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Effect of Cryosolvents and Subzero Temperatures on the Hydrolysis of L-Leucine-*p*-nitroanilide by Porcine Kidney Leucine Aminopeptidase†

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ABSTRACT: The hydrolysis of L-leucine-*p*-nitroanilide by porcine kidney leucine aminopeptidase in aqueous mixed-solvent systems containing methanol, ethanol, dimethyl sulfoxide, and dimethylformamide has been investigated in the −30 to −23 °C temperature range. At 23 °C and pH* values in the 8–10 range, the enzyme is stable for over 25 h in solutions containing 50% v/v of any of these four cosolvents. Measurements of the tryptophan fluorescence of the enzyme at pH* 9.0 confirm that the enzyme is not denatured under these conditions. K_M increases exponentially and k_{cat} decreases linearly with increasing cosolvent concentration. Methanol, in particular, has a very small effect on K_M. Ultrafiltration experiments demonstrate that there is no dissociation of monomers of the enzyme brought about by the presence of 50% v/v methanol or dimethyl sulfoxide. Preliminary tests with the partition method provide no evidence for an acyl-enzyme intermediate. The effect of pH* on k_{cat} and K_M in 50% v/v methanol is very similar to the effect of pH on these kinetic constants in aqueous solution. Lowering the temperature from 23 to 0 °C does not alter the shape of the pH* profile obtained in 50% v/v methanol. The Arrhenius plot obtained in 50% v/v methanol is linear over the −30 to −23 °C temperature range, and the calculated energy of activation, 8.2 ± 0.8 kcal/mol, is in good agreement with the value of 7.4 ± 0.7 kcal/mol found for the reaction in aqueous solution. Collectively, these data indicate that methanol is the best cosolvent for cryoenzymological studies, that ethanol and dimethyl sulfoxide are also suitable cosolvents, and that the presence of any of these cosolvents at either ambient or subzero temperatures does not perturb the catalytic pathway.

Porcine kidney leucine aminopeptidase (EC 3.4.11.1) is a hexameric zinc metalloenzyme with a subunit M_r of 54 000 (Himmelhoch, 1969; Shen & Melius, 1977; Van Wart & Lin, 1981). It hydrolyzes the N-terminal peptide bond to all L-amino acids except proline and hydroxyproline (Delange & Smith, 1971). In addition, it has both esterase (Delange & Smith, 1971) and thiolesterase (Metrione, 1972) activities. The native enzyme, represented [(LAP)Zn_6−],[1] contains one Zn^{2+} per subunit located at the active (catalytic) site and has an empty regulatory site on each subunit. The enzyme is activated by Mn^{2+} and Mg^{2+} and inhibited by Ni^{2+}, Zn^{2+}, Cu^{2+}, and Hg^{2+}. These metal ions all exert their influence on the enzyme by binding to the regulatory site. The activating and inhibiting metal ions raise and lower k_{cat}, respectively; K_M is unaltered (Van Wart & Lin, 1981). The Mg^{2+}-activated enzyme is represented [(LAP)Zn_6Mg_6].

Very little information pertaining to the mechanism of action of LAP is presently available. In order to provide such information, we have initiated a series of cryoenzymological experiments to study the hydrolysis of L-leucine-*p*-nitroanilide in aqueous organic solvents at subzero temperatures. Such studies have the potential to resolve elementary steps in the catalytic pathway and yield mechanistic information not attainable under normal conditions (Douzou, 1973, 1977; Fink & Geeves, 1979). In particular, it is possible not only to detect but also to accumulate and stabilize certain intermediates for detailed spectral characterization. Here, we report the effects of cryosolvents and subzero temperatures on the catalytic and structural properties of [(LAP)Zn_6Mg_6]. The enzyme is remarkably stable in the presence of organic cosolvents and is particularly well suited to study by the cryoenzymological approach.

Materials and Methods

Leucine aminopeptidase (type III-CP), obtained from Sigma Chemical Co. as a chromatographically purified $(NH_4)_2SO_4$ suspension, was dialyzed against 5 mM Tris, pH 8, at 4 °C for 2 days and lyophilized. Purified [(LAP)Zn_6−] was prepared by incubation of the lyophilized enzyme with 0.1 mM $ZnCl_2$ for 2 h at 37 °C, followed by affinity chromatography

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[1] Abbreviations: LAP, leucine aminopeptidase; [(LAP)Zn_6Mg_6], metalloleucine aminopeptidase where (LAP) represents the hexameric apoenzyme and the brackets indicate the firm binding of 6 g-atoms each of Zn^{2+} and Mg^{2+} at the catalytic and regulatory sites, respectively; [(LAP)Zn_6−], native leucine aminopeptidase with Zn^{2+} at the catalytic site of each subunit and the regulatory site unoccupied; Tris, tris(hydroxymethyl)aminomethane; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Caps, 3-(cyclohexylamino)propanesulfonic acid.

