cation) represents the M–O partial ionic bond character according to the Wilmshurst equation[12]; r_O is the purely ionic, single-bonded oxygen radius (176 pm)[13] and r_{Ocov} is the purely covalent oxygen radius, which has been assigned[14] values in the range 70.2 to 74 pm. Optimal values for the empirical constant γ and r_{Ocov} were obtained by iterative computation based on the best steric fit for cation and ligand site in the naturally occurring, most stable complex, Fe^{III}–MY. From the site-structural representations shown in Figure 2, it is seen that the Th cation (Figure 2*c*) largely exceeds the calculated available volume of the site configuration as established from Fe–MYS (Figure 2*b*), suggesting a resultant distortion of molecular symmetry that would expectedly lead to relative weakening of the metal–oxygen bonds. The higher degree of ionic character (~81%) calculated for the Th–O bond, together with the fact that only three of the four metal valencies can be engaged in MY complexation, may add further to this effect.

In spite of the lower stability of Th–MYS in comparison to UO_2–MYS under *in vitro* conditions, whole-cell biomass loaded with thorium cations appears to be more resistant to hydrolytic desorption than uranyl-loaded biomass. This apparently contradictory observation indicates that additional binding interactions, together with the protecting effect afforded by cellular incorporation, may be influential under complex natural conditions.

1. Brainard, J. R., Strietelmeier, B. A., Smith, P. H., Langston-Unkefer, P. J., Barr, M. E. and Ryan, R. R., *Radiochimica Acta*, 1992, 58/59, 357–363.
2. Multiauthor Review, *Experientia*, 1990, **46**, 777–878
3. Andres, Y., MacCordick, H. J. and Hubert, J. C., *Appl. Microbiol Biotechnol*, 1993, **39**, 413–417.
4. MacCordick, H. J., Hubert, J. C., Schleiffer, J. J., *J. Radioanal Nucl Chem Lett*, 1989, **135**, 349–357.
5. Andres, Y., MacCordick, H. J. and Hubert, J. C., *FEMS Microbiol. Lett.*, 1994, **115**, 27–32
6. MacCordick, H. J., *J. Radioanal Nucl. Chem Lett*, 1988, **126**, 173–181.
7. MacCordick, H. J. and Kadri, M., *J. Radioanal. Nucl. Chem Lett*, 1988, **127**, 51–58
8. Andres, Y., MacCordick, H. J. and Hubert, J. C., *Biol Metals*, 1991, 4, 207–210
9. MacCordick, H. J., *Nouv J Chim*, 1985, **9**, 535–538
10. Ratledge, C., in *CRC Handbook of Microbiology*, 2nd edn (eds Laskin, A. I. and Lechevalier, H. A.), CRC Press, Boca Raton, 1982. vol. IV, pp 575–581
11. *Gmelins Handbuch der Anorganischen Chemie*, 8. Aufl., Thorium und Isotope, Verlag Chemie, Weinheim, 1955, vol 44, p. 265
12. Wilmshurst, J. K., *J Chem. Phys*, 1959, 30, 561–565
13. *CRC Handbook of Chemistry and Physics*, 61st edn., CRC Press, Boca Raton, 1980–1981.
14. Sanderson, R. T., *J Am Chem. Soc*, 1983, 105, 2259, Wells, A. F., *Structural Inorganic Chemistry*, 4th edn., Clarendon Press, Oxford, 1975, p 236.

Received 12 December 1994, revised accepted 27 January 1995

Partial unfolding of lactate dehydrogenase in the presence of low concentrations of guanidium chloride

Abhijit Chakrabarti[†,*], Subrata Chattopadhyay and Chanchal K. DasGupta

Department of Biophysics, Molecular Biology and Genetics, University of Calcutta, 92, A P C Road, Calcutta 700 009, India
†Biophysics Division, Saha Institute of Nuclear Physics, 37 Belgachia Road, Calcutta 700 037, India

The presence of very low concentrations of guanidium chloride could partially unfold the tetrameric enzyme lactate dehydrogenase from pig muscle. The local partial unfolding in the tertiary structure of the protein was revealed from the study of quenching of the tryptophanyl fluorescence using the most widely used quenchers, acrylamide and iodide. Significant changes in the Stern–Volmer quenching constants as well as in the accessibility parameter of the tryptophan residues by the quenchers were observed in the presence of <100 mM guanidium chloride over the same in the absence of the denaturant. This indicated the presence of a partially unfolded state in the enzyme even at a low concentration of guanidium chloride which does not otherwise affect the enzymic activity or the secondary structure of the enzyme.

