Time-Dependent Effects of Trimethylamine-*N*-Oxide/Urea on Lactate Dehydrogenase Activity: An Unexplored Dimension of the Adaptation Paradigm

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ABSTRACT Given that enzymes in urea-rich cells are believed to be just as sensitive to urea effects as enzymes in non-urea-rich cells, it is argued that time-dependent inactivation of enzymes by urea could become a factor of overriding importance in the biology of urea-rich cells. Time-independent parameters (e.g. T_m, k_{cat}, and K_m) involving protein stability and enzyme function have generally been the focus of inquiries into the efficacy of naturally occurring osmolytes like trimethylamine-*N*-oxide (TMAO), to offset the deleterious effects of urea on the intracellular proteins in the urea-rich cells of elasmobranchs. However, using urea concentrations found in urea-rich cells of elasmobranches, we have found time-dependent effects on lactate dehydrogenase activity which indicate that TMAO plays the important biological role of slowing urea-induced dissociation of multimeric intracellular proteins. TMAO greatly diminishes the rate of lactate dehydrogenase dissociation and affords significant protection of the enzyme against urea-induced time-dependent inactivation. The effects of TMAO on enzyme inactivation by urea adds a temporal dimension that is an important part of the biology of the adaptation paradigm.

INTRODUCTION

The biology of adaptation involves the study of organisms that have adapted to environmental stresses such as extremes of temperature, dehydration, contact with high-salt solutions, and even the presence of intracellular concentrations of urea (Yancey et al., 1982). Organisms that have adapted to these stresses appear to concentrate small organic compounds, called osmolytes (Brown and Simpson, 1972; Stewart and Lee, 1974; Yancey et al., 1982), whose intracellular concentration in the range of several hundred millimolar is believed to have two defining characteristics: 1) the osmolytes have the ability to stabilize cellular proteins against the inactivating stress for which the osmolytes were naturally selected, and 2) the osmolytes do not greatly perturb the functional activities of proteins or otherwise upset the delicate metabolic control systems necessary to sustain life (Somero, 1986; Yancey et al., 1982; Yancey and Somero, 1980). These characteristics focus on the thermodynamic stability and function of proteins in the face of environmental stresses, and form the paradigm for discussing osmolyte involvement in the biology of adaptation. Nature's use of osmolytes in the adaptation of organisms to environmental stresses involves one of the most elusive and long-standing problems in biology, the general problem of how proteins and solvent interact to produce biologically significant effects.

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Up to the present, we have focused on one of the two defining characteristics of osmolytes, namely the mechanism by which osmolytes stabilize proteins thermodynamically against denaturing stresses. Our results provide strong evidence that the unfavorable interaction of osmolytes with the peptide backbone of protein is responsible for the thermodynamic stabilization (Wang and Bolen, 1997). But in the work described here, we address the second defining characteristic of osmolyte action. Namely, we investigate the ability of the naturally occurring osmolyte trimethylamine *N*-oxide (TMAO) to offset the deleterious effects of the 400–600 mM intracellular urea concentrations in the cells of elasmobranchs.

Many of the efforts to investigate the effects of osmolytes on protein function have focused on k_{cat}, K_m, and K_i parameters of enzymes (Burg et al., 1996; de Meis, 1988; Gopal and Ahluwalia, 1993; Lin and Timasheff, 1994; Santoro et al., 1992; Wang and Bolen, 1996; Yancey and Somero, 1979, 1980). These parameters are assumed to be time-independent, but the manner in which such data are collected can unwittingly incorporate time-dependent effects into the measurements. In the course of our investigations, we discovered time-dependent effects of both urea and TMAO on lactic dehydrogenase (LDH) activity that definitely affect evaluations of k_{cat} and K_m (Baskakov et al.; see companion paper). If catalytic activity of an enzyme is not maintained for a reasonable time while the enzyme is bathed in the milieu containing urea, TMAO, and urea/TMAO mixtures as it would be in urea-rich cells, the issues of "protein stabilization" and apparent k_{cat} and K_m effects become moot as an adaptive strategy. Clearly, the question of the lifetime of proteins in the presence of the denaturing stress and (protective) osmolytes has considerable bearing on how effective osmolytes are in the biology of adaptation.

With the goal of better understanding the requirements for TMAO stabilization of proteins in the presence of urea, we have investigated the time-dependent effects of urea and TMAO on rabbit muscle LDH stability and function. LDH is essential for a large number of organisms, and like many multisubunit proteins it is rather labile (Cho and Swaisgood, 1973). The effect of denaturing stress and osmolyte concentration on the lifetime of enzyme activity adds a different dimension to the discussion of the biology of adaptation and the issue of defining the temporal response to inactivating stress in the cell.

EXPERIMENTAL PROCEDURES

Chemicals

Rabbit muscle lactate dehydrogenase, NADH, sodium pyruvate, bovine serum albumin, and TMAO were purchased from Sigma; trisma base was from Fisher; sodium chloride was from Mallinckrodt; and ultrapure urea was from ICN. Further purification of urea and TMAO and determinations of their concentrations were performed as described in an earlier paper (Wang and Bolen, 1997). The concentration of LDH was determined spectrophotometrically at 280 nm (1.13 mg/mL/OD; supplied by Worthington).

