Trimethylamine-*N*-Oxide Counteracts Urea Effects on Rabbit Muscle Lactate Dehydrogenase Function: A Test of the Counteraction Hypothesis

Ilia Baskakov,* Aijun Wang,# and D. W. Bolen*

*Department of Human Biological Chemistry and Genetics, University of Texas Medical Branch, Galveston, Texas 77555-1052, and #Department of Chemistry and Biochemistry, University of California at San Diego, La Jolla, California 92093-0601 USA

ABSTRACT Trimethylamine-*N*-oxide (TMAO) in the cells of sharks and rays is believed to counteract the deleterious effects of the high intracellular concentrations of urea in these animals. It has been hypothesized that TMAO has the generic ability to counteract the effects of urea on protein structure and function, regardless of whether that protein actually evolved in the presence of these two solutes. Rabbit muscle lactate dehydrogenase (LDH) did not evolve in the presence of either solute, and it is used here to test the validity of the counteraction hypothesis. With pyruvate as substrate, results show that its K_m and the combined K_m of pyruvate and NADH are increased by urea, decreased by TMAO, and in 1:1 and 2:1 mixtures of urea:TMAO the K_m values are essentially equivalent to the K_m values obtained in the absence of the two solutes. In contrast, values of k_{cat} and the K_m for NADH as a substrate are unperturbed by urea, TMAO, or urea:TMAO mixtures. All of these effects are consistent with TMAO counteraction of the effects of urea on LDH kinetic parameters, supporting the premise that counteraction is a property of the solvent system and is independent of the evolutionary history of the protein.

INTRODUCTION

Many plants, animals, and microorganisms have adapted to harsh environmental stresses such as dehydration, high salt conditions, and extremes of temperature. Despite their diversity, these organisms all appear to have adopted the same strategy in protecting cellular proteins against stresses (Borowitzka, 1985; Yancey et al., 1982). That strategy appears to involve the intracellular accumulation of particular low-molecular-weight organic molecules that fall into one of three chemical classes, the polyols, certain amino acids, and particular methylamines (Brown and Simpson, 1972; Stewart and Lee, 1974; Yancey et al., 1982). These small organic molecules are known as organic osmolytes, and they protect proteins against denaturation and the loss of functional activity (Arakawa and Timasheff, 1982, 1983, 1985; Lee and Timasheff, 1981; Santoro et al., 1992; Timasheff, 1992).

Within the three chemical classes of osmolytes, distinctions have been made regarding how functional activity is maintained within the cell by particular osmolytes. This has resulted in classification of organic osmolytes either as "compatible" or "counteracting," in terms of their effects on the functional activity of proteins (Borowitzka and Brown, 1974; Brown and Simpson, 1972; Yancey et al., 1982). Compatible osmolytes are those that stabilize proteins without substantively affecting protein functional activity (Borowitzka and Brown, 1974; Bowlus and Somero, 1979; Pollard and Wyn Jones, 1979; Wang and Bolen, 1996). Representatives of this class include certain amino acids (e.g., proline and glycine) and polyols (e.g., trehalose, sucrose, and sorbitol), and the stresses that compatible osmolytes protect against include dehydration, high-salt environments, and extremes of temperature (Yancey et al., 1982). Counteracting osmolytes consist of the methylamine class of osmolytes, which are believed to have the special ability to protect intracellular proteins against the inactivating effects of urea on proteins (Lin and Timasheff, 1994; Yancey and Somero, 1979). In contrast to compatible osmolytes, which do not affect the functional activity of proteins, counteracting osmolytes are believed to cause changes in protein function that are the opposite of the effects urea has on protein function (Somero, 1986). Examples of organs and even whole animals that are rich in urea-containing cells are mammalian kidney, with betaine and glycerophosphocholine as counteracting osmolytes, and cartilaginous fishes and the coelacanth, which use trimethylamine *N*-oxide (TMAO) as the principal counteracting osmolyte (Bagnasco et al., 1986; Garcia-Perez and Burg, 1990; Nakanishi et al., 1993; Yancey, 1985; Yancey and Somero, 1980).

Cartilaginous fishes (e.g., sharks and rays) and the coelacanth have intracellular urea concentrations as high as 0.4–0.6 M, and their intracellular levels of TMAO are around half that of urea (Boyd et al., 1977; Forster and Goldstein, 1976). This approximately 2:1 to 3:2 (urea:TMAO) ratio is commonly found in all of these animals (Yancey, 1985). For a number of enzymes from sharks and rays, mammalian kidney, and non-urea-containing mammalian organs, Yancey and Somero found that urea alone generally increases K_m and decreases k_{cat}, whereas TMAO alone has the contrasting effect of decreasing K_m while increasing k_{cat} (Yancey and Somero, 1980). When urea and TMAO are combined in a 2:1 urea:methylamine ratio, the effects of both solutes on K_m and k_{cat} offset one another, giving

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Address reprint requests to Dr. David W. Bolen, Department of Human Biological Chemistry and Genetics, University of Texas Medical Branch, 5.154 Medical Research Building, Galveston, TX 77555-1052. Tel.: 409-772-0754; Fax: 409-747-4751; wbolen@hbcg.utmb.edu.