GUANIDIUM chloride (GdmCl) has been very frequently used, at a concentration >1 M, to denature proteins[1,2]. However, by spectroscopic measurements, low concentrations of GdmCl have not been shown to impart any alterations in the tertiary structure of proteins. The presence of small amounts of GdmCl in the buffer during reactivation of denatured lactate dehydrogenase (LDH), as initiated by diluting highly concentrated solutions of the denaturant, has been shown to affect the rate of reactivation of the enzyme[3]. In many studies of protein folding, ~100 mM GdmCl is often present in the buffer during reconstitution from the completely unfolded state attained by the treatment with 4–6 M of GdmCl. The present experiments were aimed at analysing the effect of low concentrations of GdmCl on the tertiary structure of the tetrameric enzyme LDH from pig muscle, which has been the subject of extensive studies, and the folding pathways of the enzyme have been elucidated[4]. In the concentration range of 10–100 mM GdmCl, the activity of the enzyme remained unaltered and the secondary structure remained intact as indicated by circular dichroism (CD) measurements. The results from studies of quenching of tryptophanyl fluorescence indicated the presence of a partially unfolded state of the protein in the presence of a very low concentration of GdmCl.

*For correspondence.

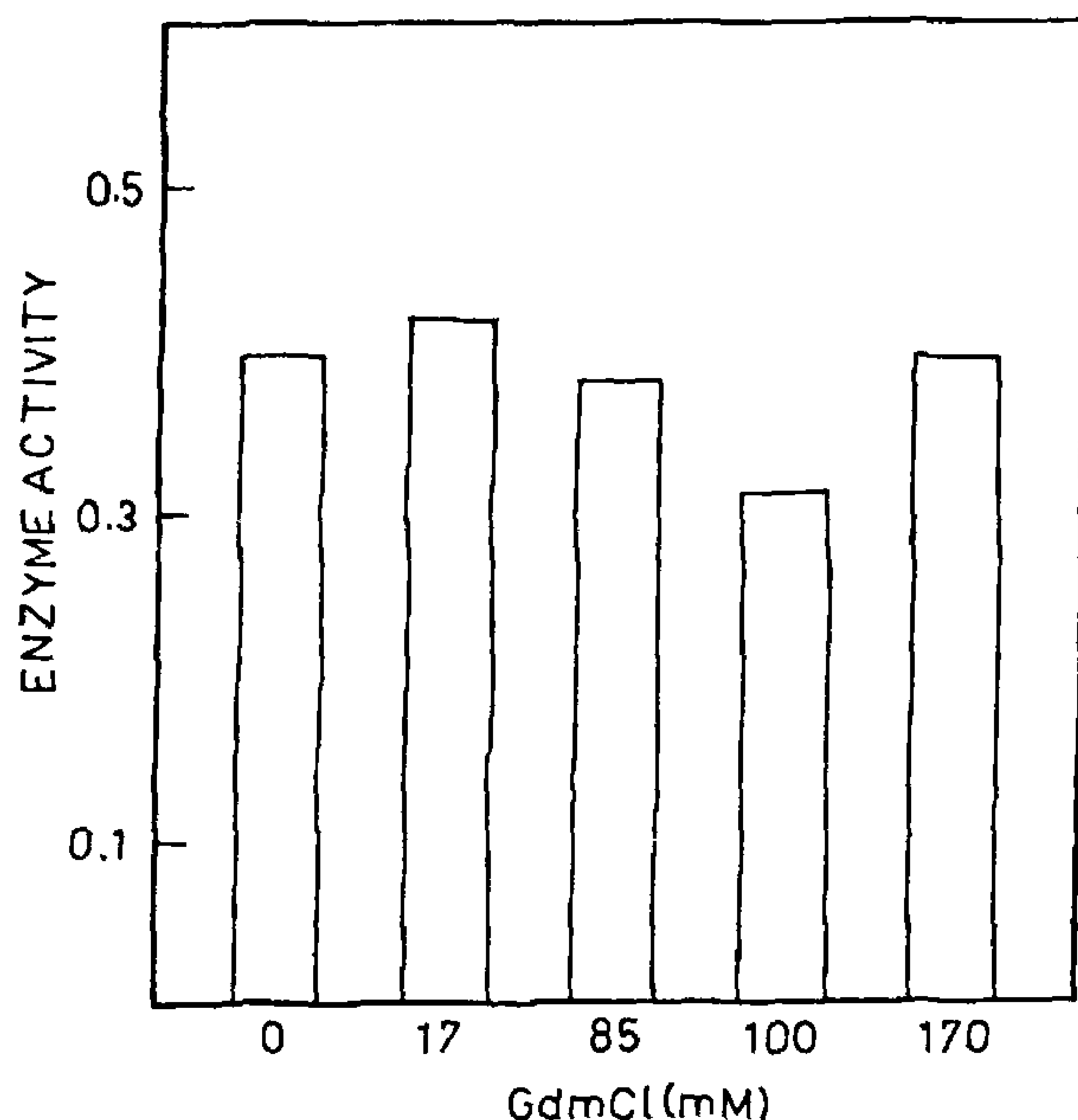

Figure 1. Histogram representation of enzyme activity of LDH versus concentration of GdmCl (mM) The enzyme activity denotes the decrease in absorbance at 340 nm in 3 min by 1 6 nM LDH subunit