Measurement of LDH activity

LDH activity was determined by following the oxidation of NADH to NAD^+ at 340 nm. The standard reaction mixture contained 2.5 mM pyruvate, 85 μM NADH in 0.2 M Tris-HCl buffer (pH 7.3) at 24°C.

Time-dependent activity measurements

Aliquots of concentrated LDH stock solution were added to solutions containing 0.2 M Tris-HCl buffer (pH 7.3), 1 mg/ml bovine serum albumin (BSA), and desired amounts of either urea, TMAO, or urea plus TMAO. These solutions were incubated on ice and, with respect to time of incubation, the LDH activity was monitored using initial velocities by addition of aliquots of the incubating solution to standard assay mixtures. The pH values of Tris-HCl buffer solutions to be used at 0°C were adjusted to pH 7.3 at 5°C, and those to be used at 24°C were adjusted to pH 7.3 at 25°C.

Reactivation of urea-inactivated LDH

Time-dependent loss of LDH activity was induced by incubation of LDH (13.4 μg/ml) in 0.2 M Tris-HCl buffer (pH 7.3) containing 0.8 M urea and 1 mg/ml BSA at 0°C. To initiate reactivation from the inactivating effects of urea, aliquots of the urea-containing incubation mixture were taken with time and diluted 10-fold into 0.2 M Tris-HCl buffer (pH 7.3), containing 1 mg/ml BSA held at 24°C. In the course of incubation at 24°C, aliquots from this solution were withdrawn and initial velocities were assayed in the standard reaction mixture. Two different procedures were used to initiate reactivation. In one, aliquots of the enzyme in 0.8 M urea at 0°C were withdrawn and diluted 10-fold into Tris-HCl/BSA buffer equilibrated at 24°C as described above. In the second procedure, aliquots of enzyme were added to the Tris-HCl/BSA buffer equilibrated at 0°C, and then rapidly (within 1 min) warmed to 24°C. No differences in the final level of LDH activity were detected with these two procedures, but the second method allowed us to follow the initial time dependence of reactivation, and this procedure, being more convenient, was used exclusively in the work described.

Determination of the apparent order of reactivation

These experiments were performed with an LDH sample that had been incubated for 24 h with 0.8 M urea (0.2 M Tris-HCl buffer, pH 7.3) at 0°C. Different amounts of this urea-containing solution were withdrawn and diluted into assay mixtures containing 5 mM pyruvate and 180 μM NADH (0.2 M Tris-HCl, pH 7.3) to obtain 340 nm absorbance versus time data, with a range of LDH concentrations from 0.0134 to 0.178 μg/ml. The first derivatives of the absorbance versus time curves show that NADH oxidation increases with time in the assay, reflecting a reactivation of LDH during the course of the assay. The initial rates of LDH reactivation, observed at different enzyme concentrations, were determined from the initial slopes of these first derivative curves and reported as the initial rates of reactivation. These data were fitted to a linearized form of the equation, velocity $= kc^n$, as suggested by van't Hoff, viz., $\log v = \log k + n \log c$, where k is the rate constant, n is the reaction order, and c is the concentration of LDH (Laidler, 1965). The reaction order is obtained from the slope of the log v versus log c plot.

Gel filtration

The experiments were carried out using a Phenomenex Biosep SEC-S3000 high-performance liquid chromatography (HPLC) gel filtration column with dimensions of 300 × 7.80 mm. LDH samples (13.4 μg/ml) were incubated in gel filtration buffer (0.10 M Tris-HCl containing 0.2M NaCl, pH 7.3) at 0°C with either 0.8 M urea alone or a mixture of 0.8 M urea plus 0.8 M TMAO. In the course of incubation, aliquots were removed, and apparent molecular masses were evaluated using the Phenomenex gel-filtration HPLC column equilibrated in the presence of either 0.8 M urea or 0.8 M urea plus 0.8 M TMAO containing gel filtration buffer at 22°C. The Phenomenex column was calibrated in the absence of urea and urea/TMAO mixture and in the presence of 0.8 M urea, using Sigma gel filtration molecular mass standards (MW-GF-200) containing blue dextran (2.000 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), and cytochrome *c* (12.4 kDa). The steel-jacketed column was operated with mechanical injection within a fully automated BioCad SPRINT HPLC system, which allowed the elution volume to be repeatable within ±0.015 ml.

RESULTS

Inactivation of LDH by urea

Fig. 1 shows data on the time-dependent inactivation of LDH in the course of incubation with different concentrations of urea. Two different protocols for testing enzyme activity in the course of incubation can be used, one in which the assay mixtures contained the same concentrations of urea as those in the incubation samples, and a second in which the common standard assay mixture did not contain urea. The two cases give different absolute velocities, but by reporting the velocity at a given incubation time divided by the velocity at zero time of incubation, the resulting percentages of initial activity for the two protocols were found to be identical. The second protocol was experimentally simpler, and the data from this protocol are reported in Fig. 1.

