Induction of a spectroscopically defined transition by guanidinium hydrochloride on a recombinant calcium binding protein from *Entamoeba histolytica*

B. Gopal[a], J.V. Krishna Rao[a], C.J. Thomas[a], A. Bhattacharya[b], S. Bhattacharya[c], M.R.N. Murthy[1,a], A. Surolia[1,a]

[a] *Molecular Biophysics Unit, Indian Institute of Science, Bangalore 560 012, India*
[b] *School of Life Sciences, Jawaharlal Nehru University, New Delhi 110 067, India*
[c] *School of Environmental Sciences, Jawaharlal Nehru University, New Delhi 110 067, India*

Received 2 November 1998

Abstract Sequence analysis and metal ion binding studies reported earlier have established that the calcium binding protein (CaBP) from the parasitic ameboid *Entamoeba histolytica* protein has four canonical EF hand motifs which bind calcium. Equilibrium denaturation studies on both the apo and the holo forms of this protein indicate the presence of stable transition intermediates at low denaturant concentrations as revealed by the binding of the non-specific hydrophobic dye ANS. Fast reaction kinetics shows that the binding of the Gdn^+ ions at or near the Ca^{2+} sites in the N-terminal domain influences metal ion binding to the sites in the C-terminal domain. Isothermal calorimetric titrations performed using low GdnHCl concentrations reveal the presence of two binding sites of low affinity, both being endothermic in nature. Thus the stabilization of CaBP observed at low GdnHCl concentration represents a native-like intermediate, with the Gdn^+ ions mimicking Ca^{2+} binding at the N-terminal domain of this protein.

Key words: Calcium binding protein; Unfolding, binding and stabilization by guanidinium ion

1. Introduction

The ion binding affinity, specificity, cooperativity and kinetics of the small, two-domain, dumbbell shaped calmodulins have been reasoned to be tuned to suit varied functional requirements [1]. Despite the low overall amino acid sequence identity of *Entamoeba histolytica* calcium binding protein (CaBP) to other calmodulins, four EF hand Ca^{2+} binding motifs are clearly discernible in this 134 residue peptide [2]. *E. histolytica* CaBP is a good model system for investigating the intrinsic structural determinants of protein folding in a metal ion binding system as it lacks disulfides, *cis*-prolyl peptide bonds or a multimeric structure, factors that could otherwise complicate the mechanism of folding. An interesting feature of this protein is its intrinsic stability [2]. It displays a dramatic change in conformation upon ion binding, a characteristic of members of the calmodulin superfamily in which large conformational changes are needed to bind to the target proteins, which are mostly kinases [2,3].

Our earlier work has shown that this protein has two low affinity Ca^{2+} specific sites, one high affinity Ca^{2+}-Mg^{2+} site and one high affinity Ca^{2+} specific site [2]. Furthermore, it was shown that the Ca^{2+}-Mg^{2+} site is located in the C-terminal domain and the N-terminal domain consists of two Ca^{2+} specific sites. It was also shown that the domain containing the lower affinity sites (N-terminal domain) is the first to unfold. A general feature of the calmodulins in solution is the mobile central linker observed as a flexible loop in NMR studies [4,5] as opposed to that in the crystal structure where it adopts a helical conformation. The putative central linker in CaBP is longer by a couple of residues and has three glycines, making it even more flexible as compared to the canonical calmodulins. Functionally, while the flexibility and increase in length could be reasoned to be tailor-made for interaction with an effector kinase from this organism, how it affects the interaction between the two domains in terms of the ion binding parameters constitutes the focus of these studies. This article reports the kinetics of metal ion binding and the changes in the dissociation rates in the presence of guanidinium hydrochloride (GdnHCl) by stopped flow kinetics and the conformational changes monitored using size exclusion chromatography and circular dichroism (CD) spectroscopy. In parallel, the binding of Gdn^+ ions at low denaturant concentrations was examined using isothermal titration calorimetry (ITC). This allowed us to dissect the roles played by the three major structural components, the N-terminal domain, the C-terminal domain and the central linker, in conferring upon this protein its observed thermostability.

2. Materials and methods

2.1. Materials

The procedure for the expression and purification of CaBP and the preparations of its solutions have been described in [2,6]. The concentrations of the GdnHCl stock solutions were determined as in [7]. The protein concentrations were determined by using its extinction coefficient ($\varepsilon^{280} = 5120\ M^{-1}\ cm^{-1}$). Ultrapure grade GdnHCl and analytical grade reagents were obtained from Gibco BRL and Sigma Chemical Co., respectively.