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apparent k_{cat} and K_m values in the combined presence of urea and TMAO that are equal to k_{cat} and K_m determined in the complete absence of the two solutes (Yancey and Somero, 1980). The selective advantage of TMAO is that it stabilizes proteins from denaturation by urea (Lin and Timasheff, 1994; Wang and Bolen, 1997; Yancey and Somero, 1979) and offsets urea functional effects, such that the kinetic character of the (enzyme-mediated) metabolic pathways is maintained to the same degree in shark cells as in cells that have neither solute (Hochachka and Somero, 1984).

Yancey and Somero's counteraction hypothesis is elegant in its simplicity, but the extent to which it holds as a general mechanism for proteins in urea/methylamine-containing cells is unclear (Mashino and Fridovich, 1987). To be completely effective and general in its action, the counteracting osmolyte TMAO would be expected to offset the effects urea has on any protein, regardless of whether that protein evolved in the presence of these two solutes. At this point, most studies of the effects of urea, TMAO, and urea/TMAO mixtures on k_{cat} and K_m have focused on enzymes from kidney or cartilaginous fishes, enzymes that have evolved in the presence of methylamines and urea (Burg et al., 1996; de Meis, 1988; Yancey and Somero, 1978, 1980). Only a very small number of studies have been conducted on enzymes that have not evolved in the presence of methylamine or urea (Mashino and Fridovich, 1987; Yancey and Somero, 1978, 1980), so the question of whether the counteraction hypothesis is general in its effects has not been extensively explored. Of the small number of enzymes studied, a significant fraction of these do not exhibit counteraction (Mashino and Fridovich, 1987; Yancey and Somero, 1978, 1980). Kinetic measurements of enzyme action in the presence of solutes like urea and TMAO present problems not normally encountered in the usual enzyme assays, and experimental precautions and considerations necessary to deal with kinetic measurements in the presence of these solutes were seldom taken in previous studies. In our experience, additional care must be taken with each enzyme to establish the authenticity of the counteracting effect or exceptions to TMAO counteractions of urea effects on enzyme activity.

Here we test the validity of the counteracting osmolyte hypothesis using rabbit muscle lactate dehydrogenase (LDH), an enzyme that did not evolve in the presence of urea and methylamines. If one avoids substrate concentrations high enough to give substrate inhibition, k_{cat} and K_m parameters of LDH can be evaluated by using a modified Theorell-Chance mechanism (Zewe and Fromm, 1965), and the effects of urea and TMAO determined. LDH from rabbit muscle is highly labile (Cho and Swaisgood, 1973), and its activity and stability should be significantly affected by urea. These features of rabbit muscle LDH present a reasonable test case for whether the counteracting hypothesis of Yancey and Somero holds for a protein with no evolutionary history of exposure to urea or organic osmolytes.

EXPERIMENTAL PROCEDURES

Chemicals

Rabbit muscle LDH, NADH, sodium pyruvate, bovine serum albumin, and trimethylamine-*N*-oxide dihydrate were purchased from Sigma; ultrapure urea was from ICN. Before use, concentrated urea solutions were treated with a mixed-bed ion-exchange resin (AG501-X8 from Bio-Rad Laboratories) for at least 1 h to get rid of ions formed through the decomposition of urea (Hagel et al., 1971). Urea solutions were then filtered through syringes equipped with 0.22-μM GV filters (Millipore Corp.). The urea concentration was evaluated by measuring the refractive index of the solution and substituting into the expression $[\text{urea}] = 117.66*\Delta n + 29.753\Delta n^2 + 185.56*\Delta n^3$, where Δn represents the difference between the refractive index of urea solution and water or buffer in which the urea was dissolved (Pace, 1986). TMAO dihydrate was recrystallized from aqueous solution and kept in a desiccator at room temperature. TMAO solutions were filtered through 0.22-μM GV filters, and TMAO concentration was determined by means of a standard curve relating refractive index to TMAO concentration. TMAO samples were prepared analytically by weight, the refractive index was measured for each analytical solution, and a plot of Δn versus [TMAO] concentrations was prepared. The functional dependence of the standard curve is given by $[\text{TMAO}] = -0.0038 + 103.3151*\Delta n - 259.43*\Delta n^2$.