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over L-leucylglycyl-AH-Sepharose (Van Wart & Lin, 1981). All experiments were carried out on [(LAP)Zn_6Mg_6], which was prepared by incubating [(LAP)Zn_6–] at 37 °C in 10 mM Tris, 5 mM $MgCl_2$, pH 9.0, for 4 h. The enzyme concentration (E_0, molarity of hexamers) was determined by using ϵ_{280} = 4.0×10^5 M^{-1} cm^{-1}.

All organic solvents were reagent grade. Dimethyl sulfoxide (Mallinckrodt) was distilled from barium oxide at reduced pressure (2–3 mmHg, bp 50 °C). Dimethylformamide (J. T. Baker) was shaken with KOH and distilled from barium oxide (bp 152 °C). Both were stored at −20 °C until further use. L-Leucine-*p*-nitroanilide, L-leucylglycine, L-leucinamide, and L-leucine methyl ester were purchased from Sigma Chemical Co.

All assays were carried out spectrophotometrically with L-leucine-*p*-nitroanilide as a substrate by continuously monitoring the appearance of *p*-nitroaniline at 405 nm with a Varian Model 219 spectrophotometer. The L-leucine-*p*-nitroanilide concentration was determined by using ϵ_{320} = 1.39×10^4 M^{-1} cm^{-1}. Initial velocities (v) were calculated from the slope of the absorbance change during the first 10% of hydrolysis and converted into units of moles per liter per minute by dividing by ϵ_{405} for *p*-nitroaniline. In the absence of cosolvents, ϵ_{405} = 9900 M^{-1} cm^{-1} (Royer & Andrews, 1973). The ϵ_{405} of *p*-nitroaniline increases linearly as a function of the percent (v/v) of each cosolvent and is given by the equations: ϵ_{405}(methanol) = 9900 + 38X, ϵ_{405}(ethanol) = 9900 + 67X, ϵ_{405}(dimethylformamide) = 9900 + 74X, and ϵ_{405}(dimethyl sulfoxide) = 9900 + 76X, where X is the percent (v/v) cosolvent.

The kinetic parameters k_{cat} and K_M were generally determined from double-reciprocal plots, on the assumption that there are six active sites per hexamer. The maximum L-leucine-*p*-nitroanilide concentration examined was limited by its solubility but always exceeded K_M by at least 2-fold, ensuring the accuracy of these parameters. Thus, in the absence of cosolvents, the maximum solubility at 23 °C is 2.5 mM while K_M is 1 mM. As the concentration of each cosolvent is increased, the solubility of substrate is increased more than K_M. Thus, in 50% v/v methanol at 23 °C, assays can be carried out at a L-leucine-*p*-nitroanilide concentration of 20 mM, while K_M is 2 mM. For the Arrhenius plot shown in Figure 7, the values of k_{cat} in the −10 to 40 °C range were obtained from double-reciprocal plots. Those in the −30 to −10 °C range were obtained at the single substrate concentration of 20 mM ([S] $\simeq 10K_M$), where v/E_0 was taken to equal k_{cat}.

The subzero kinetics were carried out by using the Cary 219 in conjunction with a specially constructed low-temperature stopped-flow instrument that is compatible with slow-scanning instruments (Van Wart & Zimmer, 1981). The temperature of the system is controlled to ±0.2 °C by pumping precooled methanol from a Neslab Model ULT-80 temperature bath through channels in the cooling block, and the exposed surfaces of the observation cell are kept free of frost with a stream of precooled dry nitrogen. The temperature of the stopped-flow module was measured with a copper–constantan grounded thermocouple (Omega, SCPSS-040G-6) and an Omega Model 2176 A-T digital thermometer. For assays, the appropriate concentrations of enzyme and substrate were prepared at 2 °C, loaded into the drive syringes, and equilibrated to the desired temperature. The absorbance change at 405 nm was used to follow hydrolysis, as described above. The absorbance at 550 nm, where neither the enzyme nor the substrate absorbs, was monitored periodically to check for optical artifacts due to protein aggregation, solute precipitation, etc. However, under the conditions reported, no problems of this type arose. Fluorescence spectra of intrinsic tryptophan residues (Timasheff, 1970) were obtained with a Perkin-Elmer Model MPF-2A fluorometer.

The apparent protonic activity in aqueous organic solutions, pH*, was measured with a Corning glass electrode at either 1 or 23 °C, as outlined by Fink & Geeves (1979). To realize a desired pH* at subzero temperature in a given buffer and cryosolvent, we used tables of the temperature dependence of pH* (Douzou, 1977) to calculate the change of pH* expected on lowering the temperature from 1 °C to the desired value. The solution was then prepared at 1 °C at a higher or lower value of pH* so that, on cooling, the desired pH* would be obtained.

