ELSEVIER

Biophysical Chemistry 89 (2001) 77–85

Biophysical Chemistry

www.elsevier.nl/locate/bpc

No effect of trimethylamine *N*-oxide on the internal dynamics of the protein native fold

Margherita Gonnelli, Giovanni B. Strambini*

Istituto di Biofisica, Consiglio Nazionale delle Ricerche, Area della Ricerca di Pisa, Via Alfieri 1, San Cataldo 56010, Ghezzano-Pisa, Italy

Received 7 July 2000; received in revised form 8 October 2000; accepted 8 October 2000

Abstract

Trimethylamine *N*-oxide (TMAO) is a natural osmolyte accumulated in cells of organisms as they adapt to environmental stresses. In vitro, TMAO increases protein stability and forces partially unfolded structures to refold. Its effects on the native fold are unknown. To investigate the interrelationship between protein stability, internal dynamics and function, the influence of TMAO on the flexibility of the native fold was examined with four different proteins by Trp phosphorescence spectroscopy. Its influence on conformational dynamics was assessed by both the intrinsic phosphorescence lifetime, which reports on the local structure about the triplet probe, and the acrylamide bimolecular quenching rate constant that is a measure of the average acrylamide diffusion coefficient through the macromolecule. The results demonstrate that for apoazurin, alcohol dehydrogenase, alkaline phosphatase and glyceraldehydes-3-phosphate dehydrogenase 1.8 M TMAO does not perturb the flexibility of these macromolecules in a temperature range between −10°C and up to near the melting temperature. This unexpected finding contrasts with the dampening effect observed with polyols as well as with the expectations based on the preferential exclusion of the osmolyte from the protein surface.

Keywords: Phosphorescence; Trimethylamine *N*-oxide; Osmolytes; Acrylamide quenching; Protein dynamics; Protein stability

Abbreviations: TMAO, trimethylamine *N*-oxide; Az, apoazurin; LADH, horse liver alcohol dehydrogenase; AP, alkaline phosphatase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; *kq*, bimolecular quenching constant; Trp, tryptophan; $\tau\alpha$, intrinsic phosphorescence lifetime

* Corresponding author. Tel.: +39-050315-3051; fax: +39-050315-2760.

E-mail address: giovanni.strambini@ib.pi.cnr.it (G.B. Strambini).

0301-4622/01/$ - see front matter
PII: S0301-4622(00)00219-2

1. Introduction

Both prokaryotic and eukaryotic cells, when subjected to harsh environmental conditions such as water, salts, cold and heat stresses, adopt a common strategy in protecting their proteins by producing low molecular weight organic substances called osmolytes [1,2]. Osmolytes can be grouped into three major classes: polyols (sugar and sugar derivatives), amino acids, methyl ammonium compounds. Often, they are further classified as 'compatible' or 'counteracting' based on their effect on the functional activity of proteins. Compatible osmolytes increase protein stability against denaturation with little or no effect on their function [1,3–5], whereas counteracting osmolytes have, in addition, the ability to offset the deleterious effects of urea on the catalytic activity [6–10].

It is now generally accepted that preferential exclusion of the osmolyte from the biopolymer surface provides the driving force for processes, like protein refolding and subunit association, that reduce the water accessible surface area (ASA) of the polypeptide [7,11]. Furthermore, the free energy of transfer of protein groups from water to osmolyte solutions points out that, at the molecular level, stabilization of the globular fold is due predominantly to the solvophobic effect of the osmolyte on the peptide backbone [12–14]. Of course, the thermodynamic equilibrium merely yields the free energy difference between folded and unfolded structures with no indication as to the extent that the structure of the individual states has been perturbed. Thus, a deeper understanding of the mechanism by which these natural compounds preserve the globular fold against adverse denaturing conditions requires knowledge of specific osmolytes effects on both native and denatured states of the protein. To date, relatively little is known of osmolytes effects on folded proteins. The osmolyte pressure to reduce the solvent accessible surface area of proteins, is expected to translate into a decrease in both the size of their internal cavities and in the number of internal water molecules; a response that in general should result in a more compact rigid structure for the native fold. Another important question related to possible osmolytes effects on the native protein structure deals with their potential modulation of biological function. The close interrelationship between stability, structural flexibility and function predicts that even subtle alterations of conformation or dynamics of enzymatic proteins by osmolytes may have large consequences on their catalytic efficiency and, in turn, important implications for the cell metabolism under osmotic stress.