2.2. CD and fluorescence equilibrium measurements of GdnHCl induced denaturation

Equilibrium unfolding as a function of GdnHCl concentration was monitored by far as well as near UV CD on a Jasco J500A spectropolarimeter. Spectra were collected at a scan speed of 10 nm/min and a response time of 16 s. Each spectrum was an average of at least eight scans. Secondary structure was monitored at 222 nm with 16 μM of CaBP using a cuvette of path length 0.1 cm. Near UV CD measurements were recorded at 276 nm using a 0.5 cm path length cuvette at a protein concentration of 80 μM. The sample temperature was maintained using a circulating water bath. For GdnHCl denaturation experiments, protein samples were prepared such that the final concentrations of denaturant, protein and buffer were achieved and the pH

[1]Corresponding authors. Fax: (91) (80) 3348535 or (91) (80) 3341683. E-mail: surolia@mbu.iisc.ernet.in

0014-5793/98/$19.00
PII: S0014-5793(98)01513-0

was maintained. Data were collected after an appropriate equilibration period (2 h).

2.3. Kinetic measurements

Kinetic measurements of the apo and holo CaBP unfolding were performed using an Applied Photosystems SX18MV SpectraKinetic stopped flow fluorescence instrument. Experiments were performed in 20 mM MOPS buffer pH 7.0. To measure the rates of dissociation, holo CaBP was mixed with 3–5 mM EDTA to achieve the required final concentrations. Following mixing, tyrosine fluorescence was measured by excitation at 278 nm using a 302 nm cut-off filter. The data were fit as double exponentials using the proKinetist software as $A(t)=A(\propto)-A_1\exp(-\lambda_1 t)-A_2\exp(-\lambda_2 t)$ where λ_1 and λ_2 are the apparent rate constants of the slow and fast phases and A_1 and A_2 are the respective amplitudes.

2.4. Size exclusion chromatography

The protein (in either form) was equilibrated in 20 mM MOPS buffer, 0.1 M KCl pH 7.0 with 5 mM EGTA or 10 mM $CaCl_2$ for 2 h prior to the chromatography run on a Superose HR 10/30 column equilibrated with the same buffer using a Pharmacia FPLC system. Typically, 80 μM of CaBP in 100 μl was injected.

2.5. ITC measurements

The titration calorimetry measurements were performed with a Microcal Omega Titration Calorimeter and the data analyzed as described earlier [2,8–10]. A typical titration consisted of injecting 5 μl aliquots of 25–1000 mM GdnHCl solutions into 0.5–1.0 mM of the protein solution after every 3.5 min to ensure that the titration peak returned to the baseline prior to the next injection. Aliquots of GdnHCl solutions were injected into just the dialysate solutions in separate ITC runs in order to measure its heat of dilution. The dilution heats so obtained were subtracted from the titration profile. Each titration was repeated at least five times.

3. Results and discussion

An inverse relation exists between the binding affinity, binding cooperativity and stability of troponin C fragments [11]. The calcium binding affinity and cooperativity are highest for the unstructured C-terminal domain and lowest for the N-domain which has the highest stability. This led to the inference that the stability of the N-domain, the N-helix and the bilobed domain organization are necessary for the fine tuning of the affinity and cooperativity of calcium binding. The N-terminal domain of CaBP, which contains the low affinity sites, was shown by us to unfold first in the presence of low concentrations of the denaturant GdnHCl [2]. Moreover, the N-terminal domain was postulated to be more structured, a finding supported further by the low resolution crystal structure of this protein [12].