The state of association of the enzyme in the presence of cosolvents was examined by ultrafiltration. In separate experiments, solutions of the enzyme in 50% v/v methanol or dimethyl sulfoxide were concentrated with an Amicon Diaflo XM100A filter (cut-off M_r 100000). These experiments, as well as a control in the absence of cosolvent, were carried out at both 2 and 23 °C. Rejection coefficients, defined as ln (C_f/C_0)/ln (V_0/V_f), were calculated for each experiment, where C_f and C_0 are the enzyme concentrations of the retentate and starting solution, respectively, and V_0 and V_f are the volumes of the starting solution and retentate, respectively.

The possibility of an acyl-enzyme intermediate was examined by the partition method in which the reaction is carried out in the presence of a nucleophile and the product distribution is subsequently determined (Epand & Wilson, 1965). Thus, in separate experiments, aliquots of the substrates (final concentration 2 mM) L-leucine-*p*-nitroanilide, L-leucinamide, L-leucylglycine, and L-leucine methyl ester were incubated overnight at 23 °C with [(LAP)Zn_6Mg_6] in 10 mM Tris, 0.1 M KCl, and 5 mM $MgCl_2$, pH 9.0, containing 1 M NH_2OH. The amount of L-leucine hydroxamate was determined by treating the mixtures with 10% $FeCl_3$ in 0.1 M HCl, followed by spectrophotometric analysis at 540 nm.

Results

Choice of Experimental Conditions. The choice of the particular metalloleucine aminopeptidase and substrate, as well as that of the cosolvent, buffer and ionic strength, to be used is critical to the success of cryoenzymological experiments. [(LAP)Zn_6Mg_6] has been chosen for study because of its high activity. While [(LAP)Zn_6Mn_6] is more active, the oxidation of unbound Mn^{2+} causes spectral interference at high pH. L-Leucine-*p*-nitroanilide was chosen because it is a good, specific substrate and its hydrolysis can be monitored at wavelengths where there is no interference from absorption of cosolvents.

The best cosolvent for use in cryoenzymology is methanol because of its low viscosity, low freezing point, and high dielectric constant. Since ethanol, dimethyl sulfoxide, and dimethylformamide have also been used with success in other systems (Fink & Geeves, 1979), all four of these cosolvents have been investigated. In the event that the enzyme-catalyzed reaction proceeds through a covalent intermediate, it would be necessary to use a cosolvent that is not nucleophilic to avoid transferase activity. For this reason, and for other presently unforeseen reasons (solubility of solutes at low temperatures, etc.), it is advantageous to investigate the use of more than one cosolvent. Near the pH optimum of [(LAP)Zn_6Mg_6] of 9.5, Tris is an appropriate buffer. Mes, Hepes, and Caps do not inhibit the enzyme and are suitable buffers for the remainder of the 6–11 pH range (Van Wart & Lin, 1981).

Table I: Stability of [(LAP)Zn_6Mg_6] in Cryosolvents under Various Conditions[a]

cosolvent (% v/v)	pH*	incubation temp (°C)	v/E_0[b] (min^{-1}) vs. incubation time (h) 0	5	10	20	25
methanol (50)	10.0	23	19	19	18	19	20
	9.0	23	24	24	24	25	25
	8.0	23	21	21	21	21	22
	7.0	23	9.0	6.0	4.0	0	0
	7.0	2	9.0	8.0	6.0	4.0	3.0
ethanol (50)	10.0	23	2.5	2.5	2.5	2.6	2.6
	9.0	23	4.1	4.0	4.0	4.2	4.0
	8.0	23	2.3	2.3	2.2	2.3	2.3
	7.0	23	1.0	0.5	0.2	0	0
dimethyl sulfoxide (50)	10.0	23	7.0	7.0	7.0	7.0	7.0
	9.0	23	9.0	8.5	9.0	9.0	9.0
	8.0	23	5.5	5.6	5.5	5.5	5.5
	7.0	23	3.5	0	0	0	0
	7.0	2	3.6	2.5	0	0	0
dimethylformamide (50)	10.0	23	6.0	5.8	6.0	6.0	6.0
	9.0	23	4.2	4.2	4.1	4.2	4.3
	8.0	23	3.8	3.8	3.6	3.8	4.0
	7.0	23	1.0	0.5	0	0	0

[a] All samples were incubated at the indicated pH*, temperature, and cosolvent in the presence of 0.1 M KCl, 1 mM Mg^{2+}, and 10 mM appropriate buffer (Hepes, pH* 7.0; Tris, pH* 8.0–9.0; Caps, pH* 10.0). [b] All assays carried out at 23 °C in 10 mM buffer (Hepes, pH* 7.0; Tris, pH* 8.0–9.0; Caps, pH* 10.0), 0.1 M KCl, and 1 mM Mg^{2+} at a L-leucine-*p*-nitroanilide concentration of 4 mM.