This work addresses the issue of osmolyte effects on the structure of the native fold of proteins by enquiring on the influence of trimethylamine *N*-oxide (TMAO), an osmolyte with an extraordinary ability to force thermodynamically unstable proteins to fold, [13–16], on the flexibility of these macromolecules. Internal protein dynamics are assessed by a sensitive spectroscopic method based on Trp phosphorescence. Trp residues buried within globular proteins exhibit a long-lived (millisecond-to-second time range) room-temperature phosphorescence lifetime (τ_0) whose magnitude depends sharply on the local flexibility of the protein matrix around the chromophore [17,18]. In addition, the bimolecular rate constant (kq), derived for the quenching of protein phosphorescence by acrylamide in solution, has recently been correlated with the structural flexibility of the entire macromolecule [19]. In this report the effects of TMAO on both parameters (τ_0 and kq) were determined across a wide temperature range and up to a concentration of 1.8 M, the highest that is normally found in living organisms [20]. The proteins chosen are monomeric apoazurin (Az), dimeric alcohol dehydrogenase (LADH) and alkaline phosphatase (AP) and tetrameric glyceraldehydes-3-phosphate dehydrogenase (GAPDH). Protein systems and temperatures were carefully selected to satisfy the following criteria: (1) full stability with respect to partial unfolding and subunit dissociation; (2) phosphorescence emission from a single and structurally identified Trp per subunit [17]; (3) protein systems providing various degrees of Trp burial within the globular structure in order to compare the effects of TMAO between superfi-

cial regions and deep cores of the macromolecule. The results demonstrate that up to the temperature of thermal unfolding, TMAO influences neither the local nor the average dynamics of these proteins, a finding that is at odds with theoretical expectations.

2. Materials and methods

Acrylamide (> 99.9% electrophoresis purity) was from Bio-Rad Laboratories (Richmond, CA). The proteins, horse liver alcohol dehydrogenase and glyceraldehydes-3-phosphate dehydrogenase, from yeast were supplied by Boheringer (Mannheim, Germany). Alkaline phosphatase from *Escherichia coli* and Trimethylamine *N*-oxide dihydrate were purchased from Sigma Chemical Co (St. Louis, MO). The latter is the highest purity grade available and was used without further purification. Copper-free azurin from *Pseudomonas aeruginosa* was a gift from Prof. Finazzi-Agrò, University of Roma (Tor Vergata, Italy). Water, doubly distilled over quartz, was purified by using a Milli-Q Plus system (Millipore Corp., Bedford, MA). All glassware used for sample preparation was conditioned in advance by standing for 24 h in 10% HCl suprapur (Merck, Darmstadt).

2.1. Sample preparation for phosphorescence measurements:

Prior to phosphorescence measurements all proteins were extensively dialyzed in Tris–HCl (10 mM, pH 7.0). For measurements of the intrinsic phosphorescence lifetime it is paramount to rid the solution of all O_2 traces. Deoxygenation of protein samples was carried out by repeated cycles of mild evacuation followed by inlet of pure nitrogen as described before [21]. The bimolecular quenching rate constant (k_q) was obtained as described before [19] from measurements of the phosphorescence decay at various acrylamide concentrations according to the equation:

$$1/\tau = (1/\tau_0) + k_q[\text{acrylamide}] \quad (1)$$

where τ_0 and τ are the phosphorescence lifetime in the absence and in the presence of a given [acrylamide]. Acrylamide quenching was not carried out with AP because, at the high concentrations (3–5 M) needed, acrylamide tends to polymerize at temperatures above 40°C.