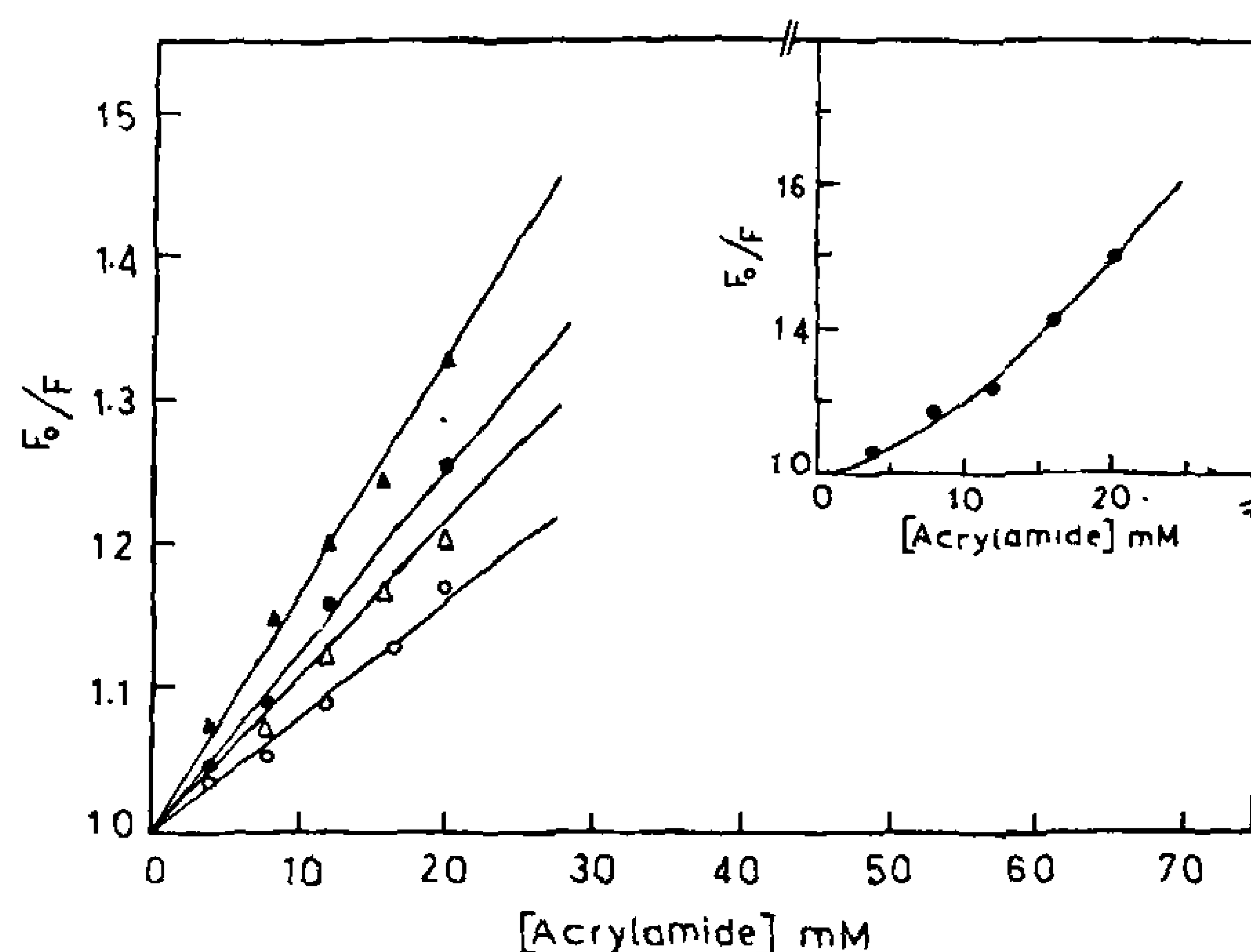

Figure 2. Stern–Volmer plots of quenching of tryptophan fluorescence by acrylamide: (○) Native LDH, 0 16 μM and after 1 h incubation with (△) 85 mM, (●) 170 mM and (▲) 850 mM GdmCl Inset shows a Stern–Volmer plot with upward curvature in the presence of 17 mM GdmCl

LDH (Sigma) was assayed at 37°C following published protocol[5]. The assay mixture contained 0.1 M Tris-HCl, pH 7.5, 5 mM sodium pyruvate and 0.25 mM NADH. The decrease in absorbance was measured at 340 nm. The enzyme assay and all other absorption measurements were carried out using a Hitachi U-2000 spectrophotometer. The concentration of LDH was determined[6] spectrophotometrically assuming an absorbance of 14.5 at 280 nm for the LDH solution of 10 mg/ml. No significant inactivation of the enzyme LDH was observed after 1 h incubation with 10–200 mM GdmCl (Sigma) at 20°C, as indicated in Figure 1. However, the enzyme activity started decreasing when 0.16 μM LDH was incubated with >200 mM GdmCl for the same period of time at 20°C. The fluorescence intensity remained unchanged at a GdmCl concentration between 10 and 100 mM although 60% of the native fluorescence was quenched in the presence of a denaturing concentration of ~1 M GdmCl (data not shown). CD spectra of 0.16 μM LDH in the absence and presence of different concentrations of GdmCl also showed no significant change in the negative peak at 222 nm, representive of the overall helix content, up to 100 mM GdmCl, indicating that the secondary structure of LDH remains intact in the presence of a low concentration of the denaturant (not shown).