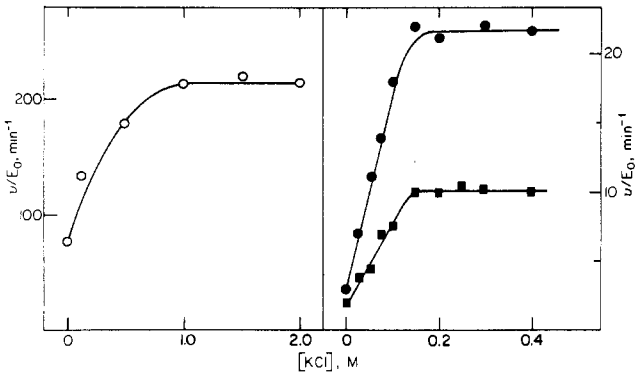

FIGURE 1: Effect of KCl concentration on activity of [(LAP)Zn_6Mg_6] in (○) 10 mM Tris–1 mM $MgCl_2$, pH 9.0, and in the same buffer at pH* 9.0 containing (●) 50% v/v methanol or (■) 50% v/v dimethyl sulfoxide. Assays were carried out at a L-leucine-*p*-nitroanilide concentration of 1 mM.

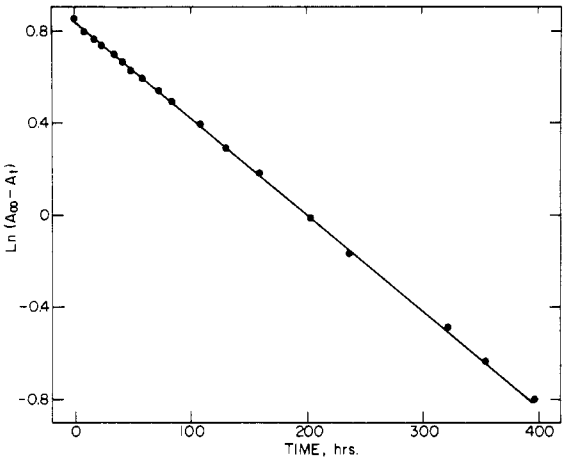

FIGURE 2: First-order plot for hydrolysis of 0.2 mM L-leucine-*p*-nitroanilide by 4 nM [(LAP)Zn_6Mg_6] in 10 mM Tris, 0.1 M KCl, and 1 mM $MgCl_2$ containing 50% v/v methanol at pH* 9.0 at 23 °C.

The ionic strength is controlled by addition of 0.1 M KCl. This was chosen on the basis of the data in Figure 1, which show the effect of KCl concentration on the activity of [(LAP)Zn_6Mg_6] in the absence of cosolvent and in the presence of 50% v/v methanol or dimethyl sulfoxide. In aqueous solution, the activity of [(LAP)Zn_6Mg_6] increases with the KCl concentration and levels off at a value of 1 M. In contrast, in 50% v/v methanol or dimethyl sulfoxide, the activity levels off near 0.15 M KCl. Due to the poor solubility of KCl in the cryosolvents employed at subzero temperatures (Douzou, 1973), 0.10 M KCl has been used in subsequent experiments to avoid precipitation. For a similar reason, 1 rather than 5 mM $MgCl_2$ has been included in the assay buffer to keep the regulatory sites saturated with Mg^{2+} (Van Wart & Lin, 1981). Thus, unless stated otherwise, the cryosolvents used all contain 10 mM buffer, 0.1 M KCl, and 1 mM $MgCl_2$.

Stability of [(*LAP*)Zn_6Mg_6] *in Cryosolvents.* The activity of [(LAP)Zn_6Mg_6] solutions containing 50% v/v of each of the four cosolvents was measured at various pH* values at 23 °C as a function of time (Table I). In all four cryosolvents, the enzyme was completely stable at 23 °C in the 8–10 pH* range for at least 25 h. At pH* 7.0, however, the enzyme progressively loses activity. This inactivation is slowed, but not prevented, by lowering the temperature to 2 °C. Hence, all subsequent experiments, except the pH* profiles, were limited to the 8–10 pH* range. The activities at lower values of pH* reported in the pH* profiles were obtained within 5 min of addition of cosolvent in order to estimate the activity.

Effect of Cosolvents on Catalytic Properties of [(*LAP*)-Zn_6Mg_6]. A stringent test as to whether a cryosolvent alters a reaction pathway is to follow the reaction for a significant portion of total hydrolysis to assess if there is any change in reaction order. A first-order plot for the hydrolysis of 0.2 mM L-leucine-*p*-nitroanilide by 4 nM [(LAP)Zn_6Mg_6] in 50% v/v methanol at pH* 9.0 and 23 °C is shown in Figure 2. The reaction follows first-order kinetics for at least the first 400 h. Since the half-life for the reaction is 165 h, the plot is linear for more than two half-lives. The slope of this plot gives $-k_{obsd} = 6k_{cat}E_0/K_M$. The calculated value of k_{cat}/K_M is 3.0×10^3 M^{-1} min^{-1}, which is in excellent agreement with the value of 2.9×10^3 M^{-1} min^{-1} obtained from a double-reciprocal plot with the full range of substrate concentrations (see below, Figure 3).