Upon incubation at 0°C in the absence of urea, LDH loses activity with a half-time of $\sim 10^5$ min. Inclusion of urea in the incubation mixture greatly accelerates the processes of inactivation, such that in 0.8 M urea, LDH is inactivated with a half-time of ~40 min.

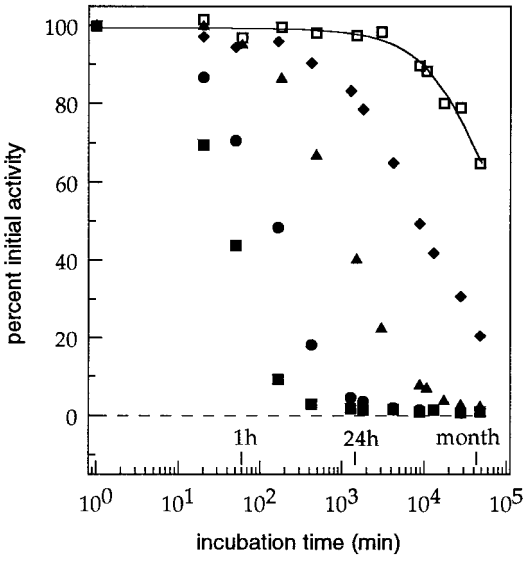

FIGURE 1 Time course of LDH inactivation in the presence and absence of urea. LDH (1.34 μg/ml) activity versus time of incubation at 0°C in 0.2 M Tris-HCl buffer containing 1 mg/ml BSA, pH 7.3, without urea (□), and with 0.2 M (♦), 0.4 M (▲), 0.6 M (●), and 0.8 M (■) urea. Residual LDH activity is presented as a percentage of the specific activity measured at zero time of incubation.

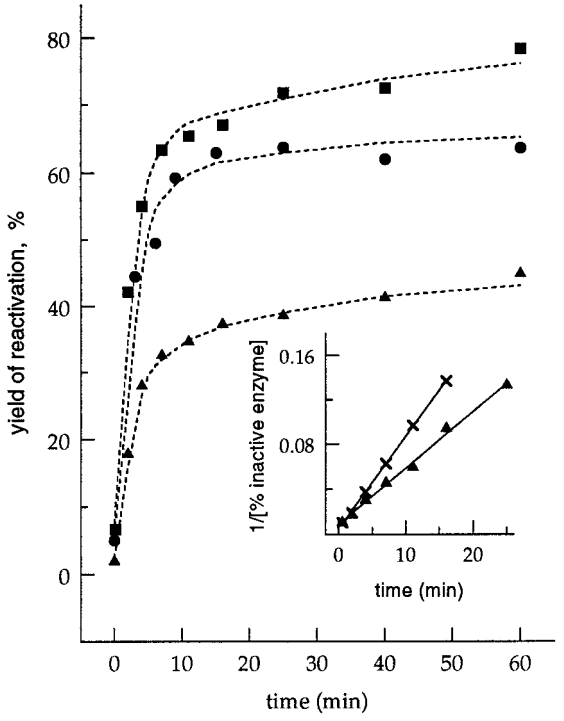

FIGURE 2 Kinetics of LDH reactivation. LDH (13.4 μg/ml) was incubated at 0°C in 0.2 M Tris-HCl buffer containing 1 mg/ml BSA, pH 7.3, in the presence of 0.8 M urea for 23, 48, and 96 h, at which times an aliquot was removed and diluted 10-fold into the buffer alone at 0°C. Then the sample was warmed to 24°C within 1 min, and aliquots were assayed with respect to the time of 24°C incubation. The results of the activity measurements are shown as the percentage yield of reactivated enzyme at 24°C after exposure to 0.8 M urea for 23 h (■), 48 h (●), and 96 h (▲) at 0°C. The inset shows a second-order plot of the 96-h LDH data (▲) given in the main figure. Also shown in the inset is a second-order plot of the reactivation of LDH that had been exposed for 96 h at 0°C to 0.8 M urea, then reactivated at 24°C in the presence of 2.5 mM pyruvate and 80 μM NADH (×).

Reversibility and reactivation kinetics

To obtain more insight into the nature of the time-dependent urea-induced LDH inactivation, the reversibility of the inactivation was studied. Reversibility was tested by withdrawing aliquots of LDH incubated for a specified period of several hours in 0.8 M urea at 0°C, then diluting the aliquots 10-fold into buffer solution at 0°C. Because reactivation was found to depend significantly on temperature, with an increase in the temperature from 0°C to 24°C promoting both the rate and the yield of reactivation (data not shown), the reactivation was initiated by warming the samples from 0 to 24°C within a period of 1 min. The activity of the enzyme in the diluted solution at 24°C was monitored with time of incubation at 24°C, using initial velocity measurements. Fig. 2 presents data on LDH reactivation kinetics as a function of LDH exposure to 0.8 M urea at 0°C for 23, 48, and 96 h. We note that after 23, 48, or 96 h at 0°C, the residual LDH activities do not exceed 2% of initial activity (see data in Fig. 1). As indicated by the data in Fig. 2, LDH inactivated by 0.8 M urea incubation at 0°C for $\geq$23 h can be recovered, but the level of reactivation is incomplete and depends upon the time LDH remains in 0.8 M urea at 0°C; the longer the incubation time, the lower the extent of reactivation.