2.2. Luminescence measurements

Phosphorescence decays were obtained with pulsed excitation as provided by a frequency-doubled flash-pumped dye laser (UV500M Candela) tuned at 292 nm. The pulse duration was 1 μs and the light energy per pulse was typically 1–10 mJ. The phosphorescence signal collected at a right angle from the excitation beam was filtered (420–460 nm band pass) and detected by an R928 photomultiplier. An electronic shutter arrangement protected the photomultiplier from the intense fluorescence pulse and permitted the delayed emission to be detected 4 ms after the excitation pulse. Alternatively, for lifetimes shorter than 10 ms, the photomultiplier was protected from the intense fluorescence pulse by a chopper blade that closes the emission slit during the excitation. The time resolution of this apparatus is typically 10 μs [18]. The photocurrent was amplified by a current to voltage converter (SR570, Stanford Research Systems), and digitized by a computerscope system (ISC-16, RC Electronics) capable of averaging multiple sweeps. All phosphorescence decays were analyzed in terms of a sum of exponential components by a non-linear least-squares fitting algorithm (Global Unlimited, LFD, University of Illinois). Lifetime data used in the analysis refers to averages of two or more sample preparations and two independent measurements on each sample. The reproducibility of phosphorescence lifetimes was typically better than 9% between different sample preparations and approximately 3% between repetitive measurements on the same sample. Lifetime measurements at different temperatures were determined on the same sample taking the precaution of checking that at the end of the temperature excursion it yielded the same lifetime as the initial measurement. Decays in super cooled

aqueous solutions, at −10°C, were determined after rapid cooling the sample (100 μl volume in 4-mm I.D. cells) for 4 min in the thermostatted sample holder, a time found to be sufficient for thermal equilibration.

3. Results

3.1. Influence of TMAO on the intrinsic phosphorescence lifetime (τ_o)

The phosphorescence decay of GAPDH, LADH, Az and AP was measured in buffer, before and after the addition of 1.8 M TMAO. An example of raw data is given in Fig. 1a for Azurin and Fig. 1b for GAPDH. The decays of GAPDH and LADH are intrinsically heterogeneous and were found to remain so even in the presence of TMAO. These are adequately fitted in terms of two lifetime components and data comparison was carried out in terms of the average lifetime, $\tau av = \Sigma \alpha_\iota \tau_\iota$, α_ι and τ_ι being the amplitude and the lifetime of each component. At 20°C, significant effects of the osmolyte are observed exclusively with GAPDH, for which the lifetime is reduced to approximately 44% of the value in buffer (Fig. 1b). A smaller τ_ι may reflect an increased flexibility of the protein matrix about the triplet probe (W84) but may also be caused by quenching reactions with trace impurities associated with the TMAO stock. A characteristic feature of quenching reactions is a linear increase in decay rate with quencher concentration. The lifetime Stern–Volmer plot ($1/\tau = 1/\tau_0 + kq$ [TMAO], τ_0 the unperturbed lifetime) for GAPDH as a function of TMAO concentration is shown in Fig. 2. The good linearity of the plot is fully consistent with impurity quenching and therefore the decrease of τ is probably not structurally based. In this respect it may be significant that a reduction of lifetime is found only with GAPDH, the protein with the phosphorescing Trp residue most accessible to long-range quenching interactions from the solvent. Another possibility is that TMAO affect the pK of a His group, the only potentially quenching side-chain within 5 A° of W84. However, the invariance of the quenching process when the solution pH is varied between five and nine apparently rules out this hypothesis.