The effect of the concentrations of methanol, ethanol, dimethyl sulfoxide, and dimethylformamide on k_{cat} and K_M for this reaction at pH* 9.0 and 23 °C is shown in Figure 3. The

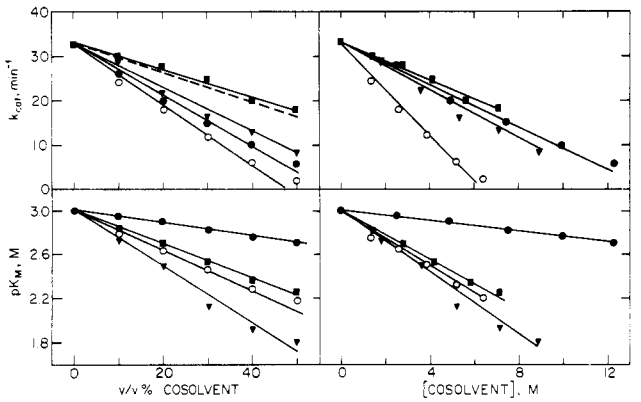

FIGURE 3: Effect of cosolvent concentration (left panel, % v/v; right panel, M) on kinetic constants for hydrolysis of L-leucine-*p*-nitroanilide by [(LAP)Zn_6Mg_6] in 10 mM Tris, 0.1 M KCl, and 1 mM $MgCl_2$, pH* 9.0 at 23 °C. The cosolvents are methanol (●), ethanol (▼), dimethylformamide (○), and dimethyl sulfoxide (■). The dashed line in the top-left panel is the value of k_{cat} calculated solely on the basis of the lowered water concentration in each cryosolvent.

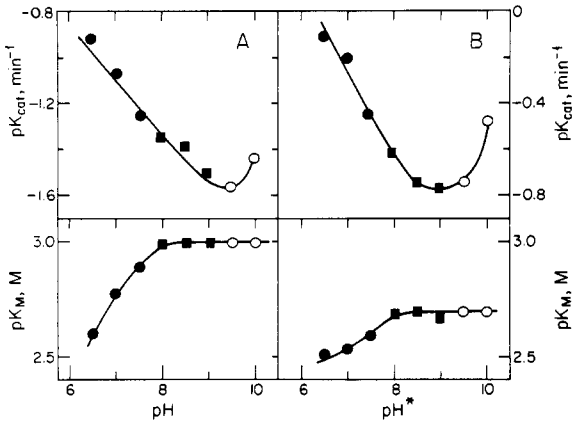

FIGURE 4: Effect of pH or pH* on kinetic constants for hydrolysis of L-leucine-*p*-nitroanilide by [(LAP)Zn_6Mg_6] at 23 °C in 10 mM buffer, 0.1 M KCl, and 1 mM $MgCl_2$ in the (A) absence and (B) presence of 50% v/v methanol. The buffers used were (●) Hepes, (■) Tris, and (○) Caps. The solid lines are included to depict the changes in the data and are not theoretical curves.

data in the left panel are plotted against the percent v/v cosolvent and those in the right panel against the more meaningful quantity, the molarity of cosolvent. Within experimental error, k_{cat} decreases linearly and to the same extent as the molarities of methanol, ethanol, and dimethyl sulfoxide increase; dimethylformamide decreases k_{cat} considerably more on a molar basis. The observed reductions in k_{cat} by all cosolvents except dimethyl sulfoxide are lower than those calculated (dashed line, left panel of Figure 3) on the basis of the lowered concentration of water and on the assumption that the water participates in the rate-determining step. Increases in the molarity of ethanol, dimethyl sulfoxide, and dimethylformamide all lower pK_M linearly (i.e., increase K_M exponentially) and to approximately the same extent. However, methanol has very little effect on pK_M.

The variation of pk_{cat} and pK_M for this reaction with pH in aqueous solution and also with pH* in 50% v/v methanol at 23 °C is illustrated in Figure 4. In both systems, pK_M increases with pH, or pH*, and levels off at a value of 8.0. In both the absence and presence of methanol, pk_{cat} decreases linearly with pH or pH*, respectively; it is minimal at pH 9.5 in the absence of methanol but at pH* 9.0 in the presence of methanol.