Assay of LDH

LDH assays were carried out at 24°C, in 0.20 M Tris-HCl buffer (pH 7.3), in the absence or presence of different concentrations of urea, TMAO, or urea-TMAO, with the latter mixtures prepared in the molar ratios 2:1 and 1:1 (urea:TMAO). Parent LDH stock solutions (38.2 μg/ml LDH in 12 mM ammonium sulfate) were prepared from a 200× dilution of commercial LDH suspension into 0.20 M Tris-HCl containing 1 mg/ml bovine serum albumin at 0°C. The concentration of LDH was determined spectrophotometrically at 280 nm (1.13 mg/ml/OD; cited by Worthington). Molar absorptivities of NADH in the presence of up to 0.6 M urea concentrations and up to 0.6 M TMAO concentrations were determined and found to be identical with the molar absorptivity in the absence of these solutes. Assays of LDH-catalyzed reactions were evaluated by following the oxidation of NADH at 340 nm, using a molar absorptivity of $6.22 \times 10^3\ M^{-1}\ cm^{-1}$ to convert rates to a molar concentration basis. The assays were performed by adding 100 μl of sodium pyruvate stock solution to the sample cuvette containing 2.75 ml of 0.2 M Tris-HCl buffer (in the presence or absence of solutes) and zeroing the baseline at 340 nm. A total of six different pyruvate concentrations were used, and at each concentration of pyruvate a 100-μl aliquot of a fixed concentration of NADH stock solution was added. A total of six different NADH concentrations were used. This gave a total of 36 assay mixtures containing different concentrations of pyruvate and NADH. Absorbances of these solutions at 340 nm were measured, and a 50-μl aliquot of a working stock solution of LDH, containing the same concentration of urea and/or TMAO as in the assay mixture, was added to initiate reaction. The working LDH stock solutions (1.91 μg/ml LDH in 0.6 mM ammonium sulfate) were prepared by adding an appropriate amount of parent LDH stock solution at 4°C to 0.2 M Tris-HCl (pH 7.3) containing 1 mg/ml bovine serum albumin and the same concentrations of urea and/or TMAO provided in the assay solution. The working LDH stock solutions lost activity with time, so up to six fresh LDH solutions containing urea or urea/TMAO mixtures were prepared in the course of each set of kinetic experiments to maintain a constant level of LDH activity during the course of the kinetic measurements.

RESULTS

Kinetic studies of rabbit muscle LDH performed by Zewe and Fromm revealed that the mechanism of LDH reaction is consistent with a modified form of the Theorell-Chance

mechanism (Zewe and Fromm, 1965). Two forms of the rate expression for this mechanism in the absence of products are given in Eqs. 1a and 1b (Wang and Bolen, 1996; Zewe and Fromm, 1965):

$$1/V = (1/k_{cat})(1 + K_{NADH})/[NADH] + K_{pyr}/[pyr] + K_{NADH\ pyr}/[NADH][pyr]) \quad (1a)$$

$$V = k_{cat}[NADH][pyr]/([NADH][pyr] + K_{NADH}[pyr] + K_{pyr}[NADH] + K_{NADH\ pyr}) \quad (1b)$$

The traditional way of estimating kinetic parameters of two-substrate enzyme-catalyzed reactions is to construct double reciprocal plots involving velocities and concentrations of one of the substrates at various fixed concentrations of a second substrate, using linearized forms of rate equations such as Eq. 1a. A more statistically appropriate means of evaluating kinetic parameters is to fit, simultaneously, a set of all data of velocity versus [substrates] in accordance with Eq. 1b, using nonlinear least-squares analyses (Johnson and Frasier, 1985). The latter method was used here with k_{cat}, K_{NADH}, K_{pyr}, and $K_{NADH\ pyr}$ as fitting parameters; a representative set of kinetic data and fitting results is shown in the inset of Fig. 1 for experiments performed in 0.6 M urea. For convenience, the results of the nonlinear least-squares fitting to the Theorell-Chance mechanism as shown in the inset are also transformed in Fig. 1 to the more conventional Lineweaver-Burk plot. Table 1 compares kinetic parameters of LDH evaluated in the present study (in the absence of urea and/or TMAO) with those of previous studies (Stambaugh and Post, 1966; Zewe and Fromm, 1962, 1965). The variation in the derived kinetic parameters is believed to be due mainly to differences in experimental conditions between the studies. Over the pH range considered, apparent Michaelis constants for pyruvate have been found to be quite sensitive to pH, with the values increasing with increasing pH (Hochachka and Somero, 1984; Yancey and Somero, 1978). The pH dependence of K_m reflects the protonation state of the imidazolium group of His^{195}, in which binding of pyruvate in the active center of the enzyme requires the protonated form of the imidazole ring (Wilson, 1977). A pK for the imidazole of His^{195} in the range of 6.8–7.0 (Holbrook and Ingram, 1973) should cause pH-dependent K_m effects consistent with observed data. Differences in K_m values might also arise if the LDH isozyme ratios differ in the studies compared in Table 1 (Wiggert and Villee, 1964). Finally, small differences in kinetic constants may arise from the different methods (nonlinear least-squares methods used here compared with linear least-squares methods used by others; Zewe and Fromm, 1962, 1965). Regardless of the cause of differences in K_m values between the various studies cited in Table 1, the differences are modest. Our goal in the present study is to test the effects of TMAO and urea on the kinetic parameters. That goal may be reached despite any real or perceived differences in K_m values obtained among various laboratories.