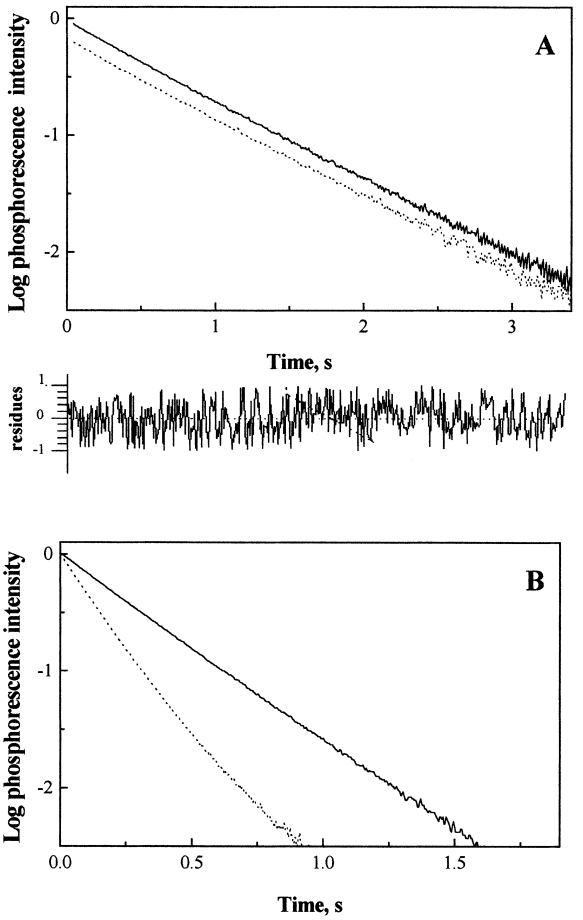

Fig. 1. Trp phosphorescence decay from 1.4 μM Az (a) and 1.2 μM GAPDH (b), in 10 mM Tris–HCl buffer pH 7.0 before (—) and after (---) the addition of 1.8 M TMAO, at 20°C. The plot of residuals refers a monoexponential fitting of Az decay. The Az decay in 1.8 M TMAO (Fig. 1a) has been displaced along the y-axis for a more convenient comparison.

Both internal dynamics and protein stability towards unfolding are highly temperature-dependent. The typical, bell-shaped unfolding free energy change of proteins indicates that in general the globular fold is destabilized by both high and

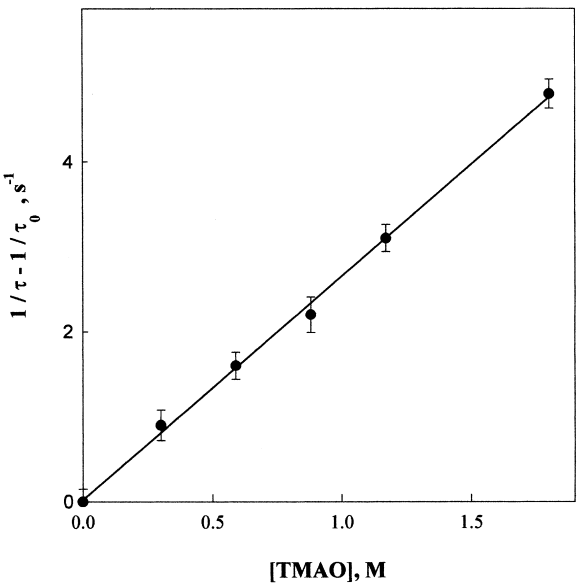

Fig. 2. Influence of TMAO concentration on the phosphorescence lifetime of GAPDH, at 20°C. Each point is the average of at least two independent experiments and the error bars indicate the range of τ variations.

low temperature. To test whether the effects of TMAO on protein structure are selective of a given temperature range and/or of rather structurally loose, unstable states of the protein, the influence of TMAO on the phosphorescence decay was measured for each protein over a wide temperature range, from -10°C (super cooled solutions) up to 40°C for LADH, to 50°C for GAPDH and Az and to 70°C for AP, temperatures in proximity of thermal unfolding. The average lifetimes at selected temperatures are reported in Table 1. The results confirm that, across the entire temperature range, and apart from the persistently smaller τav of GAPDH, the intrinsic lifetime of the other proteins is, within experimental error, unchanged by TMAO. It should be noted that the approximately 20–100-fold decrease of τ_0 across the thermal excursion entails a dramatic gain in structural flexibility of these proteins that can only derive from looser, thermally expanded structures. Therefore, the intrinsic lifetime shows that throughout, TMAO is unable to compact the native fold and render it more rigid.

