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Unusual solvent isotope effects on the aminoacylase-catalyzed hydrolysis of acetylamino acids

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The deuterium solvent isotope effect on hydrolysis of acetylamino acids catalyzed by porcine kidney aminoacylase I (EC 3.5.1.14) was studied. With Ac-L-Met, a 'standard' aminoacylase substrate, the effect was normal at low pH ($k_{cat(D)}/k_{cat(H)} = 0.7$ at pH 6), virtually absent at neutrality, and distinctly inverse ($k_{cat(D)}/k_{cat(H)} = 1.4$) at pH 9. K_m was not significantly affected. The rates of Ac-L-Phe hydrolysis in D_2O considerably exceeded those in H_2O at any pH between 6.5 and 9. We explain this unusual effect of D_2O on aminoacylase I catalysis by an inverse equilibrium effect partially cancelling or, at high pH, reversing a normal isotope effect on k_{cat}.

Aminoacylase; Kinetics; Reaction mechanism; Solvent isotope effect; (Hog kidney)

1. INTRODUCTION

Hog kidney aminoacylase I (*N*-acylamino acid amidohydrolase, EC 3.5.1.14) is widely used as a reagent to resolve amino acid racemates. Its substrate specificity and steric requirements were established many years ago [1] while systematic kinetic studies on its mechanism of action were undertaken only recently. ^{18}O exchange experiments provided evidence indicating that catalysis by aminoacylase I does not involve a covalent intermediate [2]. When examining the pH dependence of its kinetic parameters, we found that the bell-shaped pH-activity profiles published previously are mainly due to effects exerted by phosphate. In organic buffers, k_{cat} is almost independent of pH between 6 and 9 for all acetylamino acid substrates examined, while the effect of pH on K_m strongly varies among substrates [3]. Here we report results of kinetic experiments in H_2O/D_2O mixtures which reveal solvent isotope effects strikingly different from those observed with other hydrolases.

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2. EXPERIMENTAL

2.1. *Materials*

Aminoacylase I was purified from hog kidney as described [3]. Acetylamino acid substrates were obtained from Sigma or Serva, and used without further purification. D_2O (99.8%) was from Aldrich. Good buffers Mes, Mops, and Bicine (Serva) were recrystallized from water or ethanol/water before use. All buffers had a concentration of 50 mM and were adjusted to the desired p*L* (the negative logarithm of lyonium ion concentration, i.e. pH or pD) with NaOH or NaOD in D_2O. The corrections necessary to compensate for the effect of D_2O on glass electrodes were applied as in [4].

2.2. *Methods*

Aminoacylase activities were determined discontinuously at 25°C by flow injection analysis: The amino acids liberated during hydrolysis were reacted with *o*-phthalaldehyde/2-mercaptoethanol