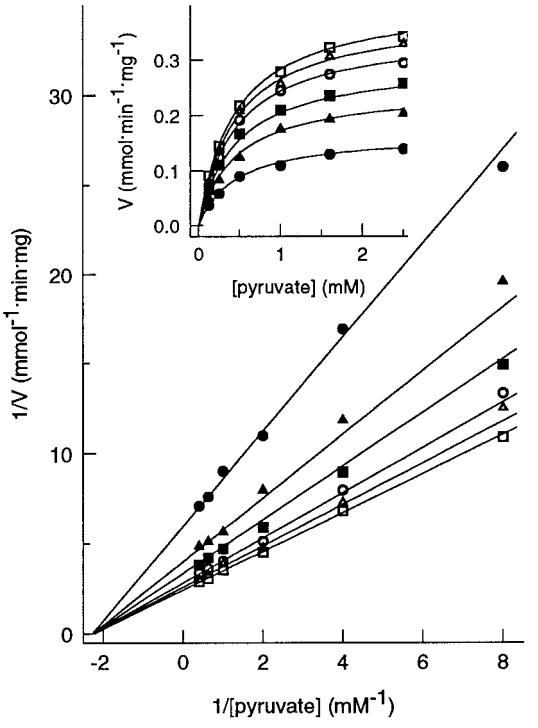

FIGURE 1 Representative Lineweaver-Burk plot of LDH kinetic data. Kinetic assays were performed at pH 7.3, 24°C, in the presence of 0.6 M urea. The initial velocites were measured as a function of pyruvate concentration in the following fixed concentrations of NADH: 9.19 (●), 18.34 (▲), 27.1 (■), 45.4 (○), 64.1 (△), 90.7 (□) μM. (*Inset*) Solid lines represent nonlinear least-squares fitting of the hyperbolic data to the modified Theorell-Chance mechanism as given in Eq. 1b. The solid lines in the Lineweaver-Burk plot represent the results of the nonlinear least-squares fit of data shown in the inset, transformed into a Lineweaver-Burk plot.

To initiate the assays under conditions suitable for evaluating kinetic parameters, LDH is prepared in solutions containing the same concentration of urea and/or TMAO as in the assay solutions for 5–10 min before assay. Preliminary experiments showed that LDH loses activity during incubation with solutions containing urea and/or TMAO. To maintain constant enzyme activity during kinetic measurements, up to six fresh LDH stock solutions were prepared in the course of the assays, the data of which are given in Fig. 1. The use of freshly prepared LDH stock solutions in the presence of solutes ensures that the activity of the enzyme is

TABLE 1 Kinetic parameters for rabbit muscle LDH

	k_{cat}	$K_{m\ pyr}$ (μM)	pH	K_{NADH} (μM)	$K_{NADH\ pyr}$ (μM)^2
Zewe and Fromm*	—	164	6.8	10.7	1380
Zewe and Fromm#	—	209	7.15	7.43	1140
NLLS§	0.498	334	7.3	11.3	2791
Stambaugh¶	—	350	7.4		

*Zewe and Fromm (1962), 28°C.
#Zewe and Fromm (1965), 28°C.
§NNLS, nonlinear least-squares analysis using Eq. 1. This work, 24°C.
¶Stambaugh and Post (1965), 25°C.

constant (decreasing by no more than 5%) during the course of all assays determined in Fig. 1.

Studies by us and others show that use of concentrations higher than 100 μM NADH or 3 mM pyruvate results in deviations from the modified Theorell-Chance mechanism, because of high-substrate inhibition (Everse et al., 1970; Fernandez-Velasco et al., 1992; Griffin and Criddle, 1970; Yancey and Somero, 1978). To avoid complications due to deviation from the mechanism in the limit of high substrate concentrations and to ensure that Eqs. 1a and 1b apply, initial velocity measurements were restricted to NADH concentrations in the range of 9–90 μM and pyruvate concentrations in the range of 0.125–2.5 mM. In the presence of TMAO, substrate concentrations were in the range of 8–65 μM for NADH and 0.125–2.5 mM for pyruvate. As a function of the concentrations of the solutes, Fig. 2 presents values for k_{cat} (Fig. 2 *A*), along with Michaelis constants K_{NADH}, K_{pyr}, and $K_{NADH\ pyr}$ (Fig. 2, *B*, *C*, and *D*, respectively). Also included in these figures are the effects on the k_{cat} and K_m parameters of urea:TMAO mixtures with ratios of 1:1 and 2:1.