The presence of an acyl-enzyme intermediate may be diagnosed by carrying the reaction out in the presence of the

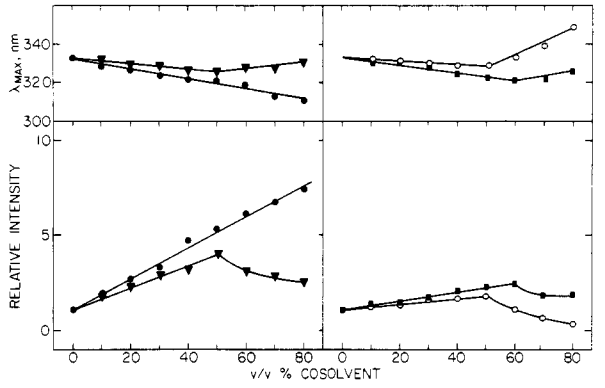

FIGURE 5: Effect of cosolvent on wavelength (λ_{max}) and intensity of tryptophan fluorescence band (λ_{ex} = 260 nm) of 16 nM [(LAP)-Zn_6Mg_6] in 10 mM Tris, 0.1 M KCl, and 0.25 mM $MgCl_2$, pH* 9.0 at 23 °C. The cosolvents are methanol (●), ethanol (▼), dimethylformamide (○), and dimethyl sulfoxide (■).

Table II: Rejection Coefficients[a] of [(LAP)Zn_6Mg_6] by an Amicon XM-100A Ultrafiltration Membrane under Various Conditions

cosolvent (% v/v)	temp (°C)	rejection coefficient
none	23	0.93
	2	0.91
methanol (50)	23	0.95
	2	0.93
dimethyl sulfoxide (50)	23	0.91
	2	0.90

[a] Rejection coefficient = $\ln (C_f/C_0)/\ln (V_0/V_f)$, where C_f = final enzyme concentration of the retentate (fluid plus filter), C_0 = initial enzyme concentration, V_0 = initial sample volume, and V_f = the final retentate volume.

nucleophile hydroxylamine and analyzing the products for the presence of L-leucine hydroxamate (Epand & Wilson, 1965). This method gives negative results for the four substrates L-leucine-*p*-nitroanilide, L-leucinamide, L-leucylglycine, and L-leucine methyl ester. Though failure to find positive evidence for an acyl intermediate does not rule out its existence, this experiment indicates that weak nucleophilic solvents like methanol or ethanol should not interfere directly with the reaction if such an intermediate were present. Dimethyl sulfoxide would be a good alternative cosolvent if it were necessary to eliminate alcohols.

Effect of Cosolvents on Structure of [(*LAP*)Zn_6Mg_6]. To assess the effects of cosolvent on the structure of the enyzme, we monitored the intrinsic fluorescence of its tryptophan residues as a function of cosolvent concentration. Excitation at 260 nm was employed to effect tyrosine to tryptophan energy transfer, since this has been reported to be extremely sensitive to small structural changes (Fink & Geeves, 1979). Both λ_{max} and relative intensities of the tryptophan emission spectra as a function of cosolvent concentration at 23 °C and pH* 9 are shown in Figure 5. The enzyme is remarkably stable in methanol; no adverse structural effects are noted up to 80% v/v. Sharp breaks in these curves, indicative of denaturation, can be seen at 50–60% v/v ethanol, dimethylformamide, and dimethyl sulfoxide. The changes in λ_{max} and emission intensity below 60% v/v of these cosolvents are smooth and linear, indicating that they are due to solvent effects on exposed residues. Hence, the enzyme remains undenatured up to 50% v/v of all four cosolvents.

To assess whether these cryosolvents alter the quaternary structure of the enyzme, we carried out ultrafiltration experiments in both 50% v/v methanol and dimethyl sulfoxide

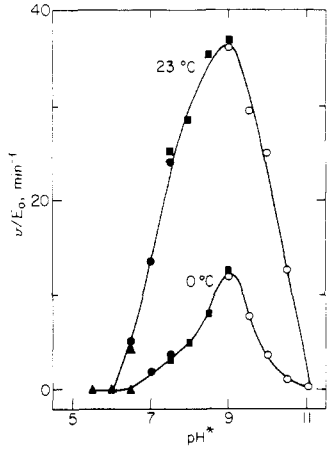

FIGURE 6: Dependence of the rate of hydrolysis of L-leucine-*p*-nitroanilide (10 mM) by [(LAP)Zn_6Mg_6] in 10 mM buffer, 0.1 M KCl, and 1 mM $MgCl_2$ containing 50% v/v methanol on pH* at 23 and 0 °C. The buffers used were Mes (▲), Hepes (●), Tris (■), and Caps (○). The solid lines are included to depict the changes in the data and are not theoretical curves.