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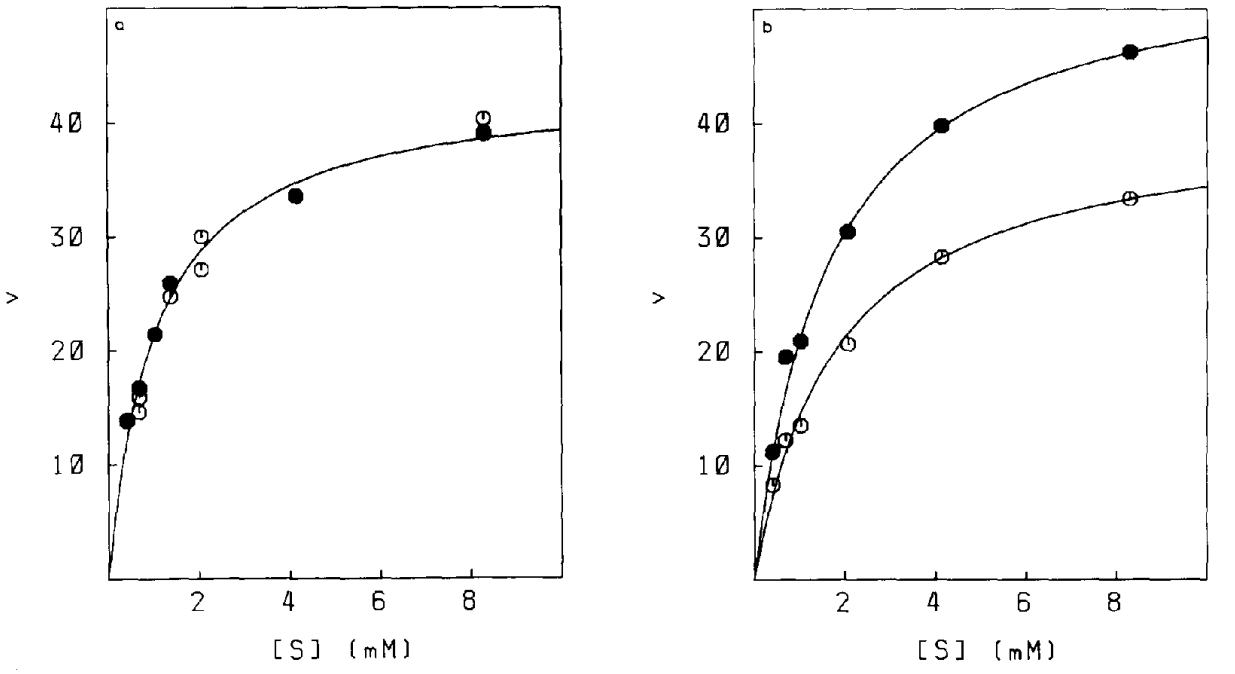

Fig.1. Kinetics of Ac-L-Met hydrolysis in H_2O (○), and in 99% D_2O (●) in 50 mM Mops, pL 7.0 (a), and 50 mM Bicine, pL 9.0 (b). Velocities are given in arbitrary units. The solid line in a is a hyperbolic fit with V_{max} = 43.4 ± 1.9, K_m = 1.05 ± 0.11 mM; the parameters in b are V_{max} = 55.4 ± 0.8, K_m = 1.62 ± 0.10 mM (upper curve), and V_{max} = 40.8 ± 1.3, K_m = 1.87 ± 0.18 mM (lower curve).

and the product detected fluorimetrically as detailed in [3,5]. Some experiments with Ac-L-Met were performed by direct ultraviolet spectroscopy at 232 or 238 nm. Both the fluorescence yield in flow injection analysis and the absorption coefficients of acetylamino acids in the ultraviolet are significantly lower in D_2O than in H_2O. All assays, therefore, were calibrated with amino acid standard solutions, or by determining the total absorption change during complete hydrolysis of the substrates. The data were evaluated by linear or nonlinear regression analysis using our KINFIT program [6].

3. RESULTS

At neutral pH the solvent isotope effects on the hydrolysis of some representative acetylamino acid

Table 1

Kinetic parameters for aminoacylase substrates in H_2O and D_2O

Substrate	$k_{cat(H)}$ (s^{-1})	$V_{max(H)}/V_{max(D)}$	$K_{m(H)}$ (mM)	$K_{m(D)}$ (mM)
Ac-L-Met	240	1.20 ± 0.09	0.99 ± 0.15	0.91 ± 0.13
Ac-L-Glu	43	1.28 ± 0.14	10.2 ± 1.5	7.0 ± 1.1
Ac-L-Phe	1.4	0.78 ± 0.04	5.5 ± 0.3	5.6 ± 0.4

Data measured in Mops/NaOH at 25°C. Buffers were made up in H_2O and 99.8% D_2O and titrated to a meter reading of 7.0 with NaOD/D_2O