3.2. Effects of TMAO on acrylamide quenching of protein phosphorescence

In addition to τ_0, an alternative and complementary way to assess the flexibility of globular proteins is by monitoring the diffusion of small quenching molecules through the protein matrix to the site of the chromophore. In particular, quenching of protein phosphorescence by acrylamide was shown to be a sensitive indicator of their flexibility, the bimolecular quenching rate constant, kq, derived from the lifetime Stern-Volmer plot ($1/\tau = 1/\tau_0 + kq$ [acrylamide]) providing a measure of the average acrylamide diffusion coefficient inside the macromolecule [19].

The addition of acrylamide to protein solutions reduces the phosphorescence lifetime of each protein examined. In these experiments the acrylamide concentration was increased until the

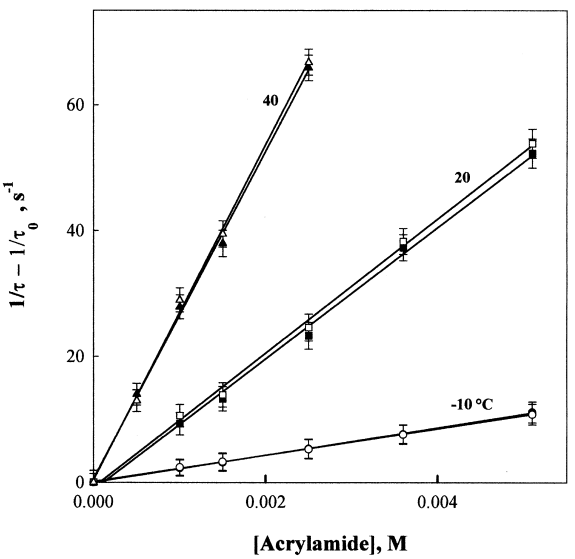

Fig. 3. Lifetime Stern–Volmer plots for the quenching of LADH phosphorescence by acrylamide, at selected temperatures, with (○, □, △) and without (●, ■, ▲) 1.8 M TMAO. Each point is the average of at least two independent experiments and the error bars indicate the range of τ variations.

Table 1
Influence of TMAO on the intrinsic, average phosphorescence lifetime and on the acrylamide bimolecular phosphorescence quenching rate constant of Az, LADH, GAPDH and AP, at selected temperatures

Protein	TMAO (M)	T (°C)	τ_0 (s)	k_q (s^{-1}, M)
AP	0	−10	4.03	
	1.8	−10	3.96	
	0	20	2.02	
	1.8	20	2.10	
	0	70	0.054	
	1.8	70	0.053	
Az	0	−10	3.43	1.2
	1.8	−10	3.52	1.5
	0	20	0.63	1.9×10^1
	1.8	20	0.62	1.9×10^1
	0	50	0.050	2.5×10^2
	1.8	50	0.051	2.4×10^2
LADH	0	−10	2.5	2.1×10^3
	1.8	−10	2.4	2.0×10^3
	0	20	0.59	1.0×10^4
	1.8	20	0.60	1.1×10^4
	0	40	0.11	2.6×10^4
	1.8	40	0.11	2.7×10^4
GAPDH	0	−10	0.734	2.1×10^2
	1.8	−10	0.320	1.9×10^2
	0	20	0.308	5.9×10^2
	1.8	20	0.135	5.9×10^2
	0	50	0.077	2.7×10^3
	1.8	50	0.036	2.7×10^3

lifetime decreased at least ten-fold. The decay of GAPDH and LADH, which are intrinsically heterogeneous, was found to remain so even when the quencher considerably reduces the average τ. A non-uniform decay reflects the presence of more than one stable conformation of the macromolecule, each with its distinct lifetime and acrylamide quenching constant, because in each protein the phosphorescence at ambient temperature is due to a single Trp residue per subunit [17]. For convenience, the lifetime Stern-Volmer plots were all constructed from the average lifetime obtained, in general, from a biexponential fitting of phosphorescence decays. Thus, the value of *kq* derived from the gradient of these plots is an average quantity.

