the protonation of histidine residue(s) of that pK_a (Edelhoch et al., 1967). The unchanged emission maximum for the two proteins in 3 M KCl, in the entire pH range used, suggested no exposure of their tryptophyls to the solvent. However, lower yields of fluorescence were obtained for the two proteins at acidic pHs. The protein structure could be more open due to repulsive forces as it becomes more positively charged. At acidic pHs, the fluorescence intensities of the two enzymes were more or less similar in the two KCl concentrations used.

In the case of *h*aldolase in low salt solution at acidic pHs, the blue emission maximum indicated that most of the tryptophyls were still confined to a hydrophobic environment. The red shifts in emission maxima and the drop in fluorescence intensity for *h*aldolase above neutral pH suggested that the enzyme extensively unfolded at higher pHs. For *hGAPDH* in dilute salt solutions from pHs above 4.0, there was a gradual red shift in emission maxima with decreasing fluorescence intensities.

In salt solutions, the charges fully exposed to the solvent may be expected to be well screened (Lanyi, 1971). However, at acidic pHs, the increased quenching of *h*aldolase and

*h*GAPDH in 3 M KCl indicated that the charge-dependent effects were expressed even at high salt concentrations.

The increase in quenching by I^- with decreasing pH for both enzymes is probably due to the additional quenching resulting from the protonation of carboxyl groups located near tryptophan residues (Cowgill, 1967). Emission spectral shifts and/or quantum yield changes have continued to prove useful as empirical measures of conformational changes induced by pH and several other parameters. Thus, information on the nature of the local charge around tryptophyls has been obtained for several proteins (Lehrer, 1971; Pelley & Horowitz, 1976; Colona et al., 1978; Das & Vithayathil, 1978).

It has been suggested by Lanyi (1971) that in the case of some halophilic proteins, charge-dependent effects prevail despite the presence of overwhelming concentrations of counterions in the surrounding solvent. The possibility of electrostatic screening in a protein will depend on its permeability to the solvent. If the solvent is permeable, electrostatic interactions are shielded above 0.2 M, whereas if the solvent is impermeable they are not shielded even at higher salt concentrations. In concentrated salt solutions, the volume of the protein decreases, which will also result in decreased charge screening (Lanyi, 1971). The exhibition of narrow pH optima for activity in high salt concentrations by aspartate transcarbamylase (Liebl et al., 1969) and polynucleotide phosphorylase (Peterkin & Fitt, 1971) are examples of the prevalence of charge-dependent effects in these enzymes at high salt concentrations (Lanyi, 1971).

Additional information on *h*aldolase conformation was obtained in the presence of DHAP and phosphate ions, that are known to affect rabbit muscle aldolase. Rabbit muscle aldolase binds to substrates, viz., DHAP, GAP, and FDP, and substrate analogs, erythrose 4-phosphate and P_i (Rose & Connell, 1969). These compounds quenched enzyme fluorescence and gave protection against the action of trypsin, suggesting that the interactions were at specific sites of the enzyme (Rose & Connell, 1969). Low concentrations of DHAP had a similar effect as millimolar phosphate ions on *h*aldolase fluorescence with no appreciable change in enzyme conformation. However, at concentrations above 1 mM DHAP, the red shift in the emission maximum indicated that binding of the substrate to *h*aldolase resulted in the exposure of tryptophyls to the solvent. The higher values of fluorescence obtained suggested a conformational change in *h*aldolase, and the affected tryptophyls could be near or away from the active site. In the case of rabbit muscle aldolase, binding of arabinito 1,5-diphosphate was shown to cause a similar exposure of tryptophyls to the solvent (Gowder et al., 1973).