3.1. Kinetic measurements

The association/dissociation rates, for the metal ions as well as the rates of unfolding of the protein obtained by monitoring the fluorescence of the tyrosine residues in CaBP, are listed along with the ion binding affinities reported earlier (Table 1, Fig. 1). The association rates for Ca^{2+} for the set of two fast binding sites could not be obtained as the event occurs within the dead time of the instrument. The binding of Mg^{2+} could be characterized completely for both the association as well as the dissociation reactions. Competitive binding experiments carried out using a Mg^{2+} saturated protein helped to ascertain the association kinetics of the Ca-Mg site for Ca^{2+} (Fig. 1(ii)). This was possible as the off-rate for magnesium slowed down the binding on-rate for calcium for the Ca-Mg site in the C-terminal domain. The reported kinetics of metal ion binding to the EF hand Ca^{2+} binding motif of the galactose binding protein, calmodulin, calbindin D_{9k} and troponin C [1,13–16] provide good parallel systems for the interpretation of the results of the present experiments. It has also been reported that the sites with diffusional on-rates are located in the N-terminal domain while the ones which exhibit slow on-rates are assigned to the C-terminal domain. While a significant part of the CaBP-Ca^{2+} interaction is over within the dead time of the instrument (1.5 ms), the slower phase of the reaction has $k_{on}=4\times10^3$ M^{-1} s^{-1} in the absence of Gdn^+ ion. Likewise the kinetics of dissociation of Ca^{2+} bound to CaBP consists of two phases – the fast component which occurs within the dead time of the instrument and the slower one with a rate constant of 3.927 s^{-1}. The high affinity sites in the C-terminal domain (one of which is Ca^{2+}-Mg^{2+}) have slower dissociation rates as compared to the lower affinity sites in the N-domain. The role of Ca^{2+} binding to the C-terminal domain thus appears to be structural in nature, providing for longer residence times for the Ca^{2+} ion. The N-terminal domain (low affinity sites) thus appears to be the regulatory domain of CaBP. The binding of the Gdn^+ ion at the N-terminal site which occurs at a concentration of 0.25 M GdnHCl appears to modulate the ion binding affinity by decreasing the dissociation rate for Ca^{2+}. The decrease in the dissociation rates for Ca^{2+} upon increase in GdnHCl con-

Table 1
Thermodynamic and kinetic parameters of Ca^{2+} and Mg^{2+} binding to CaBP at pH 7.0 in 20 mM MOPS buffer

Ligand	Site	T (°C)	K_b[a] (M^{-1})	ΔH[a] (kJ/mol)	k_{on} (M^{-1} s^{-1})	k_{off} (s^{-1})
Ca^{2+}	I/II	19	4.98 ± 1.2e3	−13.09 ± 0.7	4×10^3	2.04
			3.13 ± 0.41e3	−47.69 ± 5.46		
	III/IV		1.03 ± 0.19e4	117.57 ± 20.08		
			1.66 ± 0.1e5	−116.31 ± 10.4		
Ca^{2+} in 0.25 M GdnHCl	III/IV	20	1.9 ± 0.29e6	−25.84 ± 0.49		1.08
			1.95 ± 0.10e5	−14.57 ± 0.42		
Ca^{2+} in 0.5 M GdnHCl	III/IV	20	1 ± 0.14e6	−31.01 ± 0.75		0.90
			1.37 ± 0.10e5	−12.59 ± 0.89		
Ca^{2+} in 1 M GdnHCl	III/IV	20	3.23 ± 0.75e5	−38.49 ± 3.76		16.9
			7.18 ± 0.63e4	−10.79 ± 2.16		
Ca^{2+} (Mg^{2+}-saturated CaBP)	I/II	20	3.7 ± 0.38e4	−21.42 ± 0.16	4×10^3	3.92
			4.3 ± 0.45e2	−102.98 ± 19.27		
	III/IV		4.7 ± 0.48e5	47.45 ± 19.27	5.1×10^5	2.89
			1.07 ± 0.86e3	−24.22 ± 2.29		
Mg^{2+}	III	20	7.18 ± 0.63e4	−10.79 ± 2.16		2.18

[a]Values reported in [2].