Representative lifetime Stern–Volmer plots for LADH, in buffer and in the presence of 1.8 M acrylamide, at selected temperatures, are shown in Fig. 3. It is evident from the figure that, whereas the slope of the plot increases sharply with temperature, implying a considerable gain in structural flexibility, the addition of TMAO has negligible effects on the quenching process at any temperature investigated. This behavior is common also to Az and GAPDH, the other two proteins examined. As can be seen from a comparison of *kq* values collected in Table 1, TMAO effects are negligible throughout. The general conclusion is that over the wide temperature range of this study TMAO does not influence acrylamide migration through these proteins and, con-

sequently, that it does not attenuate the frequency of the relatively large amplitude structural fluctuations that permit its diffusion through the protein matrix, a finding in full accord with the intrinsic lifetime. Lastly, the observation that TMAO does not enhance acrylamide quenching of GAPDH supports the hypothesis that the reduction caused to the lifetime derives from impurity quenching rather than from a loosening of the structure.

4. Conclusions

The purpose of this investigation was to unveil possible perturbations of the native fold of globular proteins by TMAO at the highest concentrations found in the cytoplasm under extreme conditions. Changes in the globular fold could be induced by: (1) Structural adjustments aimed at reducing the solvent exposed surface area. These include compaction of internal cavities, withdrawal of internal water molecules and a shift to conformations with a smaller surface area. (2) Direct interaction between TMAO and protein groups. Whereas the effects of TMAO binding on the protein structure are not predictable, both the reduction in free volume and of internal hydration would necessarily lead to a more compact and rigid structure. Moreover, as the size of the protein increases with thermal expansion of its cavities, the structure compacting effect of the osmolyte would be expected to be greater at higher temperature, an effect analogous to that observed with the application of hydrostatic pressure [22,23]. Employing a natural probe that exhibits unparalleled sensitivity for subtle changes in protein structure/dynamics [17,24], this study has examined the effects of 1.8 M TMAO on the internal dynamics of four proteins both locally, at the site of the probe (τ_0), and over extended regions of the macromolecule (kq). The choice of protein systems was aimed at exploring possible correlations between osmolyte effects and structural features of the biopolymer such as: (1) The depth dependence of osmolyte pressure by varying the thickness of the protein spacer separating the probe from the aqueous interface (4.5 Å for LADH [25], 5.0 Å for GAPDH [26], 8 Å for Az [27] and 11.5 Å for AP [28]). (2) The actual flexibility of the macromolecule, as can be inferred from the wide range of phosphorescence lifetimes ($0.036\ s < \tau_0 < 4.03\ s$) and of acrylamide quenching constants ($1.2\ s^{-1} < k_q < 2.710^4\ M^{-1}\ s^{-1}$). (3) The role of the quaternary structure by comparing dimeric LADH and AP or tetrameric GAPDH to monomeric Az. Except for the intrinsic lifetime of GAPDH, all phosphorescence measurements reported here, however, consistently demonstrate that up to 1.8 M TMAO does not affect the internal dynamics of these proteins even up to temperatures close to thermal denaturation. From what has been said above this finding is rather unexpected. Moreover, it also contrasts with the distinct dampening of large amplitude structural fluctuations observed for other osmolytes such as sucrose on RNase A [5] and glycerol on myoglobin and lysozyme [29–31] (inferred from H/D exchange studies) as well as by glycerol on 3 (Az, LADH and AP) of the proteins of this study (inferred from the phosphorescence lifetime) [32]. Evidently, a major difference between TMAO and these polyols is that the latter are viscogenic compounds and, therefore, there is the possibility that the decreased flexibility of the protein be due to the coupling of protein motions to solvent viscosity [33–35].

