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Solution structure of glyceraldehyde-3-phosphate dehydrogenase from *Haloarcula vallismortis*

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Abstract

The subunit molecular mass of glyceraldehyde-3-phosphate dehydrogenase from the extreme halophile *Haloarcula vallismortis* (hGAPDH) was determined by mass spectrometry to be 35990 ± 80 daltons, similar to other GAPDHs. Complementary density, sedimentation and light scattering experiments showed the protein to be a tetramer that binds 0.18 ± 0.10 gram of water and 0.07 ± 0.02 gram of KCl per gram of protein, in multimolar KCl solutions. At low salt (below 1 M), the tetramer dissociated into unfolded monomers. This is the third halophilic protein for which solvent interactions were measured. The extent of these interactions depends on the protein, but all form an invariant particle, in multimolar NaCl or KCl solutions, that binds a high proportion of salt when compared to non-halophilic proteins.

Keywords: Halophilic protein; Glyceraldehyde-3-phosphate dehydrogenase; Solution structure; Solvent interaction; Molar mass determination

1. Introduction

Living organisms have been divided into three evolutionary kingdoms, the eubacteria, the *archae* and the eukaryotes [1]. The *archae* live in media of extreme conditions of temperature, acidity or salinity [2]. In this group, extreme halophiles require a very high salt concentration, mainly sodium chloride, for growth. But they accumulate in their cytoplasm an equally high concentration of potassium chloride as osmolyte. All their intracellular mechanisms take place in this medium, which would strongly inhibit most biochemical reactions in other organisms. Proteins from extreme halobacteria are studied not only for a better understanding of evolutionary adaptation to extreme media at the molecular level, but also because they provide models for the study of effects of the environment on protein folding, since they are inactivated and unfolded in a medium where non-halophilic proteins are usually active and stable [3,4].

Typically, halophilic proteins unfold in salt concentrations under 2 M KCl [3,4]. Their amino-acid analysis usually shows a high content of acidic

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residues ($\approx 20\%$) and, more precisely, a high ratio of acidic-to-basic residues. For halophilic proteins, whose primary sequences have been determined, the molecular weight is similar to that of non-halophilic analogs, while it appears to be significantly larger by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE). From solution studies, halophilic malate dehydrogenase (hMDH) [5] and halophilic elongation factor Tu (hEF–Tu) [6] bind rather large amounts of salt: 0.1–0.2 gram of salt per gram of protein in multimolar KCl or NaCl solutions. A stabilization model has been proposed for halophilic proteins by Zaccai et al. [7] in which a network of hydrated salt ions bound to the protein contributes to maintaining its tertiary structure in certain solvents. From homology modeling on dihydrofolate reductase from *Halobacterium volcanii*, Böhm and Jaenicke [8] suggested that clusters of negatively charged amino acids causing a high local charge density could be responsible for the unfolding of the protein at low salt. The same authors [9] suggested that for this protein, the increase in negative amino acids is of minor importance. However, we found for hEF–Tu and hMDH by the same treatment a significant increase in these amino acids (statistical significance above three units of standard deviation).