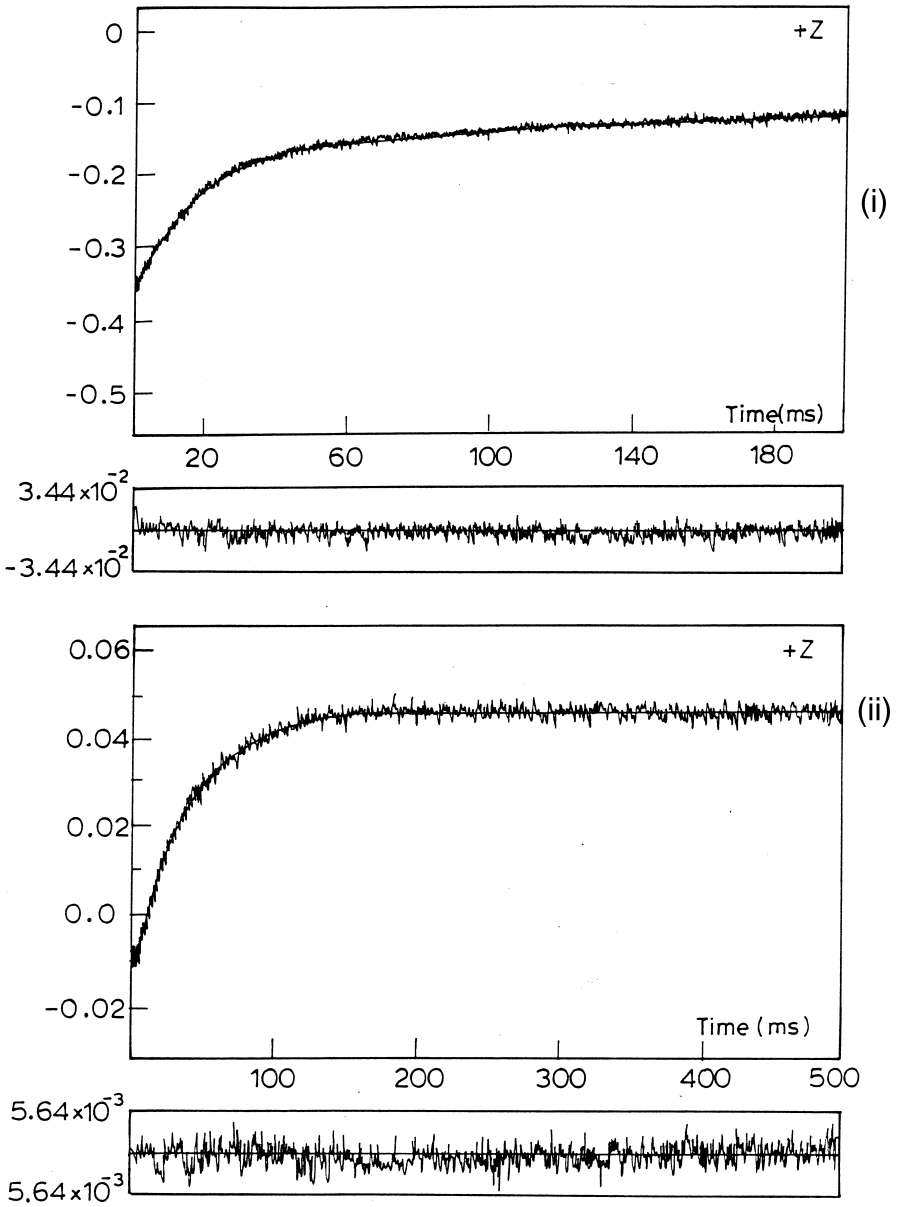

Fig. 1. Stopped flow fluorescence traces of Mg^{2+} binding to CaBP at 20°C. i: For the association reactions equal volumes of $MgCl_2$ (5 mM) and CaBP (80 μM) were mixed in the stopped flow cell. The samples were excited at 278 nm and the emission was recorded above 305 nm. The spectrum represents the average of 10 measurements. The continuous line is the non-linear least squares fit of the data. Residuals are shown in the bottom panel. ii: Stopped flow trace for Ca^{2+} association to Mg^{2+} saturated CaBP.

centration suggests that the flexibility of the C-terminal domain accommodates the effects of the denaturant and still maintains specificity for the Ca^{2+} ion. The longer residence time for the Ca^{2+} ion could be interpreted as a reduction in this flexibility. This needs to be noted in conjunction with the substantial decrease in binding affinity for Ca^{2+} and loss of the ability to bind Mg^{2+}. Thus GdnHCl acts by stabilizing the protein at low concentrations and above 1 M destabilizes the structure. Such a role of Gdn^+ ion in modulating Ca^{2+} binding to proteins has been noted earlier only for RNase T1 [17].

3.2. Equilibrium denaturation measurements

A striking feature observed during the course of measurement of the CD data in the far and near UV regions is the increase in the molar ellipticity values at low denaturant concentrations (Fig. 2(i)) which we attribute to the formation of stable transition intermediates [18,19]. The stabilization by low concentrations of GdnHCl could plausibly be a general feature of the EF hand family of proteins [2,11].