The hyperbolic nature of the activation of *h*aldolase by the addition of arsenate, pyrophosphate, and phosphate (Figure 8) indicates that it is not an allosteric effect, nor did the addition of these anions result in changes in the responses of the enzyme to KCl. According to the fluorescence data (Figure 7), these anions did not alter the buried and exposed tryptophyls; hence, the overall enzyme conformation apparently remained unaltered. Therefore, the anion activation of the enzyme may be due to local residue interactions or minor conformational changes, restricted to the enzyme active site. In certain halophilic enzymes, however, specific residue interactions were shown to result in changes in the overall structure of the enzyme molecule (Lieberman & Lanyi, 1972).

The chaotropic ions I^-, ClO_4^-, Br^-, NO_3^-, and SCN^- did not affect enzyme activity. In the case of nonhalophilic proteins, chaotropic ions are known to cause protein unfolding as shown by fluorescence studies (Altekar, 1977a). *h*Aldolase is susceptible to Gdn·HCl above 100 mM concentrations, though Gdn·HCl in moderate concentrations was shown to activate *h*MDH (Hecht & Jaenicke, 1989).

The stability of *h*aldolase and *h*GAPDH in a given set of conditions was estimated after 24-h incubation in these conditions by measuring the residual activity of the enzymes in the standard buffer. Fluorescence measurements indicated that the loss of enzyme activity was correlated to enzyme unfolding. Since the activity measurements were carried out at higher salt concentrations, the stability (or residual activity) measurements were essentially the measurements of the reversible or irreversible rate of unfolding of the proteins in the given conditions. The nature of the forces which act as barriers to the forces that disrupt the protein structure could be understood from the data shown in Figures 9 and 10. The data show that forces that stabilize the structure of *h*GAPDH in KCl and potassium phosphate are different. Since similar results were obtained in the case of the stability of *h*MDH in KCl and potassium phosphate, our results are comparable to the protein-hydrated model proposed by Zaccai et al. (1989) and Zaccai and Eisenberg (1990).

In the presence of KCl, *h*GAPDH stability continued to increase linearly with decreasing temperature, similar to the behavior of *h*MDH in different salts (Zaccai et al., 1989). From measurements of the activity and stability of the enzyme in KCl (weakly "salting out"), $MgCl_2$ ("salting in"), and potassium phosphate ("salting out") neutron-scattering and quasi-elastic light-scattering experiments, Zaccai et al. suggested that *h*MDH formed a well-structured network involving ions and the main stabilization mechanism was due to the formation of hydrate bonds between the protein and hydrated salt ions. Specific amounts of ions and water bound to the enzyme were lost upon unfolding of the molecule (Zaccai et al., 1986a,b). The enriched acidic amino acid content of *h*MDH suggested a possible mechanism, i.e., participation of carboxyl side chains of the acidic residues in the formation of the hydration network. From our data presented here and unpublished data with Zaccai's group, it appears that a protein-hydrated salt stabilization model is applicable for *h*GAPDH in KCl solutions. It has been shown that there is a large favorable enthalpic contribution to the free energy for the subunit assembly of yeast GAPDH (Ruan & Weber, 1989). This supports the hypothesis that the stability of *h*GAPDH, in KCl, may involve subunit assembly. The stability of *h*GAPDH in potassium phosphate does not show a linear dependence on temperature, but reaches the saturation limit upon lowering the temperature which is suggestive of a "salting-out" type of mechanism that occurs with nonhalophilic proteins (Privalov, 1979). Therefore, in potassium phosphate, *h*GAPDH appears to be stabilized due to hydrophobic interactions, as in the case of *h*MDH or nonhalophilic proteins (Zaccai et al., 1989).

Qualitatively similar results were obtained for *h*aldolase stabilization in potassium phosphate. Thus, the saturation limit of the stability of *h*aldolase reached upon lowering the temperature is suggestive of a "salting-out" type of mechanism discussed for *h*MDH and *h*GAPDH as well as for nonhalophilic proteins (Privalov, 1979).