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a key enzyme of glycolysis, has been well studied from a variety of sources [10–14]. It has been purified from *Haloarcula vallismortis*, characterized enzymatically and shown to be a halophilic protein by Krishnan and Altekar [15]. In contrast to non-halophilic *archae*, only a single NAD^+-specific GAPDH was found in the halobacterium. Recently, Prüß et al. [16] have confirmed most of the first results on a different strain of *H. vallismortis*. Though the amide content per se was not determined by either workers, the acidic nature of the protein is apparent from the amino-acid analysis and the isoelectric point of 4.25. The relative mobilities of the subunit in SDS and cetyltrimethylammonium bromide (CTAB) gave different values for the molecular weight, i.e. 52 ± 2 kD and 40 ± 2 kD, respectively. The relative molecular weight of the native protein found by gel filtration calibrated by non-halophilic proteins in 3.0 M KCl was 190 kD suggesting a tetramer. The corresponding values reported by Prüß et al. [16] were 70 kD and 38 kD in anionic and cationic detergents, respectively, while a value of 160 kD was found for the molecular weight of the enzyme. The enzyme is not stable at low salt: it loses almost all activity in 72 hours in 1 M KCl or in 12 hours in 0.5 M KCl. By using fluorescence techniques, Krishnan and Altekar [17] further showed clearly that the halophilic protein unfolded in dilute salt solutions. The spectral characteristics of the fluorescence spectra in 0.4 M KCl and 6 M guanidinium hydrochloride were very similar. Thermostability of the protein was investigated through the measurement of residual activity after 24 hours of incubation in a given solvent at a given temperature [17]. The behaviour in KCl solutions (weakly salting-out [18]) and in potassium phosphate solutions (strongly salting-out [18]) is quite different: in the first, stability increases with decreasing temperature, while in the second, it reaches a limiting value. Similar results were obtained on halophilic malate dehydrogenase [7]. They have been interpreted in terms of different stabilising mechanisms for the two salt solutions: one, in the phosphate solutions, dominated by an entropic contribution (hydrophobic interactions) and the other, in KCl, by an enthalpic one, which could be due to the network of hydrated ions bound to the protein. In small angle neutron or X-ray scattering experiments on dilute macromolecular solutions, the forward scattered intensity is related to the molar mass of the particle [19]. Neutron scattering experiments on hGAPDH showed, at low salt, a strong decrease in forward scattered intensity, indicating dissociation of the protein [20]. A concomitant increase in radius of gyration, a parameter related to the spatial extent of the particle in solution [21] indicated unfolding of the subunits.

At this stage of the work, it became necessary to characterise the solvent interactions of the halophilic protein, which, as mentioned above, are correlated with the mode of stabilisation of the folded structure. This information can be obtained through the determination of density increments, by very precise measurements of density, ultracentrifugation, light scattering, X-ray or neutron scattering [22,23]. However, the interpretation of these data requires a precise knowledge of the molar mass of the protein in solution and (or) of its absolute concentration [5]. In the present paper, the uncertainties encountered in

earlier work regarding the determination of molar mass of the hGAPDH subunit have been overcome by using mass spectrometry. The number of subunits in the native protein at high salt and its solvent interactions were obtained by density measurements coupled to sedimentation and light scattering experiments. The solvent interactions of this halophilic protein are compared with those of halophilic elongation factor Tu (hEF–Tu), halophilic malate dehydrogenase (hMDH) and non-halophilic bovine serum albumin (BSA).

2. Materials and methods

2.1. Sample preparation

The protein was purified according to Krishnan and Altekar [15], and stored at 4°C in a high concentration of KCl (> 3.5 M), 50 mM Tris, 50 mM phosphate, pH 8. The experiments were performed, unless specified, in 50 mM Tris–HCl, pH 8. The protein was extensively dialysed against high KCl (above 3 M) and diluted with KCl solutions in order to obtain the desired KCl concentrations.

2.2. Mass spectrometry

Mass spectrometry of the hGAPDH subunit was carried out on a Perkin-Elmer Sciex API III triple quadrupole mass spectrometer equipped with a nebulizer-assisted electrospray (ionspray) source. The instrument was calibrated using a polypropylene glycol standard solution. The spectra were recorded in the 700–1500 range of mass-to-charge (m/z) ratios in steps of 0.2 m/z. The ionspray probe tip voltage was held at 5 kV. After extensive dialysis against water and freeze–drying, the sample was dissolved at a concentration of 10 mg/ml in 50% acetonitrile, 50% water, 2 mM ammonium acetate and ammonium hydroxide to adjust its pH to 8.5. It was infused into the source using a Harvard 22 syringe pump at a flow-rate of 2 μl/min. Charge states of the observed ions and molecular mass of the hGAPDH subunit were obtained using the MacSpec software (Perkin-Elmer Sciex).