3.3. ANS binding measurements

Binding of ANS to CaBP at low denaturant concentration is consistent with the current understanding on the structure activity relationships of these proteins (Fig. 2(ii)). Most proteins in the calmodulin superfamily, which function as 'trigger' proteins, expose their hydrophobic surfaces upon calcium binding which enables these trigger proteins to recognize the effector proteins, mostly kinases. More importantly, the long central linker, which contains a stretch of hydrophobic residues, would tend to be solvent exposed as the inherently flex-

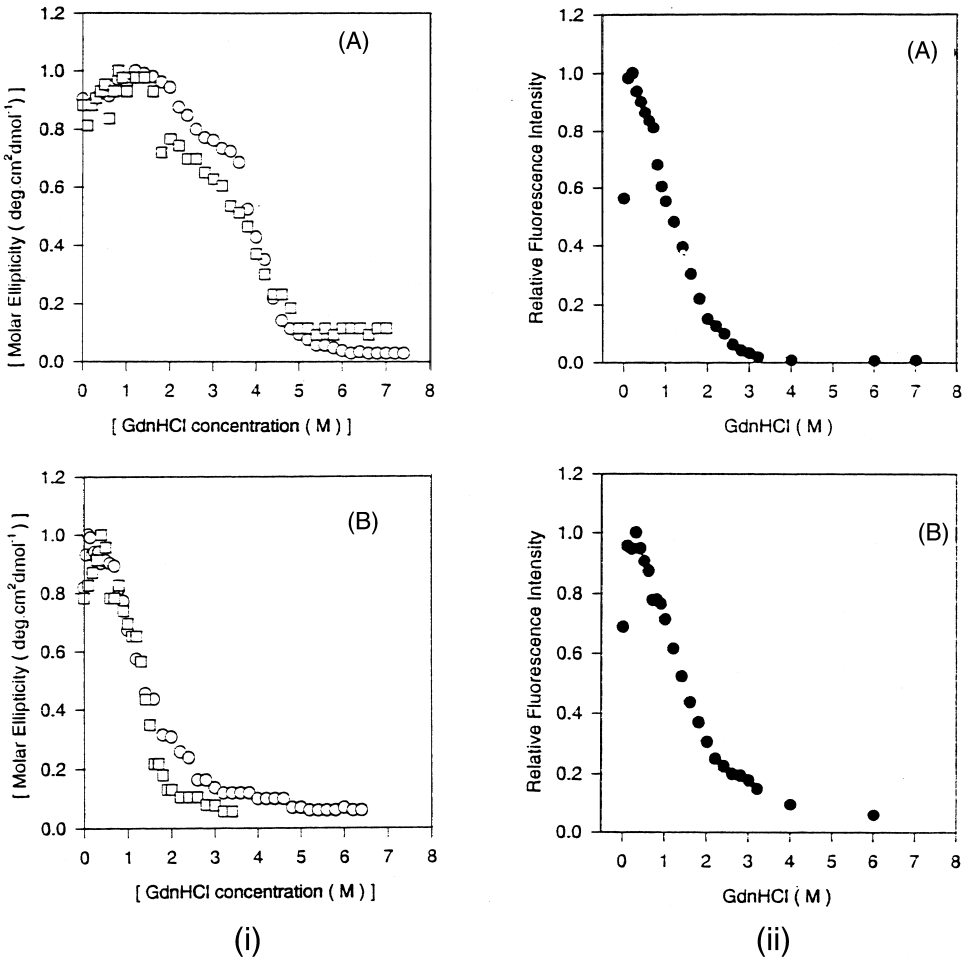

Fig. 2. i: Equilibrium denaturation profiles for CaBP in varying amounts of GdnHCl. Equilibrium denaturation curves of CaBP at 20°C, pH 7.0. GdnHCl-induced denaturation was followed by monitoring mean residue ellipticity at 222 nm (○) for the secondary structure and 276 nm (□) for the tertiary structure. A: Holo CaBP. B: Apo CaBP. ii: Binding of ANS monitored by the emission intensity at 470 nm.

ible protein spans through several conformational states. That the ANS binding peaks at 0.3 M GdnHCl shows that the stabilization could arise in part by the binding of the guanidinium ions to the cation binding sites, thus enabling the protein to mimic the calcium bound state and expose hydrophobic surfaces. The N-terminal domain, which is more structured, binds the Gdn^+ ions more readily while the C-domain retains its ability to bind calcium (Table 1).