The inset in Fig. 2 gives a replot of 96-h reactivation data in the form of a second-order kinetic plot. The percentage of inactive enzyme is evaluated from the relationship % inactive $= 100(A_\infty - A_t)/(A_\infty)$, where A_t is the activity (ΔOD/min) at time t, and A_∞ is the activity (ΔOD/min) at infinite time of reaction, here taken as 0.044 ΔOD/min, the activity after 60 min of reactivation time. The linearity of the second-order (1/[% inactive enzyme] versus time) plot in the inset of Fig. 2 suggests LDH reactivation may follow second-order kinetics. Furthermore, apparent second-order kinetics was observed regardless of whether reactivation was performed in the presence or absence of NADH and pyruvate, with the presence of the coenzyme and substrate increasing the rate of reactivation but not affecting the apparent reaction order.

A possible complication to the measurements is the presence of bovine serum albumin (BSA) in the inactivation and reactivation solutions at concentrations 100-1000-fold higher than that of LDH. BSA is commonly added to prevent LDH loss due to adsorption to glass or loss by surface denaturation. To determine the concentration dependence of LDH reactivation and to ensure that BSA does not affect the apparent order of reactivation, we performed additional experiments without BSA over a range of LDH concentrations. From such data it is possible to determine the apparent order of reactivation by using the generalized expression $\log(V) = \log(k) + n\cdot\log(C)$, where V is the initial rate of reactivation, C is LDH concentration, and n is the

apparent reaction order (Laidler, 1965). Varying concentrations of LDH inactivated with 0.8 M urea for 24 h were placed in assay mixtures containing high concentrations of pyruvate and NADH, and the 340-nm absorbance change was monitored with time as described in the Experimental Procedures. Fig. 3 *A* shows the results of such assays, and it is clear from the acceleration of absorbance change during the time of the assay that LDH was being reactivated during the course of the assay. The time derivative of these absorbance versus time curves as shown in Fig. 3 *B* defines the rate of LDH reactivation at 24°C, and the initial slope of the derivative plot at each LDH concentration is taken as the initial rate of active LDH appearance within the assay time. A ratio is taken of each of the initial velocities to the initial velocity at the lowest LDH concentration, to give the relative initial velocities for the assays. A ratio is also taken of each LDH concentration to that of the lowest LDH concentration to give the relative total LDH concentrations in the assay mixtures, and a log (relative initial velocity) versus log (relative LDH concentration) as specified by the general expression given above is shown in Fig. 4. The final LDH concentrations ranged from 0.0134 μg/ml to 0.178 μg/ml, giving corresponding relative reactivation velocities covering a 250-fold change in velocity. The slope of the plot (n = 2.05 $\pm$ 0.08) shows the reactivation to be second order, in agreement with the results in the inset of Fig. 2. Regardless of whether BSA is present (Fig. 2, *inset*) or absent (Fig. 4), reactivation appears to be second order, indicating that the order of the reactivation reaction does not depend upon the presence of BSA.

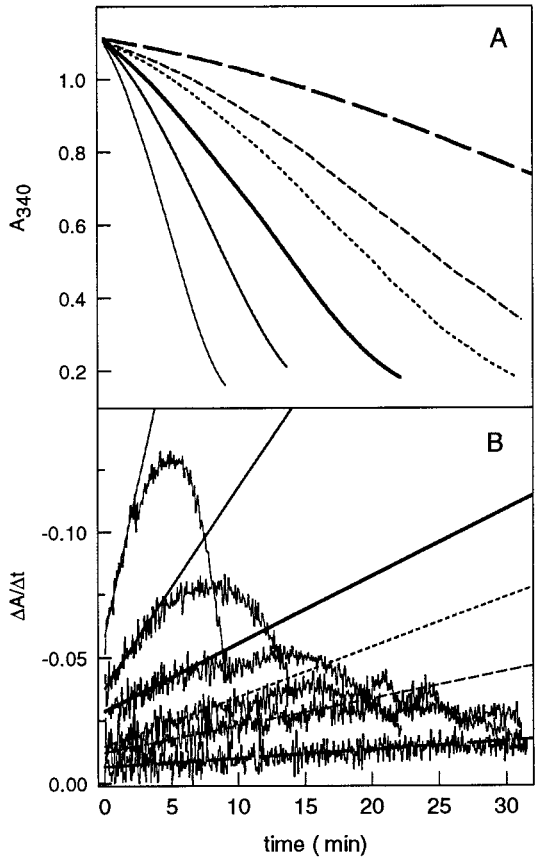

FIGURE 3 LDH reactivation dependence on LDH concentration. (*A*) Representative assays as a function of LDH concentration. The LDH solutions had been ≥98% inactivated by incubation of LDH (13.4 μg/ml in 0.2 M Tris-HCl, pH 7.3) for 24 h in 0.8 M urea at 0°C. Aliquots of this incubation were diluted from 50- to 1000-fold with the buffer at 0°C, and then assayed in the presence of high concentrations of substrates at 24°C. Final LDH concentration in μg/ml, from left to right: 0.178, 0.148, 0.0893, 0.0446, 0.0223, and 0.0134. (*B*) First derivatives of the 340 nm absorbance versus time curves of the assays in *A* were obtained, and the slopes at time 0 of the derivative plots were taken to represent the initial rates of appearance of reactivated LDH as a function of LDH concentration.