DISCUSSION

There are two separable issues in the counteraction hypothesis, one dealing with the action of TMAO in stabilizing proteins against urea-induced denaturation, and the other dealing with the action of TMAO in counteracting effects of urea on the functional activity of proteins. A broad body of work on protein stabilization has established that TMAO indeed stabilizes proteins against urea-induced denaturation (Gopal and Ahluwalia, 1993; Lin and Timasheff, 1994; Wang and Bolen, 1997; Yancey and Somero, 1979). Our

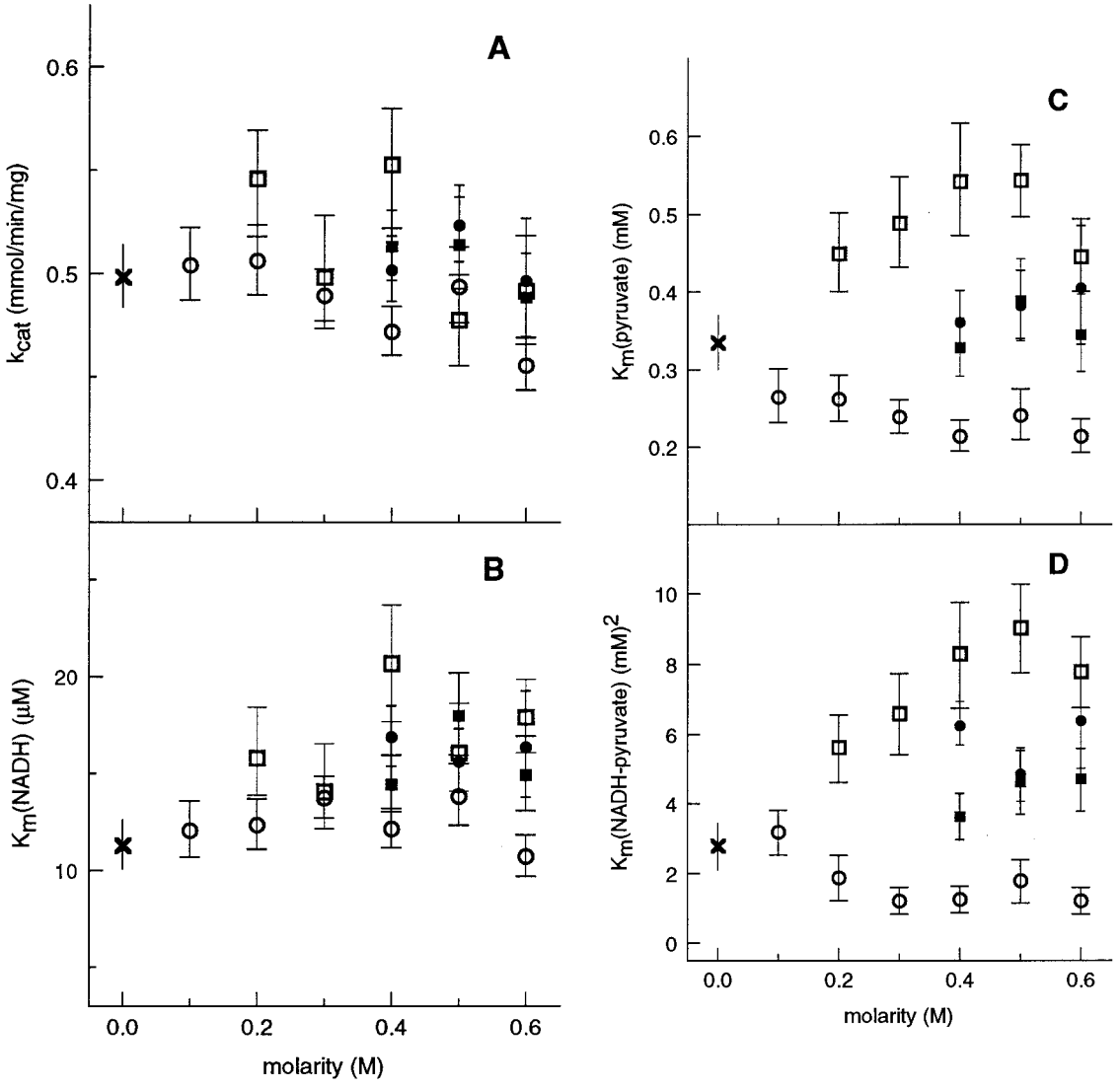

FIGURE 2 Effects of urea (□), TMAO (○), 1:1 urea-TMAO mixture (■), and 2:1 urea:TMAO mixture (●) on kinetic constants: k_{cat} (*A*), K_m values for NADH (*B*), K_m values for pyruvate (*C*), K_m values for NADH-pyruvate (*D*). Bars represent errors obtained through nonlinear least-squares fits of the velocity versus substrate concentration data to Eq. 1b. Assays were carried out in 0.2 M Tris-HCl, pH 7.3, at 24°C. Molarity values represent either concentrations of urea alone, TMAO alone, or urea concentration in urea-TMAO mixtures.

own work strongly indicates that the origin of the ability of TMAO to thermodynamically stabilize proteins against urea-induced denaturation is due to the highly unfavorable interaction of TMAO with the peptide backbone of the denatured state (Wang and Bolen, 1997). Because the peptide backbone is the most numerous grouping in a protein, the involvement of the backbone in protein stabilization ensures that stabilization by TMAO will be applicable to all proteins, regardless of whether they are derived from urea-rich cells.