2.3. Concentration determination by amino-acid analysis

The quantitative amino-acid analysis of hGAPDH samples of known absorbance in the UV, in either 3.8 or 0.75 M KCl, was performed following acid hydrolysis in 6 M HCl containing 0.1% (v/v) phenol, for 24 hours at 110°C, on a Beckman model 6300 analyzer. From the amount of amino acid, neglecting undetected Cys and Trp, the initial concentration of protein was estimated. From the slope of the plot of the absorption against the calculated protein concentration, the extinction coefficient $E_{0.1\%}$ was calculated for both the native (at 277 nm in KCl 3.8 M) and denaturated (at 275 nm in KCl 0.75 M) to be 0.85 ($\pm$0.03) cm^2/mg.

2.4. Density measurements

A dialysis bag of 300 μl of protein solution and two dialysis bags of solvent were dialysed twice, each time against 400 ml of solvent for one day in a rocking bath, in a cold room (4°C). Samples were taken directly from each bag without removing them from the bath by using a syringe, in order to minimize evaporation. The density measurements of the solutions were performed on a PAAR DMA 60 densimeter equipped with 100 μl cells, at a controlled temperature of 20 $\pm$ 0.01°C. It was verified that the two control bags gave identical values for solvent density. The protein concentrations, estimated by UV absorption, were 2.4, 4.3 and 1.5 mg/ml for KCl concentrations of 3.5, 3.0 and 2.5 M, respectively. Mass density increments at constant chemical potential of diffusible components, $(\partial\rho/\partial c_2)_\mu$ were obtained directly from the difference between the density of the protein solution r and the density of the solvent ρ^0:

$$(\partial\rho/\partial c_2)_\mu = (\rho - \rho^0)/c_2 \quad (1)$$

2.5. Sedimentation coefficients

Sedimentation velocity experiments were performed in a Beckman Spinco model E analytical ultracentrifuge at 60000 or 52000 rpm. Concentration profiles were analyzed at 280 nm with sample optical densities between 0.3 and 0.5. Data were

recorded with a DiSys Analog/Digital converter and a PC under MS/DOS. Sedimentation coefficients s were evaluated using second moment analysis of the individual concentration profiles to determine the band positions.

2.6. Dynamic light-scattering experiments

The protocol for the dynamic light scattering experiments was as described in detail by Ebel et al. [6]. The light source was a 488 nm argon laser at a typical output power of 0.5 W. The sample concentration was about 0.5 OD_{280} units/cm. Dynamic light scattering data were collected over an angular range of 25° to 140°, and the data treated by a multiexponential fitting procedure that fits directly the second-order autocorrelation function $G^{(2)}(\tau)$. The data could be treated in most cases by assuming only two components, a major one being the protein and a smaller, faster relaxation corresponding to the diffusion of salt. The measured diffusion coefficient of the protein was independent of scattering angle. The values of D reported are the result of a fitting procedure that treats the data at all angles simultaneously, as already described in [6]. The typical error in D was 2% or less. At the lower salt KCl concentrations, we had to introduce a third slow component in the fit that accounted for higher molecular weight aggregates. The scattered light intensity corresponding to this component was always less than 10% at higher angles (50° or above). The Einstein relationship is given by:

$$D = kT/f \tag{2}$$

where D is the diffusion coefficient, k is the Bolzmann constant, T is absolute temperature, and f is the frictional coefficient. The Stokes relationship is given by:

$$f = 6\pi\eta R_H \tag{3}$$

where η is the viscosity of the medium and R_H is the hydrodynamic radius, i.e. the radius of a spherical particle with the same diffusion coefficient as the particle under study.

An experimental diffusion coefficient ($D_{T,sol}$) was calculated from the data. The Einstein and Stokes relations allowed appropriate corrections to be made for viscosity and temperature. Thus the hypothetical diffusion coefficient of an identical particle in water at 20°C, ($D_{20,w}$), was calculated from $D_{T,sol}$.