Subunit dissociation

Gel filtration experiments were performed to test whether time-dependent LDH inactivation by urea is caused by dissociation of the tetrameric form of the enzyme. The gel filtration column was calibrated with molecular mass standards, conducted in the presence and absence of 0.8 M urea, and identical calibration plots relating molecular mass to elution volumes were obtained. This demonstrates that the permeation properties of the gel and the integrity of the molecular mass standards do not change significantly in the presence of 0.8 M urea.

Data on the evaluation of LDH apparent molecular mass as a function of incubation time in 0.8 M urea are presented in Fig. 5 *A*. With time of incubation, the relative amount of protein corresponding to the major peak (*left peak*) at zero

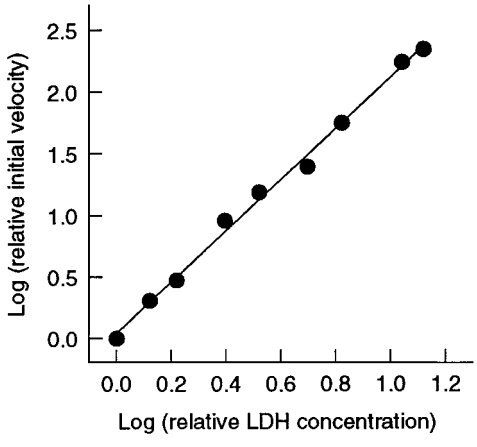

FIGURE 4 van't Hoff plot of the rate of appearance of reactivated LDH as a function of LDH concentration. The rates of appearance of reactivated LDH were obtained from Fig. 3 *B*. To convert to relative initial velocities, the rate of appearance of LDH activity at the LDH concentration in question is divided by the rate of appearance of reactivated LDH obtained for the lowest concentration of LDH (viz. 0.0134 μg/ml). The relative LDH concentration was obtained by dividing the LDH concentration in question, by the lowest LDH concentration. The highest concentration of protein represented is 0.178 μg/ml. A log-log (van't Hoff) plot is presented of the final relative concentrations of LDH present in the assays versus the initial relative rates of appearance of active LDH. The slope of the plot (n = 2.05 $\pm$ 0.08) represents the apparent reaction order of the reactivation process.

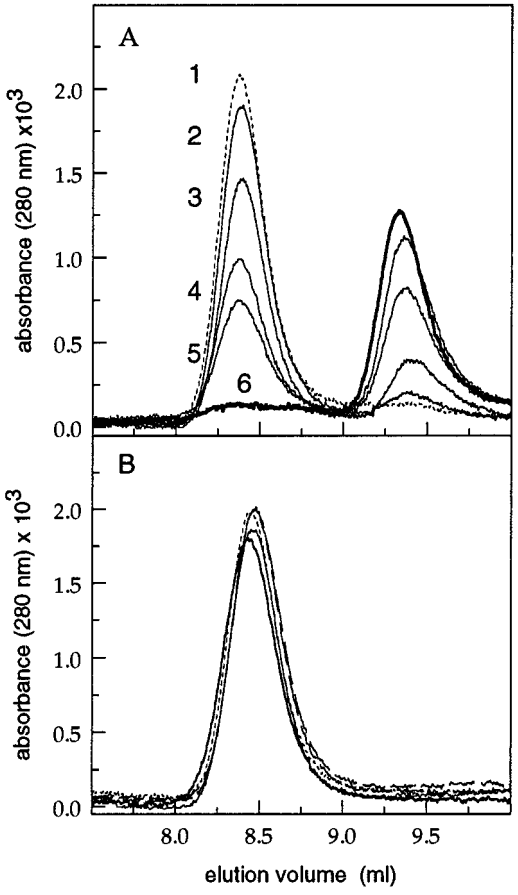

FIGURE 5 Gel filtration of LDH incubated with 0.8 M urea or a 0.8 M urea:0.8 M TMAO mixture as a function of time. The apparent molecular mass of LDH was estimated with a Phenomenex Biosep SEC-S3000 HPLC gel filtration column in gel filtration buffer (0.10 M Tris-HCl, pH 7.3, with 0.2 M NaCl) at 22°C. (*A*) The column was equilibrated with 0.8 M urea in the gel filtration buffer at 22°C, and elution volumes of LDH (13.4 μg/ml) were monitored as a function of the time of incubation in 0.8 M urea at 0°C for 5 min. (·····) (1); 40 min. (2); 80 min. (3); 160 min. (4); 240 min. (5); 1230 min. (——) (6). (*B*) The column was equilibrated with a 0.8 M urea:0.8 M TMAO mixture in 0.1 M Tris-HCl (pH 7.3) with 0.2 M NaCl at 22°C, and elution volumes of LDH (13.4 μg/ml) were monitored as a function of the indicated time of incubation in the urea: TMAO mixture at 0°C, 5 min. (·····); 80 min.(- - -); 240 min. (– – –); 375 min. (——); 1380 min. (——)