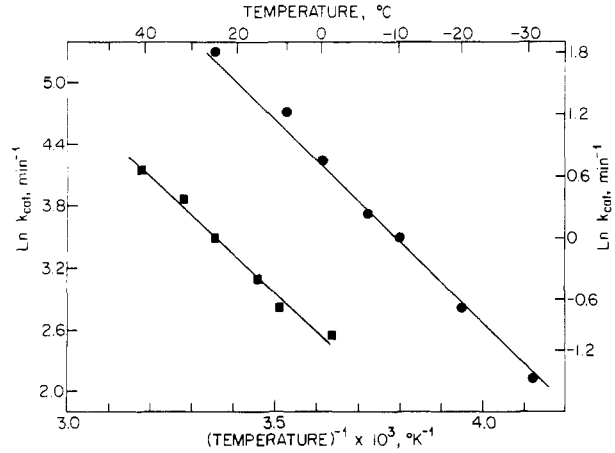

FIGURE 7: Arrhenius plots for k_{cat} values describing hydrolysis of L-leucine-*p*-nitroanilide by [(LAP)Zn_6Mg_6] in 10 mM Tris, 0.1 M KCl, and 1 mM $MgCl_2$ (■) at pH 9.0 in absence of cosolvent and (●) at pH* 9.0 in the presence of 50% v/v methanol.

Table III: Kinetic Parameters for Hydrolysis of L-Leucine-*p*-nitroanilide by [(LAP)Zn_6Mg_6] in 50% v/v Methanol, 10 mM Tris, 0.1 M KCl, and 1 mM Mg^{2+}, pH* 9.0

temp (°C)	k_{cat} (min^{-1})	K_M (mM)	k_{cat}/K_M (M^{-1} min^{-1})
23.0	5.8	2.0	2.9×10^3
0.0	2.1	2.0	1.1×10^3
−10.4	1.0	2.5	4.0×10^2

at both 23 and 2 °C. The results, expressed in terms of the rejection coefficient of the filter for the enzyme, are tabulated in Table II. The rejection coefficients in all experiments are greater than 0.90. Since the rejection coefficients for proteins of M_r 50 000 and 100 000 are approximately 0.20 and 0.80, respectively, this indicates that no monomers and probably no dimers are present. The presence of trimers would probably have gone undetected.

Effect of Temperature on Catalytic Properties of [*(LAP)*Zn_6Mg_6]. Figure 6 shows the pH* profiles for this reaction carried out in 50% v/v methanol at 23 and 0 °C. Four separate, but overlapping, buffers were used to cover this pH* range: Mes (pH 5.5–6.5); Hepes (pH 6.5–7.5); Tris (pH 7.5–9.0); Caps (pH 9.0–11.0). Both pH* profiles are bell shaped with an optimum pH* near 9.0. With the exception of the lower activity at 0 °C, lowering the temperature does not appear to alter the dependence of the reaction on protonic activity.

Arrhenius plots for k_{cat} in the presence and absence of 50% v/v methanol are shown in Figure 7. Both plots are linear over the temperature range studied. The energy of activation in 50% v/v methanol (25 → −30.4 °C) is 7.4 ± 0.7 kcal/mol, in good agreement with the value of 8.2 ± 0.8 kcal/mol measured in the absence of methanol. This agreement supports the idea that the catalytic pathway is the same in 50% v/v methanol as in the absence of methanol. The kinetic parameters for this reaction at 23.0, 0.0, and −10.4 °C are listed in Table III. Within experimental error, K_M is independent of temperature, and the reduction in activity is almost solely attributable to the lowering of k_{cat} shown in Figure 7.

Discussion

The goal of this work is to study the effects of cryosolvents and subzero temperatures on the structural and catalytic properties of [(LAP)Zn_6Mg_6] in order to assess the feasibility of using cryoenzymology to investigate the catalytic pathway of this enzyme. The results indicate that [(LAP)Zn_6Mg_6] is extremely stable in the four cryosolvent systems studied and that it is ideally suited to study by cryoenzymology.

The data in Table I and Figure 1, together with the results of previous experiments (Van Wart & Lin, 1981), reveal the best conditions for these experiments. KCl, which is added to all cryosolvents to maintain the ionic strength constant, activates the enzyme. Interestingly, a much lower concentration of KCl is required for full activation in the presence of 50% v/v methanol than in its absence. This is probably related to the lower concentration of water in the cryosolvents. The underlying basis for this activation, which is also observed for salts containing other cations and anions (S. H. Lin and H. E. Van Wart, unpublished data), is not clear. The effect of KCl is to raise k_{cat}, leaving K_M invariant (data not shown). This is the same effect that activating metals have on the enzyme.