3.4. Size exclusion chromatography

The gel elution profiles for both the holo and the apo forms of CaBP are characterized by the appearance of a second peak at low denaturant concentrations (Fig. 3). This is more so in the apo form which shows a faint shoulder even in the absence of the denaturant. Beyond a threshold denaturant concentration (1 M), the partially unfolded protein elutes at the same volume as the native. In an effort to characterize the intermediate, half saturated states of specific binding domains, the 'blocking' of the regulatory (N-terminal domain) to Ca^{2+} by Gdn^+ appeared to be a viable approach. This approach would thus lead to information on the intermediate steps in the binding process and the role of the long range effects – the contribution from the other parts of the protein. The conformational flexibility of this protein becomes apparent from an examination of the size exclusion chromatography results. The gel elution profile for the apo form shows a faint shoulder even at zero denaturant concentration. The profile separates out into two peaks up to a GdnHCl concentration of 1 M, and thereafter most of the protein elutes as a single peak. Also the holo CaBP elutes as a single peak albeit at a slightly higher volume. This feature of a single peak digressing into a shoulder and later co-eluting as a single peak is exhibited by both the apo and the holo forms. However, the denaturant concentrations required in the case of the holo protein are higher. The consistency of the appearance of the second peak irrespective of protein concentrations used, and also the absence of evidence for insoluble aggregates as determined by Rayleigh scattering, suggests that these peaks in the elution profile represent two conformational ensembles of CaBP. As the second ensemble elutes after the initial peak, this ensemble seems to have acquired a relatively more compact or globular conformation. Beyond a concentration of 0.25 M GdnHCl, the N-domain sites lose their ability to bind calcium. Consequently, the protein adopts a different conformational ensemble represented by the second peak of the elution profile. This would occur more readily for the apo form, but would occur, nonetheless, for the holo form also as the N-terminal sites have Ca^{2+} binding affinities in the millimolar range. It is pertinent to note that there has been no report of conformational heterogeneity in the NMR structures of the C-terminal frag-