The stability of *h*aldolase in KCl, in contrast to *h*GAPDH (and *h*MDH), did not show a linear dependence on temperature, but reached saturation limits at lower temperatures, the results being qualitatively similar to those obtained for the enzyme in potassium phosphate. This indicated that *h*aldolase may be stabilized in KCl by the "salting-out" type mechanism, since KCl is a weakly "salting-out" type of salt.

We have previously reported that *h*GAPDH has a higher content of acidic amino acids. Thus, being negatively charged, it also expresses faster migration rates on gels in the presence of anionic detergents (Krishnan & Altekar, 1990). Our earlier work has shown that *h*aldolase, unlike many halophilic proteins, is not a particularly acidic protein. It migrates equally well on gels in the presence of anionic and cationic detergents and is comprised of 10 subunits of 27 kDa each (Krishnan & Altekar, 1991). It was suggested that the lack of an excess of acidic amino acid residues in the case of this decameric halophilic protein would result in less interactions of its subunits with the solvent as compared to the acidic *h*GAPDH. From the time-dependent inactivation studies of the two enzymes in 0.4 M KCl, it was apparent that the two enzymes were inactivated at different rates, that of *h*GAPDH being comparatively slow. Also, the monitoring of fluorescence changes of the two proteins as a function of salt and pH indicates that *h*GAPDH denaturation occurs more slowly and gradually as compared to *h*aldolase. This would be somewhat expected, if *h*GAPDH fits the protein-hydrated model, and formed a network with water and ions. The stabilization forces important for *h*aldolase stability in KCl solutions would be the hydrophobic interactions to hold its 10 subunits together. This is supported by its somewhat higher content of borderline hydrophobic amino acids. It would appear that the acidic groups in the protein have a less significant role in the maintenance of *h*aldolase structure at higher KCl concentrations. Nevertheless, the halophilic nature of the enzyme suggests that certain areas of the protein would share the structural characteristics of halophilic proteins.

REFERENCES

Altekar, W. (1975) *Ind. J. Biochem. Biophys. 12*, 397–398.

Altekar, W. (1977a) *Biopolymers 16*, 341–368.

Altekar, W. (1977b) *Biopolymers 16*, 369–386.

Altekar, W., Paul, P., & Nadkarni, G. B. (1977) *Biochim. Biophys. Acta 495*, 203–211.

Baxter, R. M. (1959) *Can. J. Microbiol. 2*, 599–606.

Bencze, W. L., & Schmid, K. L. (1957) *Anal. Chem. 29*, 1193–1196.

Chen, R. F. (1967) *Fluorescence: Theory, Instrumentation and Practice*, (Guilbolt, G. G., Ed.) pp 433–443, Marcel Dekker, New York.

Christian, J. H. B., & Waltho, J. A. (1962) *Biochim. Biophys. Acta 65*, 502–506.

Colona, G., Irace, G., Parlato, G., Aloj, S. M., & Valestrieri, S. M. (1978) *Biochim. Biophys. Acta 532*, 354–367.

Das, M. K., & Vithayathil, P. K. (1978) *Biochim. Biophys. Acta 533*, 43–50.

Ebel, C., Krishnan, G., Altekar, W., & Zaccai, G. (1991) *Physica B 174*, 306–308.

Ebel, C., Guinet, F., Langowski, J., Urbanke, C., Gagnon, J., & Zaccai, G. (1992) *J. Mol. Biol. 223*, 361–371.

Edelhoch, H., Brand, L., & Wilcheck, M. (1967) *Biochemistry 6*, 547–559.

Eftink, M. R., & Ghiron, G. A. (1976) *J. Phys. Chem. 80*, 486–493.

Eisenberg, H., & Wachtel, E. (1987) *Annu. Rev. Biophys. Chem. 16*, 69.

Ginsburg, J., Sachs, L., & Ginsburg, B. Z. (1970) *J. Gen. Physiol. 55*, 187–207.

Gochin, M., & Degani, M. (1985) *J. Inorg. Biochem. 25*, 151–162.