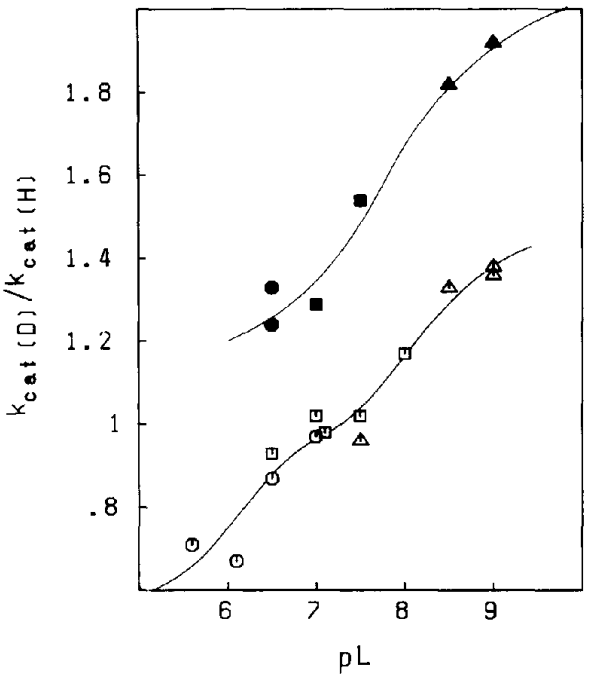

Fig.2. pH dependence of the solvent isotope effects on k_{cat} for Ac-L-Met (open symbols), and Ac-L-Phe (filled symbols). Ratios of k_{cat} in 96–99% D_2O over k_{cat} in H_2O are plotted vs pL (see text). The buffers were Mes (circles), Mops (squares), and Bicine (triangles), 50 mM each.

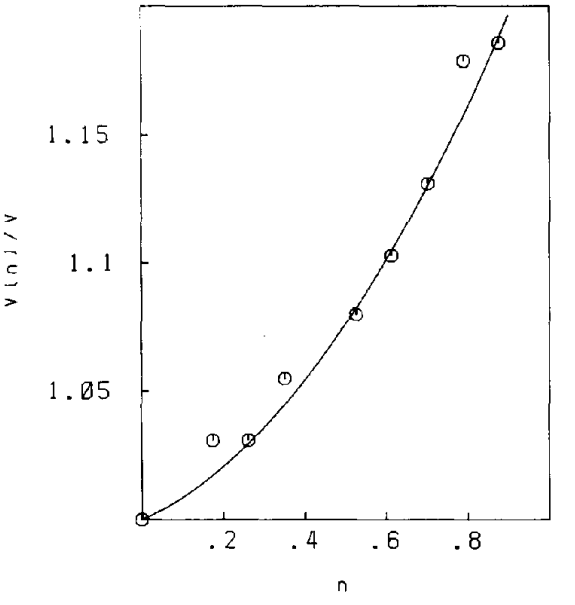

Fig.3. 'Proton inventory' of Ac-L-Met hydrolysis at pL 8.0. Rates v_n in mixed isotopic solvents containing various atom fractions n of deuterium are given relative to the rate v in pure H_2O. The substrate concentration was 8.8 mM in 50 mM Mops buffer.

substrates were small (table 1). With Ac-L-Met, an exceptionally good substrate, and with Ac-L-Glu hydrolysis was slightly faster in H_2O while with Ac-L-Phe a distinctly inverse isotope effect ($k_{cat(H)} < k_{cat(D)}$) was observed. The slightly lower $K_{m(D)}$ for Ac-Glu is due to the fact that, in the experiment shown, buffer was adjusted to the same meter reading rather than to the same pL. The Michaelis constants for Ac-Met and Ac-Phe which are almost independent of pH around 7 were not significantly affected by that circumstance.