LDH at 0.16 μM with respect to the monomer was incubated with different concentrations of GdmCl for 1 h at 20°C in a final volume of 1 ml before each quenching experiment was carried out. The buffer used in the study contained 20 mM Tris-HCl, 50 mM KCl, pH 7.5, containing 5 mM 2-mercaptoethanol. In case of quenching with iodide anions, the buffer contained 1 mM $Na_2S_2O_3$ in addition, to avoid I_3^- formation. Steady-state fluorescence was measured using a Hitachi F3010 spectrofluorometer. Tryptophan fluorescence was measured upon excitation at a wavelength of 290 nm. The emission was maximum at 345 nm. The sample temperature was maintained at 20°C. Fluorescence quenching experiments were carried out by recording the intensities (EX: 290 nm; EM: 345 nm) after successive addition of small aliquots of the quencher stock solution. The quenching data were analysed by plotting according to the Stern–Volmer equation[7],

$$F_0/F = 1 + K_{sv} \cdot [Q], \qquad (1)$$

where F_0 is the fluorescence intensity in the absence of the quencher, F is the same at a quencher concentration of Q and K_{sv} is the Stern–Volmer quenching constant. The linear range of the plot of F_0/F versus the final quencher concentration was used to determine K_{sv}. Here K_{sv} is the quenching constant, which can be interpreted in different ways depending upon the assumed mechanism of quenching. The fraction of Trp residues that are accessible to the quencher molecule was estimated from the modified Stern–Volmer equation:

$$F_0/(F_0 - F) = 1/(K_{sv} \cdot f_e \cdot [Q]) + 1/f_e, \qquad (2)$$

where f_e is the fraction of Trp residues accessible to the quenchers, acrylamide or iodide. Stern Volmer plots of