2.7. Calculation of mass density increments and their interpretation in terms of solvent interactions [19]

The sedimentation coefficients are related to the molar mass of the species in solution M_2, to its frictional coefficient f, and to its mass increment density at constant chemical potential of diffusible components, $(\partial\rho/\partial c_2)_\mu$:

$$s = M_2(\partial\rho/\partial c_2)_\mu/N_A f \tag{4}$$

(N_A is Avogadro's number).

$$(\partial\rho/\partial c_2)_\mu = RTs/DM_2 \tag{5}$$

In a thermodynamic analysis for a three-component solution, the mass density increments can be expressed as a function of a solvent interaction parameter ξ_1, expressed in gram of water per gram of protein:

$$(\partial\rho/\partial c_2)_\mu = (1 - \rho^0\bar{v}_2) + \xi_1(1 - \rho^0\bar{v}_1) \tag{6}$$

ρ^0 is the solvent density (g/cm^3), $\bar{v}_1$ and $\bar{v}_2$ (cm^3/g) are the partial specific volumes of water and protein respectively. In a model in which one gram of protein binds B_1 gram of water and B_3 gram of salt:

$$\xi_1 = B_1 - B_3/w_3 \tag{7}$$

$$(\partial\rho/\partial c_2)_\mu = 1 + B_1 + B_3 + \rho^0(\bar{v}_2 + B_1\bar{v}_1 + B_3\bar{v}_3) \tag{8}$$

where w_3 is the salt-to-water ratio (in gram salt/gram water) in the solvent, and $\bar{v}_3$ the partial specific volume of salt.

The values taken for $\bar{v}_1$ and $\bar{v}_3$ are respectively 1.000 and 0.414 cm^3/g (from the density of the salt solution [24]).

3. Results and discussion

3.1. Determination of the molecular mass of the subunit of HGAPDH by mass spectrometry

The subunit of hGAPDH, like most of the other halophilic proteins studied shows a high content of acidic residues. The high ratio of acidic on basic

residues explains our first choice of trying to analyze this protein in negative-ion mode in a basic solvent. No significant peak was obtained in these conditions. Conversely, a good response was obtained in positive-ion mode with the same solvent. The hGAPDH subunit ESI mass spectrum is presented in Fig. 1. Using the MacSpec software, 22 adjacent peaks were labelled and their charges were then automatically calculated. A coherent series of multiply-charged ions, related to molecules bearing from 21 to 44 protons was clearly observed with dotted bars displayed on the spectrum. These bars indicate the predicted position for the ion series. Each labelled peak generated an estimate of the molecular mass of the protein and a mean value was obtained: 35990 ± 80 daltons. Each peak is rather broad. This is due to the strong ionic interactions of potassium ions which were not completely eliminated in the dialysis and which led to a rather poor accuracy in the mass measurement.

Positive ions produced by electrospray ionization from proteins in solution are usually obtained by proton binding to basic sites (N-terminus, arginines, lysines or histidines) [25,26]. The number of basic sites on the hGAPDH subunit was estimated from its amino-acid composition to be 27 [16] or 36 [15]. These estimates are lower than the maximum number of charges observed in the mass spectrum (44). This high number of charges could be explained by protonation of other amino acids such as glutamines (through their amide function), as observed by Moore et al. [27]. Other authors [28] suggest that additional charges might be due to gas-phase higher reactivities of other sites such as amide bonds.

Considering the previous uncertainty in the value of the subunit molecular mass of hGAPDH obtained through empirical approaches, the accuracy of the measurement (still greater than 1%) shows the power of mass spectrometry for the determination of the molar mass of halophilic polypeptide chains of unknown primary sequence.

3.2. Molar mass of the native enzyme by densimetry, ultracentrifugation, and light scattering

The analytical centrifugation and light scattering experiments allowed us to define a range of KCl concentrations, between 3.8 and 2 M KCl, where the hGAPDH could be studied in its native form: sedimentation velocity experiments performed in high KCl show one boundary, suggesting a homogeneous material above 2 M KCl; the diffusion coefficients