In contrast to showing that TMAO protects proteins against urea-induced denaturation and establishing a molecular-level explanation for this behavior, the counteraction by TMAO of urea effects on protein function is not at all simple to explain. The reason is that unlike urea-induced denaturation, which involves molecular interactions common to all proteins, protein function involves a large variety of different kinds of reactions, and it is more difficult to imagine how TMAO can offset the myriad effects urea might have on the active site, effects on subunit interactions, and specific interactions of urea with substrates of differing chemical character. In addition, there are numerous nonspecific effects that can influence protein function, such as solute-induced attenuation of hydrophobic interactions important in substrate-protein interactions or attenuation of electrostatic interactions between substrate and protein (Bolen and Fisher, 1969). To sort out the counteraction effects of TMAO and urea on enzyme activity, it is important first to establish the effects these solutes have on protein function and then to determine the extent to which the counteracting effects are general. This in itself is not easy, because literature data reporting examples of counteraction as well as examples contrary to the counteraction hypothesis have not always been performed under experimental conditions sufficiently controlled to authenticate either result.

Counteraction hypothesis—experimental issues

In the course of our kinetic studies with rabbit muscle LDH, we found that the manner in which the experiments are conducted is critical to both the analysis of the data and conclusions that may be drawn from the studies. First, kinetic measurements are made by adding enzyme to an assay mixture containing the solute of interest, but this can be done with or without preincubation of the enzyme with solute for a period of time before the assay. In many instances, it makes considerable difference in the initial velocity measurement as to whether the enzyme has been pre-exposed to solute before the initial velocity measurement. Preincubation of enzyme with solute ensures that any initial shock, transient effects, or time-dependent effects of solute on the enzyme are not reflected in the initial velocity measurements. These issues, along with issue of reversibility of solute-induced effects, are discussed in the preceding companion paper. Second, it is important to recognize that the kinetic parameters k_{cat} and K_m are model dependent, and that it matters what substrate concentration ranges are used in the evaluation of k_{cat} and K_m and how the kinetic data are collected. In a two-substrate system, determination of the K_m of one substrate (K_{m1}) by "saturating" with high concentrations of the second substrate can lead to erroneous values in K_{m1} due to high substrate inhibition (see below). Furthermore, some reported studies of urea/TMAO effects show nonlinear Lineweaver-Burk plots, but the data have still been analyzed by using Michaelis-Menten kinetics (Yancey and Somero, 1980). The k_{cat} and K_m values reported in these instances make it difficult to establish whether counteraction is authentic. In summarizing literature results concerned with the question of counteracting phenomena, it is important to establish whether appropriate controls and precautions have been taken in collecting and evaluating the data. If not, the results may arise from effects quite different from what the original interpretation suggests.

TMAO counteraction of urea effects on rabbit muscle LDH

Our results of the effects of TMAO and urea on the kinetic parameters of rabbit muscle LDH correspond well to the premise of the counteraction hypothesis of Yancey and Somero (1980); the K_m changes caused by urea are counteracted by TMAO. Fig. 2, *c* and *d*, shows that $K_{m\ pyr}$ and $K_{m\ NADH\ pyr}$ are both increased by urea and decreased by TMAO. However, although it is evident that a mixture of 2:1 urea:TMAO brings the K_m values closer to what they would be in the absence of both solutes, this mixture is not as effective as a 1:1 ratio in this regard. $K_{m\ NADH}$ appears to be slightly increased in urea, but TMAO has a marginal if any effect on this kinetic parameter. A 2:1 or 1:1 ratio of urea:TMAO, however, does move the urea-perturbed $K_{m\ NADH}$ very close to the value expected in the absence of both solutes, giving an apparent K_m that approximates the average of the effects of urea and TMAO individually. Finally, k_{cat} for LDH exhibits essentially no dependence on either urea or TMAO; thus there is no change in k_{cat} for TMAO to counteract. For mammalian muscle and elasmobranch LDHs, k_{cat} is independent of urea concentration as long as the concentration of pyruvate does not exceed substrate inhibition levels of ~2.5 mM (Lushchak and Lushchak, 1994; Rajagopalan et al., 1961; Withycombe et al., 1965; Yancey and Somero, 1978).

The changes in K_m parameters brought about by TMAO or urea and the attenuation of the urea effects by TMAO represent a maximum change in K_m of threefold, with solute concentrations in the physiological range occurring in elasmobranchs (sharks and rays). The effects of urea and TMAO alone on kinetic parameters are not large, and it is legitimate to question whether counteraction effects are important physiologically. Considering that cellular metabolism is a finely tuned system of regulation, even small changes in kinetic parameters with the large number of

enzymes involved can disrupt the intricacies of metabolic control, leading to metabolic impairment or cell death. Consequently, the ability of TMAO to offset the effects of urea and move kinetic parameters much closer to values unperturbed by solutes should provide a strong selective advantage for counteraction. The fact that rabbit muscle LDH has not evolved in the presence of either urea or TMAO, and yet it fulfills the essential features of the counteraction hypothesis, is supportive of the premise that (with this enzyme), counteraction is a property of the urea/TMAO system and is independent of the evolutionary history of the protein.