Gowder, A. L., Barker, R., & Swenson, C. A. (1973) *Biochemistry 12*, 2078–2083.

Guinet, F., Frank, R., & Leberman, R. (1988) *Eur. J. Biochem. 172*, 687.

Hecht, K., & Jaenicke, R. (1989) *Biochemistry 28*, 4979–4985.

Holmes, P. K., & Halvarson, H. O. (1965) *J. Bacteriol. 90*, 312–315.

Hubbard, J. S., & Miller, A. B. (1969) *J. Bacteriol. 99*, 161–168.

Hubbard, J. S., & Miller, A. B. (1970) *J. Bacteriol. 102*, 677–681.

Krishnan, G. (1990) Ph.D. Thesis, Bombay University.

Krishnan, G., & Altekar, W. (1990) *J. Gen. Appl. Microbiol. 36*, 19–32.

Krishnan, G., & Altekar, W. (1991) *Eur. J. Biochem. 195*, 343–350.

Kronman, M. J., & Holmes, C. G. (1971) *Photochem. Photobiol. 14*, 113–134.

Lanyi, J. K. (1974) *Bacteriol. Rev. 38*, 272–290.

Lanyi, J. K., & Stevenson, J. (1970) *J. Biol. Chem. 245*, 4074–4080.

Lehrer, S. S. (1971) *Biochemistry 10*, 3254–3263.

Leicht, W., Weber, M. M., & Eisenberg, H. (1978) *Biochemistry 17*, 4004–4010.

Lieberman, M. M., & Lanyi, J. K. (1971) *Biochim. Biophys. Acta 245*, 21–33.

Lieberman, M. M., & Lanyi, J. K. (1972) *Biochemistry 11*, 211–216.

Liebl, V., Kaplan, J. G., & Kushner, D. J. (1969) *Can. J. Biochem. 47*, 1095–1097.

Mevarech, M., Eisenberg, H., & Neuman, E. (1977) *Biochemistry 16*, 2781–2785.

Pelley, R., & Horowitz, P. (1976) *Biochim. Biophys. Acta 427*, 359–363.

Peterkin, P. I., & Fitt, P. S. (1971) *Biochem. J. 121*, 613–620.

Peterson, G. L. (1983) *Methods Enzymol. 91*, 95–105.

Privalov, P. L. (1979) *Adv. Protein Chem. 33*, 73–165.

Pundak, S., & Eisenberg, H. (1981) *Eur. J. Biochem. 118*, 463–470.

Rao, J. K. M., & Argos, P. (1981) *Biochemistry 20*, 6536–6543.

Rose, I. A., & Connell, E. L. (1969) *J. Biol. Chem. 244*, 126–134.

Ruan, K., & Weber, G (1989) *Biochemistry 28*, 2144–2153.

Sibley, J. A., & Lehninger, A. L. (1949) *J. Biol. Chem. 117*, 859–878.

Stern, O., & Volmer, O. (1919) *Phys. Z. 20*, 183.

Tanford, C. (1988) *Adv. Protein Chem. 23*, 121–282.

Van Duuren, B. L. (1961) *J. Org. Chem. 26*, 2954–2960.

Weber, M. M., & Mevarech, M. (1978) *Arch. Biochem. Biophys. 187*, 447–456.

Zaccai, G., & Eisenberg, H. (1990) *Trends Biochem. Sci. (Pers. Ed.) 15*, 333–337.

Zaccai, G., Bunicle, G. J., & Eisenberg, H. (1986a) *J. Mol. Biol. 192*, 155–157.

Zaccai, G., Watchel, E., & Eisenberg, H. (1986b) *J. Mol. Biol. 190*, 97–106.

Zaccai, G., Cendrin, F., Haik, Y., Borochov, N., & Eisenberg, H. (1989) *J. Mol. Biol. 208*, 491–500.