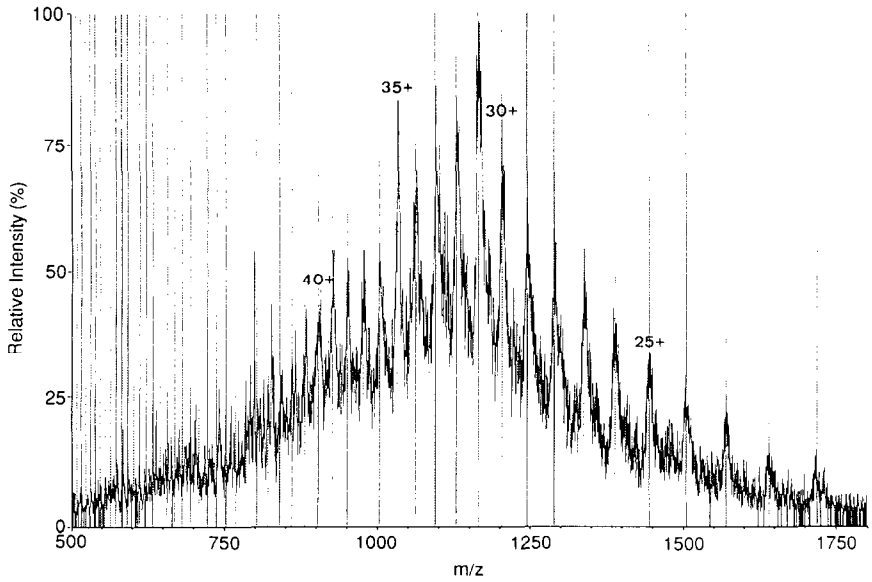

Fig. 1. Electrospray mass spectrum of the subunit of hGAPDH. The net charge of some of the ions is indicated above the peaks which correspond to multiprotonated forms of the subunit of hGAPDH.

$D_{20,w}$ can be considered as constant for KCl above 1.8 M. The molar mass of the native enzyme was determined without any assumption (Eq. 5) by using the combined values of $(\partial\rho/\partial c_2)_\mu$, s and D, obtained from densimetry, ultracentrifugation and light scattering, respectively, at 3.5, 3.0 and 2.5 M KCl (Table 1). The absolute concentration of the protein, which is needed for the determination of $(\partial\rho/\partial c_2)_\mu$ from densimetry experiments was determined by UV absorption with the value of 0.85 cm^2/mg for the extinction coefficient, determined by amino-acid analysis. We calculated a molar mass M_2 of the native enzyme similar for the three salt concentrations (Table 1) of 140000 ± 17000 g/mol. This corresponds to a tetramer (144000 g/mol calculated from the mass spectrometry subunit determination).

The molar mass of hGAPDH is thus very similar to that of non-halophilic GAPDHs [29]. Since the molar mass found previously for hGAPDH by gel filtration with non-halophilic markers was significantly larger, we performed a gel filtration experiment in high salt with two halophilic markers, hEF–Tu and hMDH, whose molar masses were obtained from the amino-acid sequences (respectively 45609 [30] and 130552, this last value corresponding to a tetramer [31,5]). The column (Superose 12 HR 10–30 supplied from Pharmacia) was eluted by KCl 3.3 M, Tris–HCl 50 mM, pH 7.6 with a flow-rate of 0.4 ml/min. Despite this approach to be empirical, the elution volumes (hEF–Tu: 13.0 ml; hMDH: 11.2 ml; hGAPDH: 11.1 ml) were consistent with a molar mass for hGAPDH of 144000 g/mol.

3.3. Evidence for dissociation of the enzyme at low salt by analytical ultracentrifugation and light scattering

The dissociation of the protein in low salt is clearly shown: in 1 M KCl, a second boundary is seen in the sedimentation profiles, corresponding to a smaller species. Very similar observations were made on hMDH [32]. The existence of a well defined sedimentation boundary indicated that the rate of association of the monomers is slow compared to the measurement time. The relative amount of dissociated protein increased with time after lowering the salt concentration in the solvent to 1 M KCl by dilution. It was 36%, 49% and 77% a few minutes after dilution, 4 hours and 21 hours later, respectively. Light scattering experiments in 1 M KCl also

Table 1
Sedimentation coefficients, diffusion coefficients, mass density increments and interaction parameters for hGAPDH in KCl solutions