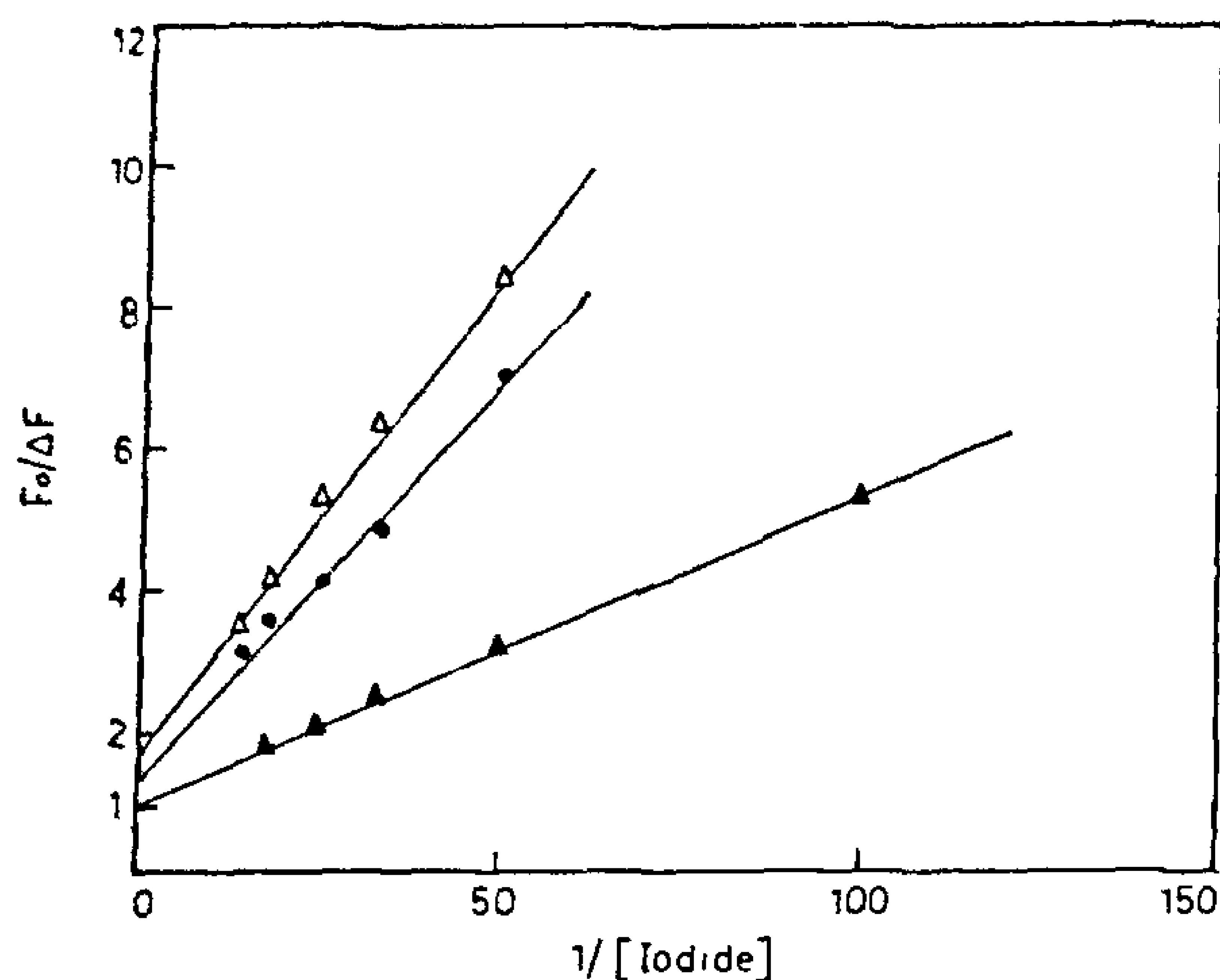

Figure 3. Lehrer plots of iodide quenching of tryptophanyl fluorescence in LDH (△) Native LDH, 0 16 μM and after 1 h incubation with (●) 85 mM and (▲) 850 mM GdmCl.