In conclusion, the above results emphasize that at physiological concentrations TMAO does not perturb the dynamics of the native protein fold. This could be a peculiar feature of this osmolyte that was selected for preserving the catalytic efficiency of enzymes in conditions of osmotic stress. Such inability of TMAO to compact globular proteins is, however, at odds with the expectations based on a preferential exclusion mechanism. The different behavior of polyols raises the possibility that the mechanism of protein stabilization by different osmolytes may not be universal. Finally, as the structure of the folded state is not detectably affected by TMAO its remarkable stabilizing action against protein unfolding must be attributed predominantly to a selective solvophobic effect on the open, denatured state.

References

[1] P.H. Yancey, M.E. Clark, S.C. Hand, R.D. Bowlus, G.N. Somero, Living with water stress: evolution of osmolytes system, Science 217 (1982) 1214–1222.

[2] L.J. Borowitzka, in: R. Gilles, M. Gilles-Baillien (Eds.), Transport processes, iono- and osmoregulation, glycerol and other carbohydrate osmotic effectors, Springer-Verlag, Berlin, 1985, p. 437.

[3] A. Pollard, R.G. Wyn Jones, Enzyme activities in concentrated solutions of glycinebetaine and other solutes, Planta. 144 (1979) 291–298.

[4] R.D. Bowlus, G.N. Somero, Solute compatibility with enzyme function and structure: rationales for selection of osmotic agent and end-product of anaerobic metabolism in marine invertebrates, J. Exp. Zool. 208 (1979) 137–151.

[5] A. Wang, A.D. Robertson, D.W. Bolen, Effect of a naturally occurring compatible on the internal dynamics of ribonuclease A, Biochemistry 34 (1995) 15096–15104.

[6] A. Wang, D.W. Bolen, Effect of proline on lactate dehydrogenase activity: testing the generality and scope of the compatibility paradigma, Biophys. J. 71 (1996) 2117–2122.

[7] T.Y. Lin, S.N. Timasheff, Why do some organisms use a urea-methylamine mixture as osmolytes: thermodynamic compensation of urea and Trimethylamine N-oxide interactions with protein, Biochemistry 33 (1994) 12695–12701.

[8] A. Wang, D.W. Bolen, A natural occurring protective system in urea-rich cells: mechanism of osmolytes protection of protein against urea denaturation, Biochemistry 36 (1997) 9101–9108.

[9] I. Baskakov, D.W. Bolen, Time-dependent effects of Trimethylamine N-oxide/urea on lactate dehydrogenase activity: an unexplored dimension of the adaptation paradigm, Biophys. J. 74 (1998) 2655–2658.

[10] I. Baskakov, A. Wang, D.W. Bolen, Trimethyl-N-oxide counteracts urea effects on rabbit muscle lactate dehydrogenase function: a test of the counteraction hypothesis, Biophys. J. 74 (1998) 2666–2673.

[11] T. Arakawa, S.N. Timasheff, The stabilization of proteins by osmolytes, Biophys. J. 47 (1985) 411–414.

[12] Y. Liu, D.W. Bolen, The peptide backbone plays a dominant role in protein stabilization by naturally occurring osmolytes, Biochemistry 34 (1995) 12884–12891.

[13] Y. Qu, C.L. Bolen, D.W. Bolen, Osmolyte-driven contraction of a random coil protein, Proc. Natl. Acad. Sci. U.S.A. 95 (1998) 9268–9273.

[14] I. Baskakov, D.W. Bolen, Forcing thermodynamically unfolded proteins to fold, J. Biol. Chem. 273 (1998) 4831–4834.