KCl (mol/l)	ρ^0 (g/cm^3)	$1/w_3$ (g/g)	s (22.3°C) (S) ($\pm$0.1)	$D_{22.3,sol}$ (10^{-11} m^2 s^{-1}) ($\pm$0.1)	$D_{20,w}$ (10^{-11} m^2 s^{-1}) ($\pm$0.1)	$(\partial\rho/\partial c_2)_\mu$ measured by densimetry ($\pm$0.03)	M_2 (kg mol^{-1}) ($\pm$30)	$(\partial\rho/\partial c_2)_\mu$ calculated from s and D ($\pm$0.007)	ξ_1 (g/g)
3.8	1.164	3.12	4.58	4.82	4.70			0.162	-0.139 ± 0.04
3.5	1.151	3.42	4.46			0.16	145	0.157	-0.053 ± 0.05
3.0	1.129	4.05	5.11	4.99	4.63	0.20	130	0.178	-0.097 ± 0.05
2.5	1.109	4.95	5.71			0.21	140	0.199	-0.165 ± 0.06
2.0	1.087	6.29	6.21	4.92	4.57			0.216	-0.220 ± 0.08
1.8				4.97	4.60				
1.0	1.043	13.0	7.54 [b]	5.20	4.82			0.260 [e]	-0.707 ± 0.23
1.0 [a]			*1.95* [c]						
0.75				5.35 [d]	5.00				

[a] The line in *italics* corresponds to the dissociated species.
[b] $\pm$0.2 S.
[c] $\pm$0.4 S.
[d] The diffusion coefficient measured at 20.2°C was corrected to 22.3°C for insertion in Table 1.
[e] $\pm$0.01.
The sedimentation coefficients s measured at temperature in the range 22.3–24.7°C have been reduced to 22.3°C and averaged at each salt concentration. M_2 is the experimental molar mass of the protein in solution obtained from the combination of the experimental values of s, D, and $(\partial\rho/\partial c_2)_\mu$ (measured by densimetry). The two last columns, $(\partial\rho/\partial c_2)_\mu$ and ξ_1, result from calculations involving $M_2 = 144000$ g/mol (see the text).

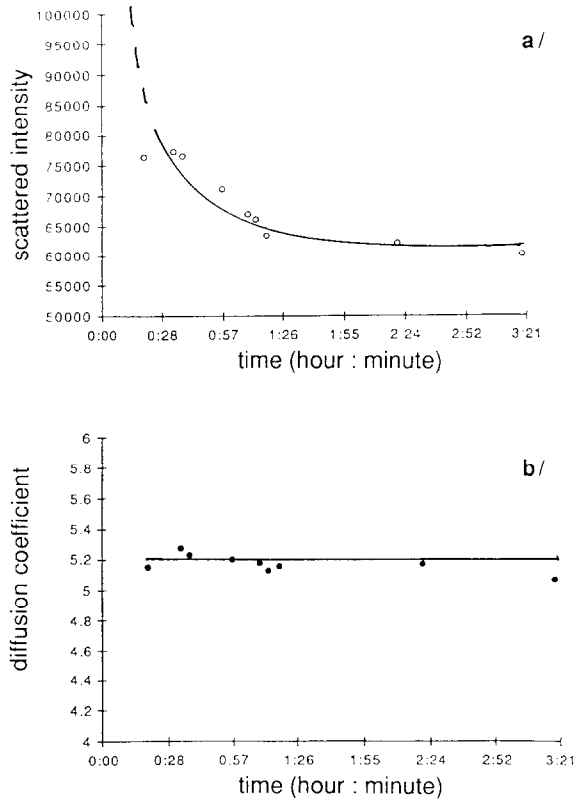

Fig. 2. Light scattering in 1 M KCl. (a) Relative scattered intensity (arbitrary unit); (b) diffusion coefficient (10^{-11} m^2 s^{-1}), both measured at 90° scattering angle as a function of time (hour:minute).