Table 1. Quenching of tryptophan fluorescence of LDH by acrylamide and iodide in the presence of GdmCl

Quencher	GdmCl (mM)	K_{sv} (M^{-1})	f_e (%)
Acrylamide	0	8 20	100 0
	17	9 40	84 0
	85	10 80	79 0
	170	12 90	84 0
	850	16 40	100 0
Iodide	0	6 15	58 0
	17	7 80	67 0
	85	7 80	71 5
	170	7 90	64 5
	850	22 70	100 0

Standard error (SEM) for three independent quenching experiments is between 2 and 5% for K_{sv} and ~5% for f_e.

quenching of tryptophan fluorescence in LDH by acrylamide are shown in Figure 2. The linear range of plots of F_0/F against the final acrylamide concentration indicated local structural differences in the LDH molecules at different GdmCl concentrations. The inset shows a representative curvilinear plot when 17 mM GdmCl was present in the buffer. The fraction of accessible tryptophan (f_e) residues was estimated from the modified Stern–Volmer plot, commonly known as the Lehrer plot[8], where $F_0/(F_0 - F)$ is plotted against $1/[Q]$. In the presence of low concentrations of GdmCl, the accessibility is decreased to 80% from the value of 100% in the native as well as in the completely unfolded LDH when acrylamide was the quencher. The Lehrer plots of iodide quenching, shown in Figure 3, indicated an increase in the fractional accessibility of Trp residues in the presence of 85 mM GdmCl from that in its absence. In the presence of 850 mM GdmCl the accessibility became 100%, indicating complete unfolding of LDH at that concentration of the denaturant. Both f_e and K_{sv} values for the quenchers are summarized in Table 1.

Acrylamide and iodide are the frequently used quenchers of protein fluorescence. The bimolecular quenching constants are known to be sensitive and vary among various proteins. At 10–100 mM GdmCl, there were significant increases in the Stern–Volmer quenching constants for both the quenchers, acrylamide and iodide, compared to those in the absence of GdmCl (Table 1). All the six Trp residues present in one subunit of the tetrameric LDH from pig muscle[9] were accessible to acrylamide when the protein was either in native state, i.e. in the absence of GdmCl, or in its completely unfolded state. In the presence of 17–170 mM GdmCl concentrations, one of the six Trp residues became inaccessible (f_e ~ 80%) to acrylamide, indicating the occurrence of a partial restructuring of the enzyme on binding to GdmCl, leading to a more compact molten globule-like intermediate. Stern–Volmer plots for acrylamide quenching have been found to be upward curving in the presence of no or very small amounts of GdmCl. Such a curvature in the Stern–Volmer plot could be attributed to the presence of two types of emitting centres in the protein[10]. At >120 mM GdmCl the secondary structure started getting affected due to the unfolding of LDH. As a consequence, the microheterogeneity in the two emitting centres was lost and the Stern–Volmer plots became linear at a higher GdmCl concentration. In the case of quenching with iodide anion no such curvature was observed in the Stern–Volmer plot. A lower degree of accessibility of tryptophan residues was observed in the case of native LDH (f_e < 60%), which was increased in the presence of 85 mM GdmCl (f_e ~ 70%). Linearization of Stern–Volmer plots in iodide quenching experiments indicated that only the more exposed tryptophans or the residues involved either in hydrogen bonding or in the neighbourhood of basic amino acid residues (e.g. Lys or His) were quenched by the bulky, anionic iodide. An f_e value of < 60% could account for three such Trp residues in native LDH, which increased to 70% in the presence of 85 mM GdmCl when one more Trp residue possibly moved to a favourable ionic atmosphere due to partial unfolding in LDH becoming then accessible to iodide anion.

The mechanism of action of GdmCl on protein denaturation is still obscure. It is also not well understood whether the action of this widely used denaturant is through direct binding or is indirect and involves a change in the water structure[11, 12]. The interaction of guanidium chloride with proteins has been recently studied calorimetrically by Makhatadze and Privalov[13]. They described the observed heat effects in terms of a simple binding model and estimated the number of apparent GdmCl binding sites for three proteins, ribonuclease A, lysozyme and cytochrome c. According to the

estimate, 40–50 molecules of GdmCl were bound per native protein molecule and around 80 GdmCl molecules were bound to the unfolded protein. This suggests that even at submolar concentrations of GdmCl (10^{-2} – 10^{-1} M) similar binding may take place at 0.16 µM protein, used in the present study. The estimated affinity constants at this concentration were 10 M^{-1} for the native and 50 M^{-1} for the denatured LDH[3]. The present study indicated that even millimolar concentrations of the denaturant could perturb the tertiary structure of a protein through a possible direct binding, which is generally assumed to be 'innocent' in many studies of protein folding.