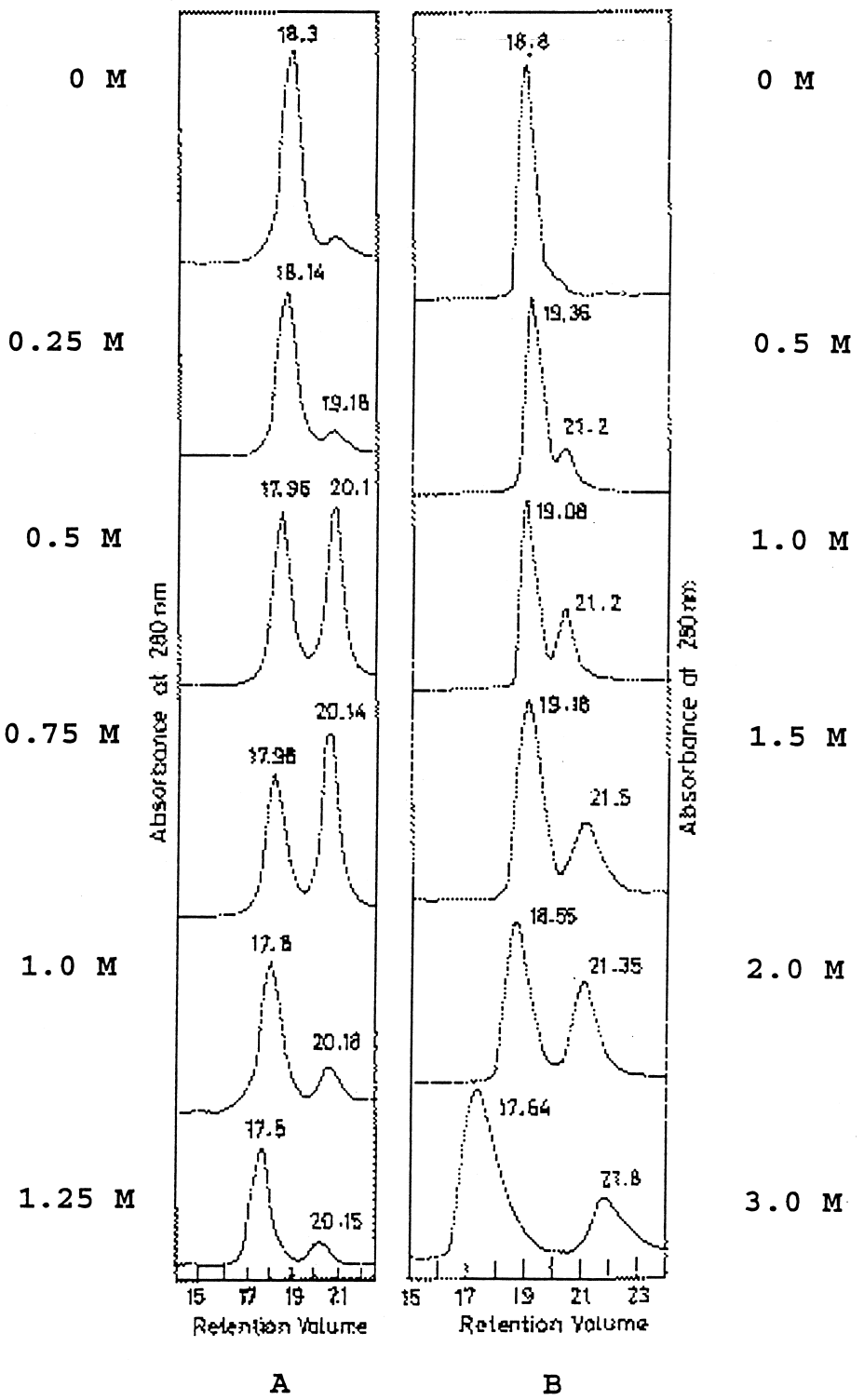

Fig. 3. Size exclusion chromatography profiles of (A) apo and (B) holo CaBP performed in varying concentrations of GdnHCl. The flow rate used was 0.3 ml/min (see text for details).

ment of calmodulin [20]. Thus the effect of conformational heterogeneity must be correlated with the central linker possibly involving the N-terminal domain. Beyond 1 M GdnHCl concentration, unfolding takes over and the two different conformational ensembles merge to elute at similar volumes. Thus the stabilization observed at low GdnHCl concentration represents a native-like intermediate observed in the course of unfolding and can be partially characterized.

3.5. Calorimetric measurements of Gdn^+ binding

A typical titration profile for the binding of Gdn^+ ions to CaBP is depicted in Fig. 4. The number of binding sites for

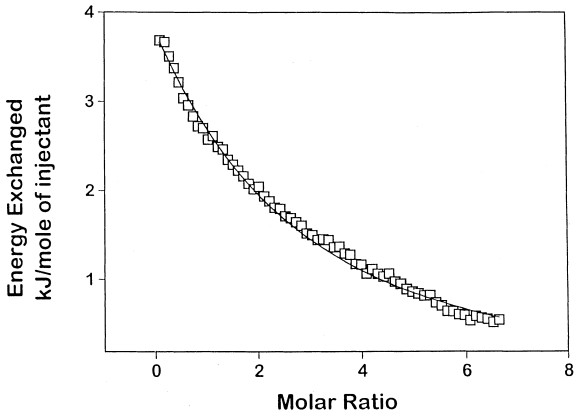

Fig. 4. A calorimetric titration of 5.0 μl aliquots of 25 mM GdnHCl into 1.0 mM of apo CaBP in 20 mM MOPS buffer at 19°C. A least squares fit of the data to the heat absorbed per mol of titrant versus the ratio of the total concentration of ligand to the total concentration of protein.

Gdn^+ as evaluated from this profile is 2.01 (±0.031), with a K_b of 368 (±18) M^{-1} and an enthalpy of binding, ΔH_b, of 5.022 (±0.2) kJ/mol. Titrations performed at higher concentrations of GdnHCl showed additional sites, with much lower K_b and exothermic ΔH_b. The latter sites are apparently distributed throughout the polypeptide chain and binding to them accompanies the denaturation process. The hypothesis that Gdn^+ ions preferentially attach themselves at or near the cation binding sites in the N-terminal domain was proven by ITC measurements. ITC data show that GdnHCl binding indeed occurs with the K_bs in the range of 368 (±18) M^{-1} and a titration profile so obtained can be fitted for two sites (Fig. 4). As expected, the titration performed with a higher concentration of GdnHCl shows more binding sites for the apo than the holo form per polypeptide chain, with the binding affinities lower as compared to the specific binding mentioned earlier (data not shown). The number of binding sites obtained for the holo form is ~70/polypeptide chain which is in agreement with those reported for a protein of similar size [21]. Moreover, the ΔH value for binding to these sites is exothermic in contrast to the endothermic value of ΔH observed for binding to the specific sites. Thus the sites which the Gdn^+ ions share with the Ca^{2+} ions on the apo protein are distinct from the sites at which this denaturant binds and unfolds the protein.