[15] I. Baskakov, R. Kumar, G. Srinivasan, Y. Ji, D.W. Bolen, E.B. Thompson, Trimethylamine N-oxide induced cooperative folding of an intrinsically unfolded transcription-activating fragment of human glucocorticoid receptor, J. Biol. Chem. 274 (1999) 10693–10696.

[16] O. Gursky, Probing the conformation of a human apolipoprotein C–1 by amino acid substitutions and Trimethylamine-N-oxide, Protein Sci. 8 (1999) 2055–2064.

[17] M. Gonnelli, G.B. Strambini, Phosphorescence lifetime of tryptophan in proteins, Biochemistry 34 (1995) 13847–13857.

[18] G.B. Strambini, M. Gonnelli, Tryptophan phosphorescence in fluid solution, J. Am. Chem. Soc. 117 (1995) 7646–7651.

[19] G.B. Strambini, P. Cioni, Acrylamide quenching of protein phosphorescence as a monitor of structural fluctuations in the globular fold, J. Am. Chem. Soc. 121 (1999) 8337–8344.

[20] R.E. MacMillen, A.K. Lee, Australian desert mice: independence of exogenous water, Science 158 (1967) 383–385.

[21] G.B. Strambini, Quenching of alkaline phosphatase phosphorescence by O_2 and NO. Evidence for inflexible regions of protein structure, Biophys. J. 52 (1987) 23–28.

[22] P. Cioni, G.B. Strambini, Pressure-temperature effects on protein flexibility from acrylamide quenching of protein phosphorescence, J. Mol. Biol. 291 (1999) 955–964.

[23] G.B. Strambini, P. Cioni, Pressure-temperature effects on oxygen quenching of protein phosphorescence, J. Am. Chem. Soc. 121 (1999) 8337–8344.

[24] V. Subramaniam, N.C. Bergenhem, A. Gafni, D.G. Steel, Phosphorescence reveals a continued slow annealing of the protein core following reactivation of *Escherichia coli* alkaline phosphatase, Biochemistry 34 (1995) 1133–1336.

[25] H. Eklund, B. Nordstrom, E. Zeppezauer, Three-dimensional structure of horse liver alcohol dehydrogenase at 2.4 angstroms resolution, J. Mol. Biol. 102 (1976) 27–59.

[26] T. Skarzynski, P.C.E. Moody, A.J. Wonacott, Structure of holo-glyceraldehyde-3-phosphate dehydrogenase from Bacillus stearothermophilus at 1.8 resolution, J. Mol. Biol. 193 (1987) 171–187.

[27] E.T. Adman, L.H. Jensen, Structural features of azurin at 2.7 angstroms resolution, Isr. J. Chem. 21 (1981) 8–12.

[28] E.E. Kim, H.W. Wyckoff, Reaction mechanism of alkaline phosphatase based on crystal structures. Two-metal ion catalysis, J. Mol. Biol. 218 (1991) 449–464.

[29] D.G. Knox, A. Rosenberg, Fluctuations of protein structure as expressed in the distribution of hydrogen exchange rate constants, Biopolymers 19 (1980) 1049–1068.

[30] D.B. Calhoun, S.W. Englander, Internal protein motions, concentrated glycerol, and hydrogen exchange studied in myoglobin, Biochemistry 24 (1985) 2095–2100.

[31] R.B. Gregory, The influence of glycerol on hydrogen

isotope exchange in lysozyme, Biopolymers 27 (1988) 1699–1709.
[32] M. Gonnelli, G.B. Strambini, Glycerol effects on protein flexibility: a tryptophan phosphorescence study, Biophys. J. 65 (1993) 131–137.
[33] B. Gavish, M.M. Werber, Viscosity-dependent structural fluctuation in enzyme catalysis, Biochemistry 18 (1979) 1269–1275.
[34] B. Gavish', Position-dependent viscosity effect on rate coefficient, Phys. Rev. Lett. 44 (1980) 1160–1163.
[35] W. Doster, Viscosity scaling and protein dynamics, Biophys. Chem. 17 (1983) 97–103.