The independence of k_{cat} of urea, TMAO, and 2:1 urea:TMAO mixture described above for rabbit muscle and elasmobranch LDHs is very different from the results of Yancey and Somero, who found that urea activates (~15% increase in k_{cat}) and TMAO inhibits (~15% decrease in k_{cat}) guitarfish (ray) LDH (Yancey and Somero, 1980). The differences can readily be explained by the fact that, unlike Yancey and Somero, who used substrate inhibitory concentrations (3 mM) of pyruvate in all of their studies on k_{cat} and K_m, we varied the pyruvate concentration over a range that avoided the phenomenon of high-substrate inhibition. The apparent urea activation observed by Yancey and Somero was attributed by them to an abortive complex, a phenomenon described by Fernandez-Velasco et al. as an enzyme-NAD-pyruvate ternary complex (Fernandez-Velasco et al., 1992; Yancey and Somero, 1978). The abortive complex is promoted whenever the pyruvate concentration in the assay is high (>2.5 mM), resulting in high-substrate inhibition observed in LDHs from a variety of species, including mammalian muscle and elasmobranchs (Everse et al., 1970; Fernandez-Velasco et al., 1992; Fromm, 1963; Stambaugh and Post, 1966; Yancey and Somero, 1978). It has been shown previously that urea diminishes the concentration of the abortive complex, and relief of substrate inhibition results in the apparent "activation" of LDH activity by urea at high concentrations of pyruvate (Fernandez-Velasco et al., 1992; McQueen, 1974). In the presence of both TMAO and urea, high substrate inhibition is apparently not prevented, leading to a lower LDH activity in the mixture of TMAO plus urea than in urea alone. The fact that very different K_m and k_{cat} results are obtained, depending on the pyruvate concentration, underscores the fact that k_{cat} and K_m are model-dependent parameters. The evaluation of k_{cat} and K_m values must take into account all enzyme species appearing under the experimental conditions used.

What is the molecular origin of counteraction?

In a well-controlled kinetic study, Burg and Peters find that methylamines do not counteract the effects of urea on kidney aldose reductase (Burg and Peters, 1997), and there are other examples of enzymes that do not appear to exhibit counteraction (Burg et al., 1996; de Meis, 1988; Yancey and Somero, 1979, 1980). Although some of these examples may also have their own experimental problems, it is reasonable to acknowledge that counteraction may not occur with all enzymes (Mashino and Fridovich, 1987; Yancey and Somero, 1980). But for the significant number of enzymes that do exhibit counteraction, how may this phenomenon be explained as a feature of the osmolyte/urea solution? An explanation offered by Machino and Fridovich is that urea loosens and expands protein volume, whereas TMAO is presumed to compact protein structure (Mashino and Fridovich, 1987). They believed the protein in the presence of TMAO and/or urea to be in a continuum of structural compactness of the native state ensemble of species, ranging from a most compact structure (in the presence of TMAO) to the highly expanded native state species D, with gradations of compactness at intermediate concentration mixtures of these solutes as given in the model below:

$$\text{Most compact} \leftrightarrow \text{A} \leftrightarrow \text{B} \leftrightarrow \text{C} \leftrightarrow \text{D} \leftrightarrow \text{Random coil}$$

In the presence of denaturing concentrations of urea, the protein cooperatively unfolds to a random coil-like species, a protein species included in the model for the sake of completeness. From the equilibria shown in the model, these authors imagine a case in which the principal protein species may be species A or B in the absence of urea and/or TMAO, but they hypothesize that the most active form of the enzyme is the maximally compact form. In this case, urea by itself would decrease functional activity by shifting the equilibria away from A and B and toward more open (and less active) structures (e.g., C, D, and random coil), whereas TMAO by itself would increase the activity above that in water by shifting the equilibrium from A and B toward the most compact and therefore the most active enzyme form. Clearly, a decrease in enzyme activity brought about by the addition of urea (shift to C or D) could be counteracted by the addition of sufficient TMAO to shift the equilibrium back to B, A, or the compact form of the protein. This case is consistent with the observations of Yancey and Somero, who observed that urea nearly always increases K_m and decreases k_{cat} values of a number of enzymes from urea-rich cells of elasmobranchs (Yancey and Somero, 1980), whereas TMAO was found most often to have the opposite effect of decreasing K_m while increasing k_{cat}. The two solutes are viewed as having opposing effects on k_{cat} and K_m because of the functional characteristics of the protein species each solute promotes.