time (elution volume 8.36 ml) is observed to decrease concomitantly with an increase in the area of the peak at the larger elution volume (*right peak in figure*). The left peak essentially disappears after 1280 min of incubation with urea (Fig. 5 *A*) at 0°C. The sum of the areas of the two peaks decreases with time, suggesting the species at the larger elution volume has a lower absorptivity than the species with small elution volume. The position of the left peak does not change with time of incubation, whereas the right peak shifts slightly with time in a direction consistent with higher molecular mass species.

The same type of gel filtration experiment was performed with LDH incubated in the presence of a 0.8 M TMAO:0.8 M urea mixture (Fig. 5 *B*). Here LDH incubated for up to 1380 min in the TMAO:urea mixture appears as a single peak with an elution volume essentially the same as that of the initial chromatogram in Fig. 5 *A*. These results suggest that at LDH concentration as low as 13.4 μg/ml, urea causes time-dependent changes in the quaternary structure of LDH, whereas the addition of TMAO to the urea prevents these changes from occurring.

To define the elution positions of the enzyme in the highest and lowest states of association, the dependencies of apparent molecular mass on LDH concentration in the presence and absence of 0.8 M urea were studied. In both experiments (with and without urea), the major peak (V_e = 8.36 ml) contained more than 99% of total absorption, and two very minor peaks were detected (Table 1), one with an apparent molecular mass approximately that of an octamer, and the other with an apparent molecular mass near that of a single subunit. The species composing the major peak has an apparent molecular mass of ~100 kDa, which is between the molecular mass of a tetramer (144 kDa) and a dimer (72 kDa). An apparent intermediate molecular mass is suggestive of a tetramer-dimer equilibrium that is rapid relative to the chromatographic time scale, with an average elution volume composed of the weighted averages of the forms in equilibrium. If this interpretation applies, the elution volume will shift toward the dimer elution volume with decreasing LDH concentration. It was found, however, that the elution volume and the ratio of the major peak to the minor peak corresponding to a monomer do not depend on the concentrations of enzyme in the range 5.2–670 μg/ml either with or without urea (see Table 1). This suggests that the reason for deviation of the molecular mass is not due to rapid equilibrium between dimer and tetramer, but apparently arises from other effects.

TABLE 1 Elution volumes of LDH species obtained after 5 min of incubation at 0°C in the presence of the solutes indicated

Conditions	Concentrations of LDH	Elution volume (ml) and corresponding (kDa)* of		
		1st minor peak[#]	Major peak	2nd minor peak
No solutes	5–670 μg/ml[§]	7.673 ± 0.005 (230)	8.442 ± 0.007 (97.6)	9.640 ± 0.010 (24.2)
0.8 M urea	5–670 μg/ml[§]	7.50 ± 0.005 (277)	8.352 ± 0.009 (103)	9.315 ± 0.015 (34)
0.8 M urea + 0.8 M TMAO	13.4 μg/ml	—	8.464 ± 0.014 (93.1)	—

*Average elution volumes corresponding to different LDH concentrations. Values of molecular masses determined from calibration plots are presented in parentheses.

[#]The two minor peaks become apparent only on the most sensitive absorbance scale (0.001 OD at 280 nm).

[§]Eight different concentrations between 5 and 670 μg/ml were used.

The minor peak reported in Table 1 with an apparent molecular mass of 24 kDa is lower than the known mass (36 kDa) of a LDH subunit. This observation suggests that column sorption effects may be responsible for the anomalously low molecular masses. The fact that apparent molecular masses of the three detectable chromatographic species in the presence of urea are higher than the corresponding peaks in the absence of urea (see Table 1) could be due to 0.8 M urea partially abolishing the adsorption effects and/or inducing swelling of the protein species. A swelling effect caused by urea on the native forms of proteins has been observed by means of size exclusion chromatography (Corbett and Roche, 1984).

Effect of TMAO on urea-induced time-dependent inactivation of LDH

At issue is the question of how TMAO affects the time-dependent inactivation of LDH by urea. Fig. 6 shows results of the same type of experiment as shown in Fig. 1, but with incubations carried out in the presence of TMAO alone and in mixtures of urea:TMAO. Upon incubation of LDH in 0.6 M TMAO, it is found that the enzyme is inactivated by a measurable but relatively small extent, with a half-time of inactivation that is about twofold less than that of the control. Urea (0.6 M) inactivates the enzyme at a rate that is 300-fold faster than that of the control, but when 0.3 M TMAO is present with 0.6 M urea, the half-time of the inactivation rate is only 60-fold faster. If TMAO concentration is increased to give a mixture of 0.6M urea:0.6M TMAO, the half-time of inactivation is only ~10-fold faster than that of the control. Clearly, TMAO provides significant protection of LDH from time-dependent activity loss in the presence of urea, but it is unable to completely prevent loss of activity in the concentration range and ratios of 3:2 or 2:1 urea:TMAO found in sharks and rays (Yancey and Somero, 1979).