The finding of an inverse deuterium isotope effect led us to examine the pH dependence of the effect on Ac-Met and Ac-Phe in more detail. In this series of experiments the buffers in D_2O and H_2O were adjusted to the same pL. Substrate saturation curves obtained with Ac-Met at pL 7 and 9 are shown in fig.1. At pL 7 both k_{cat} and K_m in D_2O did not significantly differ from those in H_2O as judged by the t-test [6]. At pL 9, $K_{m(D)}$ was slightly lower than $K_{m(H)}$, the difference still being insignificant at the 95% level. In contrast, the maximal velocity in D_2O was substantially higher than in ordinary water ($k_{cat(H)}/k_{cat(D)} = 0.74$). The pH dependence of the isotope effect on k_{cat} for Ac-Met is shown in fig.2. At pH 5.5 we observed a normal effect of about 1.4 which became progressively lower with increasing pH, changing its sign at pH 7–7.5. Similar results were obtained with Ac-Phe. Again, $K_{m(H)}$ and $K_{m(D)}$ were not significantly different at any pL between 6.5 and 9 while the effect on k_{cat} became more inverse with increasing pL, reaching 2 at pH 9.

Studies on the solvent isotope effects in a series of isotopic solvent mixtures containing different atom fractions n of deuterium (so-called 'proton inventories' [4]) often provide valuable information on the underlying mechanism of reaction. Depending on the number of protonic sites involved and on the particular reaction mechanism, plots of v_H/v_D vs n may be linear or exhibit upward or downward curvatures. The proton inventory data for Ac-Met at pL 8.0 (fig.3) suggest a non-linear behaviour with an upward curvature of the plot.

4. DISCUSSION

Whenever protons are transferred in the rate-limiting step of a reaction pathway, large deuterium solvent isotope effects (>2) are expected to occur. This, for instance, holds for L-asparaginase, another amidohydrolase [7], as well as for several serine proteases and other hydrolytic enzymes. We show here that with aminoacylase I the solvent isotope effects on k_{cat} for good substrates are normal but small at moderately acidic and neutral pH; at higher pH they become distinctly inverse.

As yet, inverse solvent isotope effects on enzyme-catalyzed reactions have rarely been encountered. A well-documented example is the oxidation of aldehydes by yeast alcohol dehydrogenase [8] where $k_{cat(H)}/k_{cat(D)}$ was as low as 0.5–0.6. Usually such observations are explained by the assumption that protonic equilibria involving acids with fractionation factors well below unity precede the rate-limiting step in catalysis (the fractionation factor ϕ is defined as the ratio of deuterium to protium present in a given protonic site relative to the ratio in bulk water, cf. [4]). In such a case the inverse preequilibrium effect may cancel a small normal effect in a subsequent rate-limiting step [9]. Among the species with low fractionation factors are OH^- (ϕ = 0.47–0.56), sulfhydryl groups (ϕ = 0.40–0.46) and, possibly, metal-coordinated water molecules [10].

The active site of hog kidney aminoacylase I harbours both a Zn^{2+} and essential cysteine residues [11]. One of the cysteines appears to be involved in Zn^{2+} binding while the other is accessible to solutes. From the pH dependence of the reaction of the latter group with *N*-ethylmaleimide an apparent pK_a of 8.3 was obtained [12]. Very similar pK_a values were derived from K_m-pH profiles for acetylamino acid substrates [3]. The increase of K_m with pH is particularly steep with Ac-Glu [3] which indicates that direct electrostatic repulsion between the thiolate formed at high pH and the side chain carboxylate of this substrate is involved. In that instance, the essential sulfhydryl group should be close enough to the catalytic site to be able to participate in syncatalytic proton transfer reactions, and the inverse solvent isotope effects observed may be the result of such a process. The small effects of D_2O on K_m are also consistent with a sulfhydryl group being responsible for the observed K_m-pL profiles [10].

On the other hand, we cannot exclude that Zn^{2+}-bound water or OH^- are involved. The data on fractionation factors for metal-coordinated water are scanty and, in part, contradictory [10]. The apparent pK_a values for the ionization of metal-bound water are 7–9 [13]; thus, at least the alkaline limb of the profiles shown in fig.2 could be due to such a process. However, the steady change of the isotope effect on Ac-Met hydrolysis over more than three pH units shows that a single ionization event cannot account for the observed pH dependence.

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