indicated a dissociation of the native protein (Fig. 2): the scattered intensity at 90° scattering angle decreases significantly over a time course of several hours when the protein is diluted into 1 M KCl. Since for a particle with dimensions much smaller than the reciprocal scattering vector (as in this case) the 90° scattering intensity at constant weight concentration is proportional to the molecular weight, this result is consistent with a dissociation. At the same time the dissociated monomer is unfolded with respect to its associated state, since the diffusion coefficient does not change during the dissociation (Fig. 2). The value of the diffusion coefficient in 0.75 M KCl was measured after three days of incubation in this buffer, in order to characterise the fully dissociated and unfolded state. As can be seen on Table 1, at low salt (1 and 0.75 M), $D_{20,w}$ increases significantly (the hydrodynamic radius is decreased) indicating a structural change in the protein. The moderate increase of the $D_{20,w}$ coefficient and the strong decrease of the sedimentation coefficient during dissociation are arguments in favor of the unfolding of the dissociated protein at low salt, previously suggested by neutron scattering [20], and in agreement with the fluorescence study [17]. From the sedimentation coefficient determined for the smaller species, and from the diffusion coefficient of the fully denatured protein, assuming a monomer, the mass density increment was estimated for the dissociated protein in 1 M KCl. A value for $(\partial\rho/\partial c_2)_\mu$ of 0.25 ± 0.03 was found, close to that of the tetramer (see below) but unfortunately not precise enough to give information on the state of solvation of the unfolded polypeptide chain.

3.4. Solvent interactions from density increments and interaction parameters

Information on solvent interactions can be obtained from density increments (Eqs. 6 to 8). These can be obtained directly from density measurements (Eq. 1), or via hydrodynamic measurements, if the molar mass of the protein in solution is known (Eq. 5). The values of $(\partial\rho/\partial c_2)_\mu$ from densimetry were not used for the determination of the solvent interactions of hGAPDH since it is difficult to obtain accurate values with limited amounts of protein (Table 1). However, since it was shown that the protein is a tetramer in its native state, mass density increments were calculated from the experimental diffusion and sedimentation coefficients and the Svedberg equation, by assuming a molar mass of 144000 g/mol. This was clearly valid for salt concentrations between 3.8 and 2 M KCl where the protein is fully in its native state. In 1 M KCl, however, the protein was partially dissociated since the sedimentation profiles showed two boundaries. We could nevertheless extract a value for the sedimentation coefficient of the tetramer alone from its sedimentation boundary. The diffusion coefficient of the tetramer in this condition was calculated from $D_{20,w}$, since this value was seen to be reasonably constant for the native protein and it increased only slightly when the protein dissociated at low salt.

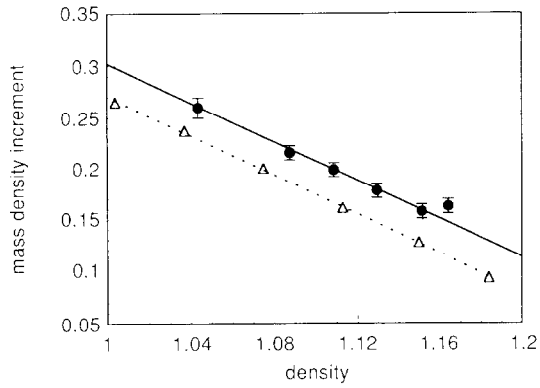

Fig. 3. Mass density increments of hGAPDH in KCl as a function of mass density of the solvent. (●) hGAPDH in KCl; the straight line corresponds to a model of an invariant particle with $B_1 = 0.18$ gram of water per gram of protein and $B_3 = 0.07$ gram of salt per gram of protein. The dotted line corresponding to BSA in NaCl solutions [5] was plotted for comparison.

The partial specific volume $\bar{v}_2$ of hGAPDH estimated from its amino-acid composition is 0.739 cm^3/g [33]. The values of $(\partial\rho/\partial c_2)_\mu$ and ξ_1 (calculated from Eq. 6) are given in Table 1. Figs. 3 and 4 are the plots of $(\partial\rho/\partial c_2)_\mu$ versus ρ^0, the solvent density, and ξ_1 versus $1/w_3$, the salt-over-water ratio in the solvent. Since a reasonable straight line could be fitted to these two sets of data, we assumed a particle invariant in composition and volume with respect to solvent composition [34] for the