A quantitative assessment of how the half-time for LDH inactivation varies with urea, TMAO, and 1:1 and 2:1 ratios of urea:TMAO is given in Fig. 7. At both 1:1 and 2:1 ratios of urea:TMAO, as urea concentration increases TMAO is seen to provide greater and greater improvement in staving off inactivation, compared to activity loss that would occur if TMAO were not present.

DISCUSSION

Urea-rich cells such as those in elasmobranches and kidney pose the problem that intracellular enzymes must reside in the presence of the denaturant (urea), yet must also maintain functional activity. Enzymes in urea-rich cells are believed to be just as sensitive to the deleterious effects of urea as those occurring in non-urea-containing cells (Yancey et al., 1982; Yancey and Somero, 1978). What protects the enzymes in urea-rich cells and enables them to function is the additional presence of organic osmolytes such as TMAO in elasmobranches and glycerolphosphocholine in kidney cells (Bagnasco et al., 1986; Garcia-Perez and Burg, 1990; Yancey, 1985; Yancey and Somero, 1978). Much of the emphasis in the biology of adaptation of urea-rich cells has been on the magnitude of the effects of urea and of

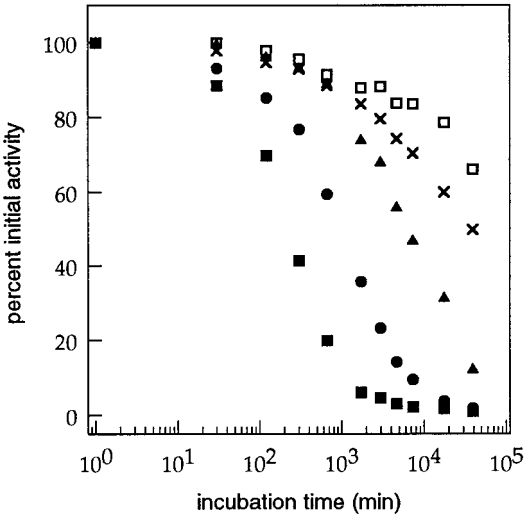

FIGURE 6 Time course of LDH activity loss during incubation with urea, TMAO, or urea-TMAO mixtures. LDH (1.34 μg/ml) was incubated in 0.2 M Tris-HCl buffer containing 1 mg/ml BSA (pH 7.3) at 0°C without solute (□), in the presence of 0.6 M TMAO (×), 0.6 M urea, and 0.6 M TMAO (▲), 0.6 M urea and 0.3 M TMAO (●), 0.6 M urea (■). Residual LDH activity is presented as a percentage of the specific activity measured at zero time of incubation.

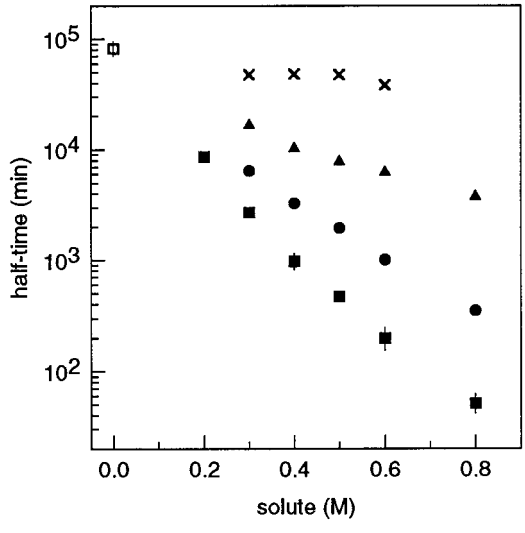

FIGURE 7 Dependence of the half-time of LDH activity loss as a function of urea and/or TMAO concentration. The half-times of activity loss were evaluated from incubations conducted at various concentrations of urea, TMAO, and urea:TMAO mixtures from data such as those presented in Fig. 6. Represented are incubations of LDH (1.34 μg/ml) in 0.2 M Tris-HCl containing 1 mg/ml BSA (pH 7.3), carried out in the absence of solutes (□), in the presence of TMAO (×), 1:1 urea:TMAO (▲), 2:1 urea:TMAO (●), and urea alone (■). The concentration scale represents the solute concentration or, in the case of urea:TMAO mixtures, the concentration of the urea component.

counteracting osmolyte (e.g., TMAO) on the stability and function of enzymes (Burg et al., 1996; de Meis, 1988; Gopal and Ahluwalia, 1993; Lin and Timasheff, 1994; Santoro et al., 1992; Wang and Bolen, 1996; Yancey and Somero, 1979, 1980). Accordingly, the parameters generally sought are changes in T_m as a function of urea and/or TMAO and changes in k_{cat} and K_m of enzymes as a function of these solutes. Such parameters are truly time-independent parameters if the experiments for determining T_m, k_{cat}, and K_m are performed in a manner that excludes time-dependent effects (Hand and Somero, 1982; Withycombe et al., 1965). However, in many reports, precautions to exclude time-dependent effects have not been implemented, and the possibility exists that such evaluations of the above-mentioned parameters may not entirely reflect the interpretation offered for them. Given that enzymes in the urea-rich cells of sharks and rays are continuously bathed in the urea:TMAO milieu, it is important to evaluate time-dependent effects of these solutes on enzyme function, because such dependencies are an integral part of adaptation phenomena.