1 Tanford, C , *Adv Protein Chem* , 1968, **23**, 122–282
2 Tanford, C , *Adv Protein Chem* , 1970, **24**, 2–95
3 Zettlmeissl, G., Rudolph, R and Jaenicke, R , *Eur J Biochem* , 1979, **100**, 593–598
4 Jaenicke, R., *Prog Biophys Mol Biol* , 1987, **49**, 117–237
5 Badcoe, I G , Smith, C. J , Wood, S , Halsall, D J., Holbrook, J J , Lund, P and Clarke, A R , *Biochemistry*, 1991, **30**, 9195–9200
6 Jaenicke, R and Knof, S , *Eur. J Biochem* , 1968, **4**, 157–163
7 Lakowicz, J R , *Principles of Fluorescence Spectroscopy*, Plenum Press, New York, 1983
8 Lehrer, S S , *Biochemistry*, 1971, **10**, 3254–3263
9 Kiltz, H -H , Keil, W , Griesbach, M , Petry, K and Meyer, H , *Hoppe Seyler's Z Physiol Chem* , 1977, **358**, 123–127
10 Eftink, M R and Ghiron, C A , *Anal Biochem* , 1981, **114**, 199–227
11 Arakawa, T. and Timasheff, S N , *Biochemistry*, 1984, **23**, 5924–5929
12 Creighton, T E , *Curr Opin Struct Biol* , 1991, **1**, 5–16
13 Makhatadze, G I and Privalov, P L , *J Mol Biol* , 1992, **226**, 491–505

ACKNOWLEDGEMENTS We thank Dr D. DasGupta for helpful discussions CDG acknowledges financial supports from CSIR, DAE and DBT, Government of India SC is a UGC Senior Research Fellow

Received 15 December 1994, revised accepted 21 March 1995

Analysis of fall in serum ferritin after chelation of iron with Deferiprone (L1) in β-thalassaemia and haemoglobin E β-thalassaemia

D. Adhikari*,†, T. Basu Roy*, S. Chandra* and S. K. Adhikari§

*Department of Haematology, Kothari Medical Centre, 8/3 Alipore Road, Calcutta 700 027, India

§Instituto de Fisica Tiorica, Rua Pamplona no 145, São Paulo, Brazil

Serum ferritin reflects body iron store. It is increased in thalassaemias due to many reasons. The chelation of iron with deferiprone (L1) causes fall of serum ferritin. This is found to be biexponential when chelated by hydrophilic α-hydroxypyridones such as deferiprone *in vivo*.

FERRITIN is a protein which stores iron in the body and is found in the serum as well as inside the cell. It is composed of 24 subunits of at least two types: L (or light 19,700 M_r) and H (or heavy 21,100 M_r)[1]. Those tissues functioning as major iron storage depot (like liver and spleen) have a preponderance of L subunits while other tissues have a higher proportion of H subunits[2].

The amount of serum ferritin is usually proportional to the amount of intracellular ferritin[3]. Serum ferritin is increased due to iron overload in β-thalassaemia (BT) and haemoglobin E β-thalassaemia (EBT) patients This is partly due to repeated transfusions and partly due to increased intestinal absorption of iron[4]. Effective reduction of serum ferritin was obtained by use of Deferiprone (DFP) or L1 at a dose of 75 mg/kg/day on BT and EBT patients ($n = 20$) in this study over a period of 15 months after signing the informed-consent forms. DFP is a new oral chelator which is undergoing trial in various countries in the world[5].

In BT patients receiving DFP, the mean serum ferritin dropped significantly from initial 3763 ± 1404 ng/ml to 1956 ± 851 ng/ml during the study ($p < 0.005$). In EBT patients receiving DFP, the mean serum ferritin dropped significantly from 2948 ± 1771 ng/ml to 1166 ± 894 ng/ml ($p < 0.005$).