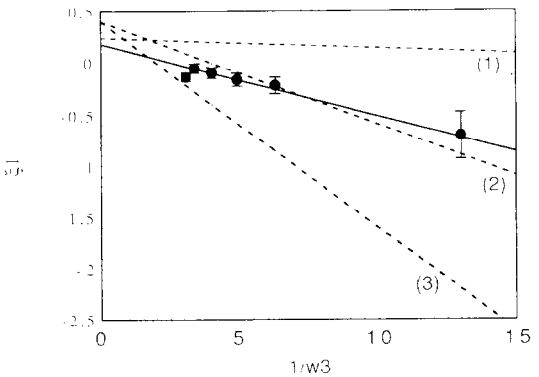

Fig. 4. Plot of the interaction parameter ξ_1 as a function of $1/w_3$ for hGAPDH in KCl. Values for hGAPDH (●) are from Table 1. The straight line corresponds to an invariant particle with $B_1 = 0.18$ gram water per gram of protein (intercept) and $B_3 = 0.07$ gram of salt per gram of protein (slope). The dotted lines correspond to BSA in NaCl (1), hMDH in KCl or NaCl (2) [5], and hEF-Tu in KCl (3) [6].

hGAPDH tetramer in solution between 1 M and 3.5 M KCl and calculated its composition (Eq. 7). $B_1 = 0.18 \pm 0.07$ gram of water bound per gram of protein and $B_3 = 0.07 \pm 0.02$ gram of salt bound per gram of protein. This result did not change significantly when $\bar{v}_2$ was allowed to vary from 0.73 to 0.75 cm^3/g (B_1 varies from 0.19 to 0.16 g/g and B_3 varies from 0.05 to 0.085 g/g).

The line corresponding to this invariant particle was plotted in Fig. 3 by using Eq. 8 and is in agreement with the experimental values of the density increments. The volume occupied by the solvated protein (protein plus bound water and salt per gram of protein) for hGAPDH is 0.94 ± 0.08 cm^3/g, similar to that of BSA in NaCl solutions [5], while the values found for the two previously studied halophilic proteins were larger: 1.16 cm^3/g in NaCl and KCl for hMDH [5], 1.2 cm^3/g in KCl for hEF-Tu [6]. The density increments for hGAPDH, however, are larger than those of BSA, indicating that solvated hGAPDH is significantly more dense than solvated BSA.

On Fig. 4 were plotted also the results obtained for BSA in NaCl, hMDH in NaCl or KCl and hEF-Tu in KCl. Clearly, the behaviour of hGAPDH is intermediate between that of BSA and hEF-Tu and closer to that of hMDH. We note that the value found for water binding by hGAPDH, $B_1 \approx 0.2$ g/g, is lower than for other halophilic proteins (≈ 0.4 g/g for hMDH or hEF-Tu) and similar to that of BSA: 0.2 g/g. On the same figure, however, an important qualitative difference is seen between the non-halophilic protein, which binds no or very little salt and the halophilic ones, for which there is a notable amount of salt binding. The B_3 values are 0.07 g/g for hGAPDH, 0.1 g/g for hMDH and 0.2 g/g for hEF-Tu. Even if the solvation of the three halophilic proteins is different, therefore, the ratio between salt and water is always high, between 0.25 and 0.5 corresponding to a very high salt concentration: for KCl, between 3.1 M and supersaturation.

In conclusion, from a precise value for the molecular mass of the hGAPDH subunit calculated by mass spectrometry, the active native protein was shown to be a tetramer in multimolar KCl solution, by complementary densimetry, ultracentrifugation and light scattering experiments. The protein is inactivated by dissociation and unfolding below 2 M

KCl. The knowledge of the native protein mass in solution allowed a determination of its mass density increments at various KCl concentrations from sedimentation and diffusion coefficients. The solvated tetramer could be considered as an invariant particle between 1 and 3.5 M KCl, with the protein binding 0.18 ± 0.07 gram of water and 0.07 ± 0.02 gram of KCl per gram. Comparing these values to those of two other previously studied halophilic proteins, hEF–Tu and hMDH, it appears that the solvation of halophilic proteins is not the same. Water binding and salt binding varies by a factor of about two, but in the three cases, salt binding is significantly larger than for non-halophilic proteins. The hydration of hGAPDH presented here combined with the thermostability measurements performed in different solvent conditions by Krishnan and Altekar [17] show strong similarities with measurements on hMDH [5,7].

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