As shown in Fig. 1, LDH held at 0°C in the absence of urea loses activity with a half-time of ~60 days, but in 0.6 M urea, a concentration reported in the cells of rays (Forster and Goldstein, 1976), the loss of activity increases by several orders of magnitude and occurs with a half-time of ~3 h. Antidiuretic rat kidney has been reported to have urea concentrations of 1.5 M (Garcia-Perez and Burg, 1990), and 5 M urea has been reported in desert mice under dehydration stress (MacMillen and Lee, 1967). Given the sensitivity of activity loss of LDH in urea, time-dependent effects on activity must be an exceedingly important issue in understanding the biochemistry of urea-rich cells.

Fig. 2 illustrates that LDH inactivated in the presence of 0.8 M urea at 0°C can be reactivated at 24°C, but reversibility of reactivation is dependent on the length of time of inactivation at 0°C. These data also suggest that reactivation is a second-order process, and this implies that inactivation by urea involves the dissociation of LDH. More than 98% of LDH activity is lost when it is incubated with 0.8 M urea at 0°C for 24 h, but when the temperature is shifted to 24°C, reactivation occurs rapidly enough to see the velocity increase during the course of the assay for LDH activity (Fig. 3 *A*). The time derivatives of the assays (Fig. 3 *B*) can be used to evaluate the rate of reactivation as a function of LDH concentration, and these data permit an evaluation of the reactivation reaction as second order by using the van't Hoff (log-log) plot (Fig. 4) (Laidler, 1965). Again, the results suggest that urea has the effect of dissociating LDH and that reassociation of dimers to active tetramers is rate determining.

Different molecular mass species can be separated with time of LDH incubation in 0.8 M urea at 0°C (Fig. 5 *A*), and it is clear that urea causes dissociation of LDH, presumably to monomeric species. It is also clear that TMAO prevents urea from dissociating LDH (Fig. 5 *B*), although the tetrameric species appears to change its elution properties slightly with time of incubation. TMAO in a 1:1 ratio with urea does not give complete protection of LDH from inactivation after 24 h incubation in the mixture (Figs. 6 and 7), so the absence of lower molecular mass species (Fig. 5 *B*) at >24 h incubation indicates that loss of activity does not have to result entirely from LDH dissociation.

Although the effects are exceedingly slow, TMAO itself causes LDH inactivation, decreasing the half-time of inactivation in the absence of solutes from ~60 days to ~30 days at 0°C (see Fig. 6). Nevertheless, TMAO is quite effective in extending the half-time of the enzyme in the presence of urea, decreasing the urea-induced rate of loss by fivefold in 0.6 M urea:0.3 M TMAO and by ~30-fold in 0.6 M urea:0.6 M TMAO (Fig. 6). Thus it appears that TMAO has the ability to greatly diminish time-dependent loss of LDH activity, presumably by preventing urea-induced dissociation of LDH.

TMAO has also been reported to decrease time-dependent loss of phosphofructokinase activity due to cold-induced tetramer dissociation, but it does not protect the enzyme from inactivation due to urea-induced dissociation (Hand and Somero, 1982). There is an important biological reason why this enzyme may be an exception. Because phosphofructokinase activity regulates glycolytic flux, it has been hypothesized that the urea-induced dissociation of tetrameric enzyme prevents build-up of damaging acidic conditions, thereby playing an important role in the urea-rich cellular environment occurring in hibernating animals (Hand and Somero, 1982).

Time-dependent processes have not been emphasized in the context of the biology of adaptation, but their importance becomes evident when effects of urea on multisubunit proteins are considered. Urea-induced dissociation of multisubunit proteins can occur in the presence of very low concentrations of urea (Hand and Somero, 1982). If it is true that enzymes in urea-rich cells are just as sensitive to urea effects as enzymes in non-urea-rich cells (Yancey et al., 1982), the importance of time-dependent effects becomes inescapable. In fact, with some enzymes it could be more important than the counteracting effect on k_{cat} and K_m.

Enzyme turnover is a key factor in metabolic control, and loss of enzyme activity as an important part of the turnover phenomenon will affect the survivability of cells stressed by urea. From the results with LDH, it is indeed likely that the ability of counteracting osmolytes like TMAO to slow down or prevent dissociation of proteins in urea-rich cells is an essential part of the protection and counteraction it offers against urea-induced inactivation.

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