In conclusion, we postulate the sequence of events to be as follows. At low GdnHCl concentrations, guanidinium ions attach themselves more readily to two sites, probably the cation binding motifs, in the N-terminal domain than the non-specific sites on the polypeptide chain. Binding of Gdn^+ at the sites in the N-terminal domain precludes Ca^{2+} binding to these sites. Nevertheless, this promotes their interactions with the sites in the C-terminal domain as evidenced by the diminution of the off-rates for the Ca^{2+} ions. Thus, following the binding of the guanidinium ion to the N-terminal domain, the protein mimics the structure of the calcium bound form acquiring a more compact conformation. These studies suggest that in some situations Gdn^+ ions can influence the overall conformation/activity of proteins by binding to specific sites. This is attested in the case of CaBP by the thermodynamic and kinetic parameters of the binding of Gdn^+ to the N-terminal domain. This then modulates the overall conformation of CaBP and the orientations of the two domains in a manner reminiscent of its binding to Ca^{2+}.

Acknowledgements: We wish to thank Mr. C.P. Swaminathan for conducting the isothermal titration calorimetry experiments and Dr. R. Varadarajan and Mr. Girish S. Ratnaparkhi for their comments on the manuscript. This work has been supported by grants from the Department of Science and Technology, Government of India to A.S. and M.R.N.M. and from the Department of Biotechnology, Government of India to A.S.

References

[1] Falke, J.J., Drake, S.K., Hazard, A.L. and Peersen, O.B. (1994) Q. Rev. Biophys. 27, 219–290.
[2] Gopal, B., Swaminathan, C.P., Bhattacharya, S., Bhattacharya, A., Murthy, M.R.N. and Surolia, A. (1997) Biochemistry 36, 10910–10917.
[3] Yadava, N., Chandok, M.R., Prasad, J., Bhattacharya, S., Sopory, S.K. and Bhattacharya, A. (1997) Mol. Biochem. Parasitol. 84, 69–82.
[4] Kuboniwa, H., Tjandra, N., Grzesiek, S., Ren, H., Klee, C.B. and Bax, A. (1995) Nature Struct. Biol. 2, 768–776.
[5] Gagne, S.M., Tsuda, S., Li, M.X., Smillie, L.B. and Sykes, B.D. (1995) Nature Struct. Biol. 2, 784–789.
[6] Prasad, J., Bhattacharya, S. and Bhattacharya, A. (1993) Cell Mol. Biol. Res. 39, 167–175.
[7] Pace, C.N., Shirley, B.A. and Thomson J.A. (1989) in: Protein Structure: A Practical Approach (Crieghton, T.E., Ed.), pp. 311-330, IRL Press, Oxford.
[8] Wiseman, T., Williston, S., Brandts, J.F. and Lin, L.N. (1989) Anal. Biochem. 79, 131–137.
[9] Schwarz, F.P., Puri, K. and Surolia, A. (1991) J. Biol. Chem. 266, 24344–24350.
[10] Waltersson, Y., Linse, S., Brodin, P. and Grundstrom, T. (1993) Biochemistry 32, 7866–7871.
[11] Fredricksen, R.S. and Swenson, C.A. (1996) Biochemistry 35, 14012–14026.
[12] Gopal, B., Suma, R., Murthy, M.R.N., Bhattacharya, A. and Bhattacharya, S. (1998) Acta Crystallogr. D (in press).
[13] Martin, S.R., Linse, S., Johansson, C., Bayley, P.M. and Forsen, S. (1990) Biochemistry 29, 4188–4193.
[14] Forsen, S., Linse, S., Thulin, E., Lindegard, B., Martin, S.R., Bayley, P.M., Brodin, P. and Grundstrom, T. (1988) Eur. J. Biochem. 177, 47–52.
[15] Rosenfeld, S.S. and Taylor, E.W. (1985) J. Biol. Chem. 260, 242–251.
[16] Martin, S.R., Andersson Teleman, A., Bayley, P.M., Drakenberg, T. and Forsen, S. (1985) Eur. J. Biochem. 151, 543–550.
[17] Mayr, L.M. and Schmid, F.X. (1993) Biochemistry 32, 7994–7998.
[18] Matthews, C.R. (1993) Annu. Rev. Biochem. 62, 653–683.
[19] Fersht, A.R. (1993) FEBS Lett. 325, 516.
[20] Finn, B.E., Evenas, J., Drakenberg, T., Waltho, J.P., Thulin, E. and Forsen, S. (1995) Nature Struct. Biol. 2, 777–783.
[21] Makhatadze, G.I. and Privolov, P.L. (1992) J. Mol. Biol. 226, 491–505.