By applying a different case for consideration, using the model presented by Machino and Fridovich, it is possible to explain exceptions to the counteraction hypothesis (Mashino and Fridovich, 1987). For example, if it is assumed that species A or B is the most active enzyme form with the most compact species being the predominant form in water, the action of urea would activate the enzyme, whereas TMAO would inhibit. Thus, depending on which enzyme form predominates in water and which is most active, the model can accommodate cases in which the general observations of Yancey and Somero apply, as well

as cases that are exceptions to the generalizations given by Yancey and Somero.

Our studies on the ability of TMAO to stabilize proteins thermodynamically lends support to the proposal by Mashino and Fridovich. We have found that the unfavorable interaction between TMAO and the peptide backbone provides a strong force for minimizing exposure of the polypeptide backbone to this solute (Wang and Bolen, 1997). Because of this, TMAO will tend to dampen backbone exposure of structural fluctuations arising from the native state ensemble, resulting in an apparent compaction of the native state. It is quite possible that the very same force responsible for thermodynamic stabilization of proteins by TMAO is responsible for TMAO's effects on protein function.

There are other possible explanations for the somewhat general effects of osmolytes on enzyme K_m values that do not require solute-induced shifts in native and denatured ensembles as offered by Machino and Fridovich. Urea acts as a competitive inhibitor of organic substrates of most enzymes (Lushchak and Lushchak, 1994; Rajagopalan et al., 1961; Withycombe et al., 1965; Yancey and Somero, 1978), although there are a small number of reports that urea is a non- or uncompetitive inhibitor of some enzymes, especially of enzymes that use inorganic substrates (Rajagopalan et al., 1961). As follows from transfer free energy measurements of compounds from water to aqueous urea solution, almost all organic compounds interact favorably with urea, regardless of whether they are hydrophobic, polar, or charged (Kundu and Das, 1979; Nozaki and Tanford, 1963; Wang and Bolen, 1997). The propensity of urea to interact favorably with all manner of organic functional groups explains why urea interacts favorably with the native states of proteins and very likely interacts favorably with the vast majority of substrate molecules. In contrast, TMAO does not interact favorably with native protein; it has essentially no interaction with aliphatic hydrophobic amino acid side chains, it interacts favorably to a small extent with charged side chains, and it is very unfavorable in its interaction with the peptide backbone (Wang and Bolen, 1997). Thus the propensities of TMAO and urea to interact with a variety of chemical groupings are basically the opposite of each other, with urea being a better solvent than water for a large number of functional groups, whereas TMAO is generally a poorer solvent than water. If urea interacts favorably with a substrate molecule, it increases the solubility of the substrate. This diminishes the driving force of the substrate to bind to the enzyme active site and should translate into an increase in K_m, as is commonly observed. TMAO, being a poor solvent, will interact unfavorably with that substrate and decrease substrate solubility. The decrease in substrate solubility increases the propensity of substrate to get out of water, and this can be accomplished by binding to the enzyme active site, thus causing an apparent decrease in K_m. As a result, the action of urea and TMAO on substrate solubility alone is sufficient to explain the tendencies of urea and TMAO to induce opposing effects on substrate K_m. Solubility effects, however, do not readily account for any alterations in enzyme k_{cat} values these solutes might cause.

It is useful to point out that the two explanations of counteraction are derived from the same principle. That is, the model by Machino and Fridovich and the effects of urea and of TMAO on substrate solubility are both manifestations of the effects of these solutes on solvation. A poorer solvent than water (e.g., TMAO solution) will favor more compact protein conformations than are favored in water, because compactness restricts exposure to the poorer solvent. In addition, a poorer solvent than water will favor greater affinity of substrate to enzyme, because the bound state restricts exposure of the substrate to solvent. In contrast, a better solvent than water (e.g., urea solution) promotes more expanded protein conformations than water does, because a better solvent will favor greater exposure of protein fabric to solvent. A better solvent than water also promotes substrate dissociation from the enzyme, again because the dissociated state provides greater surface exposure of both the protein fabric and substrates to favorable interactions with the solvent. To differentiate between these possibilities, further work is necessary to explore the effects TMAO and urea have on solvation at the surface of proteins and on substrate solubilities.

Counteraction is not due to nullification of urea effects by the formation of a complex between TMAO and urea. Although it is possible to show by proton NMR that TMAO and urea form a complex in anhydrous organic solvents like acetonitrile, the addition of 1% v/v water to this solution completely abolishes the complex (Baskakov, Qu, and Bolen, unpublished results).

The significance of the counteraction hypothesis of Yancey and Somero is that in the biology of adaptation it is the intracellular environment (the solution) that has evolved, not the intracellular proteins themselves. The premise is that mutational changes in the vast number of intracellular proteins are not necessary for adaptation of the organism to the environmental stress—simply providing an appropriate intracellular solution is enough to protect the organism against the environmental stress while permitting adaptation to such extreme conditions as dehydration, low or high temperature, or the intracellular presence of urea. Our work tests whether counteraction holds for an enzyme that we know has not undergone mutational changes that confer counteraction properties on the enzyme. The results make a good case that solution properties alone play an important role in modulating enzyme activity in a manner helpful in the biology of adaptation.

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