Mean fall of serum ferritin in all the patients was plotted on a semilog paper for the period of study (Figure 1). The fall in serum ferritin fits well in a biexponential curve whose equation can be written as

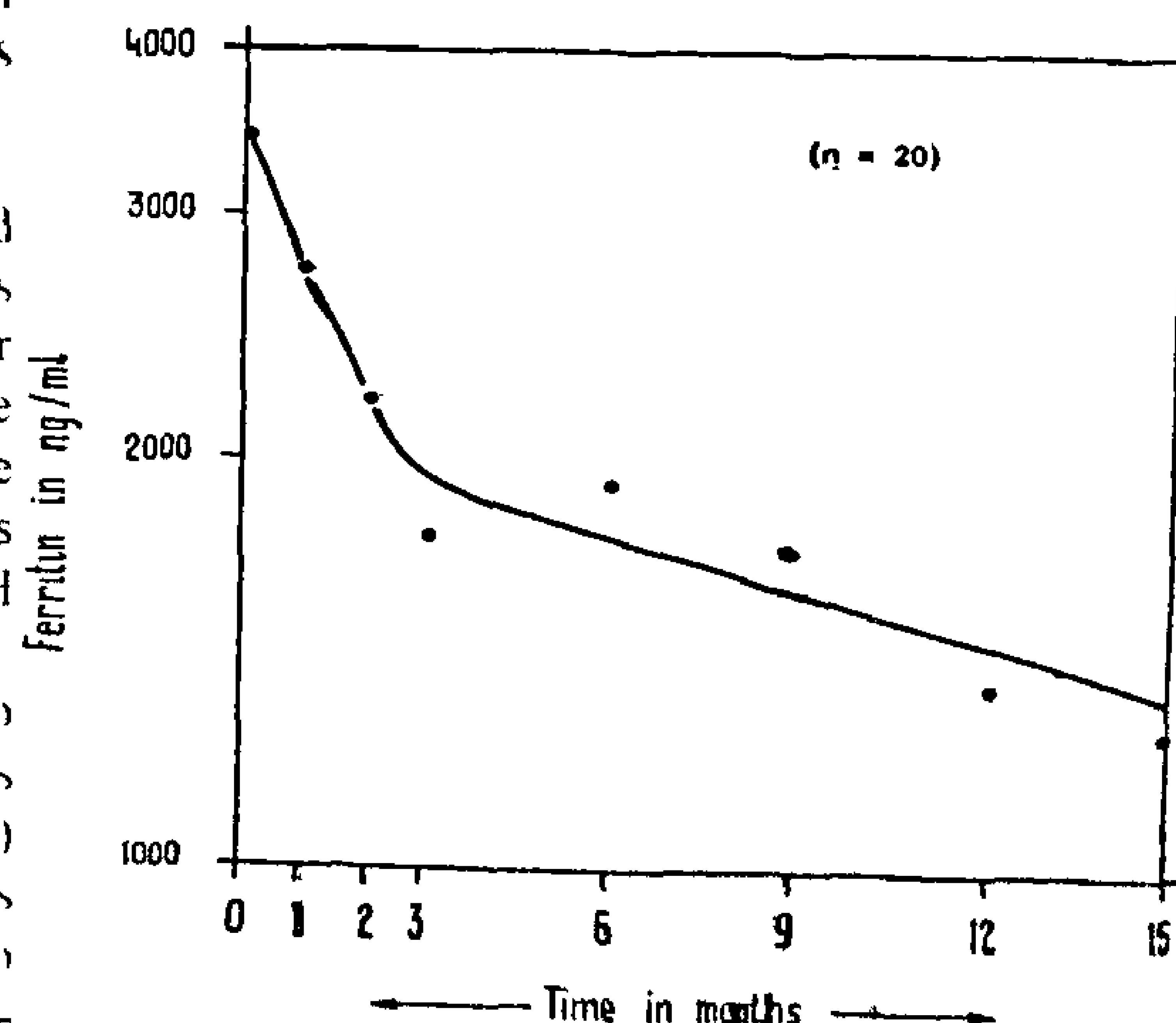

Figure 1. Fall of serum ferritin (ng/ml) over months in thalassaemia patients treated with Deferiprone (L1) at a dose of 75 mg kg/day

†For correspondence