Table I establishes that the enzyme is stable in the presence of 50% v/v of all four cosolvents, provided that the pH* is in the 8–10 range. The progressive decrease in activity below this pH* as a function of time is likely due to loss of the catalytic zinc from the active site. Himmelhoch (1969) has demonstrated that the native enzyme loses activity at pH 6.0 due to loss of zinc. Presumably, organic cosolvents destabilize the zinc binding site, and loss of zinc can occur at values of pH* near 7.0. In practical terms, this is not a limitation to the study of the enzyme by cryoenzymology, since such experiments focus on the pH* 8–10 region, where activity is maximal. Since the activity of the enzyme is probably the most sensitive criterion for whether or not adverse effects are occurring, the time-independent activities observed in the pH* 8–10 region strongly imply that these aqueous organic media are acceptable cryosolvents. This conclusion is supported by the observation that the hydrolysis of L-leucine-*p*-nitroanilide in 50% v/v methanol follows first-order kinetics for over 400 h and that the activity calculated from the half-life agrees with that obtained from initial rates measured as a function of substrate concentration.

The effects of all four cosolvents on k_{cat} and K_M for the hydrolysis of L-leucine-*p*-nitroanilide by [(LAP)Zn_6Mg_6] are similar to those reported for other enzyme-catalyzed reactions (Fink & Geeves, 1979). The linear decrease in pK_M with cosolvent concentration has been attributed to the hydrophobic partitioning of the substrate between the cryosolvent and the active site of the enzyme, leading to decreased binding. It is

noteworthy that methanol increases K_M very little; the presence of 50% v/v methanol at 23 °C only increases K_M from 1.0 mM to 2.0 mM. The other three cosolvents all increase K_M to a much greater extent. It is not clear why methanol alone has so little effect on K_M, but this property makes it an ideal cosolvent for cryoenzymology. All cosolvents except dimethylformamide produce about an equal linear decrease in k_{cat} as the molarity is increased. With the exception of dimethyl sulfoxide, the decrease exceeds that calculated on the basis of the decrease in the water concentration. Hence, these cosolvents do have some effect on the efficiency of the enzyme. The basis for the larger inhibitory effect of dimethylformamide is not obvious, but the shift in the pH* optimum to above 10 (Table I) confirms that it has an effect on the reaction and, hence, is not a good choice. The retention of activity in 50% v/v cosolvents is in agreement with the fluorescence measurements (Figure 5), which confirm that [(LAP)Zn_6Mg_6] is not denatured until the concentration of cosolvent exceeds 50% (v/v). In fact, for methanol, there is no evidence for denaturation at 23 °C in as high as 80% v/v methanol. The ultrafiltration data for the enzyme in the presence of either methanol or dimethyl sulfoxide indicate that no monomers are formed. Hence, these four cosolvents do not appear to cause any major structural alteration in the enzyme. Methanol is clearly the best cosolvent, but either ethanol or dimethyl sulfoxide is also suitable.

Further proof that methanol does not alter the catalytic properties of the enzyme comes from the similarity of the pH profiles in the presence and absence of 50% v/v of this cosolvent (Figure 4). The magnitudes of pk_{cat} and pK_M are affected by methanol, but the variations in these quantities with pH* are almost identical with the variations in pH found in aqueous solution, with the exception that k_{cat} is maximal at pH 9.5 in aqueous solution but at pH* 9.0 in 50% v/v methanol. This similarity is probably due to the fact that the dielectric constant of 50% v/v methanol (65) is almost as high as that of water (80), leading to similar electrostatic interactions in both systems. The shift in the pH* optimum from 9.5 to 9.0 in the presence of methanol is probably related to the pK of L-leucine-*p*-nitroanilide, since this decreases from 7.7 in aqueous solution to 7.3 in 50% v/v methanol.

Lowering the temperature does not appear to alter the catalytic reaction, other than by lowering the overall rate. The pH* profile has a very similar shape and the same pH* optimum at both 23 and 0 °C (Figure 6). The reduction in activity with temperature is caused primarily by a reduction in k_{cat}; K_M does not seem to vary appreciably as a function of temperature (Table III). The Arrhenius plots for the reaction carried out in the presence and absence of 50% v/v methanol are linear and nearly parallel (Figure 7). This indicates that there are no adverse effects on the structure of [(LAP)Zn_6Mg_6] and that there is no change in the rate-determining step over this temperature range. Furthermore, the fact that the energies of activation are nearly equal in the presence and absence of methanol provides additional proof that the reaction is following the same pathway as under normal conditions.

Collectively, the previous observations indicate [(LAP)-Zn_6Mg_6] is ideally suited to study by cryoenzymology. In particular, cryosolvents containing methanol are tolerated particularly well by the enzyme. There is no evidence for any adverse effects on catalysis up to greater than 50% v/v methanol down to at least −30 °C. In addition, there does not seem to be any problem with transferase activity involving the alcohol due to the presence of an acyl intermediate. Should it be necessary to use other cosolvents for presently unforeseen reasons (solubility of enzyme or substrate at very low temperatures, etc.), dimethyl sulfoxide and ethanol are suitable alternatives. Studies designed to search for catalytic intermediates in this reaction carried out at subzero temperatures are currently under way.

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