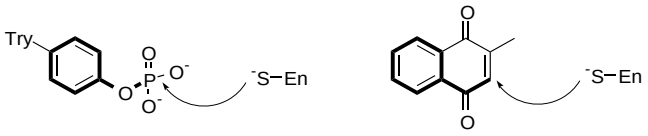

Figure 4. Structural similarity of phosphotyrosine and menadione.

cept on the vertical axis is 95. From the data plotted in accordance with equation 1, the values found for the dissociation constant K_i and the inactivation rate constant k_{inact} were 38 ± 4×10^{-6} M and $1.05 \pm 0.08 \times 10^{-2}$ sec^{-1}, respectively. These values compare very favorably with those for mechanism-based inhibitors of phosphatases.[9,10]

As shown in Figure 4, menadione shares critical structural features with phosphotyrosine. Therefore, menadione might have the favorable dissociation constant. For example, incubation of cdc25 phosphatase with 1,4-benzoquinone did not show inhibition, suggesting that the aromatic ring of inactivator is necessary for its action.

In conclusion, the active site of cdc25A phosphatase could be irreversibly inactivated by menadione. We have also been able to provide data, K_i and k_{inact}, that show the efficiency of inactivation. Based on these data, modifying the inactivator is matter of future interest.

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Affirmation of a general base of *Hafnia alvei* Aspartase by Organic Solvent Perturbation Method

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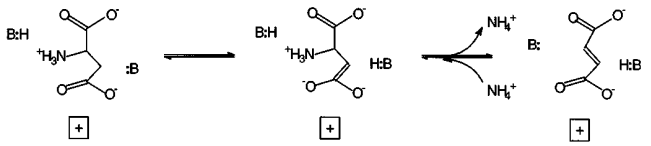
Aspartase (L-aspartate ammonia-lyase, EC4.3.1.1) catalyzes the reversible conversion of L-aspartate to fumarate and ammonia.[1] The enzyme is specific for aspartate and fumarate, but NH_2OH can be substituted for ammonia as a substrate.[2,3] A variety of divalent metal ions, such as Mg^{2+}, Mn^{2+}, Zn^{2+} and Co^{2+} activate the reaction.[4] Initial velocity studies obtained for the enzyme from Hafnia alvei are consistent with a rapid equilibrium kinetic mechanism in which Mg^{2+} binds prior to aspartate, but with a random release of Mg^{2+}, NH_4^+, or fumarate.[5]

An acid-base chemical mechanism for *Hafnia alvei* aspartase has been proposed using pH studies and deuterium wash-in[6,7] (scheme 1). Data are consistent with a proton which is abstracted from C-3 of the monoanionic form of aspartate by an enzyme general base with a p*K* of 6.3-6.6 in the absence and presence of Mg^{2+}. The resulting carbanion is presumably stabilized by delocalization of electrons into the β-carboxyl with the assistance of a protonated enzyme group in the vicinity of the β-carboxyl. Ammonia is then expelled with the assistance of a general acid group that traps an initially expelled NH_3 as the final NH_4^+ product. The p*K* for the general acid is about 7 in the absence of Mg^{2+}, but is increased by about a pH unit in the presence of Mg^{2+}. Since the same p*K* values are observed in the $pKi_{succinate}$ and V/K pH profile, both enzyme groups must be in their optinum protonation state for efficient binding of reactant in the presence of Mg^{2+}. At the end of a catalytic cycle, both the general base and general acid groups are in a protonation state opposite that in which they started when aspartate was bound. When the aspartase reaction is run in D_2O to greater than 50% completion no deuterium is found in the remaining aspartate, indicating that the site is inaccessible to solvent during the catalytic cycle.[7]

Construction of a reaction mechanism for an enzyme requires a knowledge of its amino acid or other residues involved in the catalytic process. One method for identifying the charge types at the active site of an enzyme is based on the use of organic solvents perturbation.[8] The solvent pertur



Scheme 1.

bation method depends on the different behavior of neutral and cationic acids when organic solvents are added to water. This method follows from the fact that neutral acids either increase or have a constant pK as water is replaced by an organic solvent such as ethanol, while cationic acids either decrease or have a constant pK under similar conditions.[9]

Efforts to identify the amino acid or other residues involved in the catalytic function of an enzyme on the basis of pK values of ionizing groups derived from kinetic measurements are valid, only no conformational changes occur in the enzyme under the conditions of the experiment. If such changes occur, then it can be argued that an ionization which affect V_{max} or K_m or both does so by changing the conformation of the enzyme and does not involve any residue which directly participates in the catalytic process. No such conformational changes have been shown to occur in the case of aspartase in the pH range 5.0 to 9.5. Thus, absorption spectra of the enzyme in the 225 nm to 330 nm range under these conditions (data not shown) have been found to be identical and superimposable indicating lack of alteration either in the environment of aromatic residue or in the peptide backbone structure of the enzyme. An estimate of the variation of the kinetic mechanism over the pH range to be studied is required in order to ensure that the enzyme complex is being titrated. Initial velocity patterns were obtained at pH 5.0 and and pH 9.0 by varying aspartate at a fixed level of Mg^{2+}. The results at pH 9.0 are shown in Figure 1. These results are in agreement with the kinetic mechanism data (rapid equilibrium ordered addition of Mg^{2+} prior to aspartate) of Nuiry *et al.*[5] Absence of disturbance in the environment of aromatic chromophores in solvent-water and kinetic mechanism detailed above, strongly indicates that the direction of shifts in

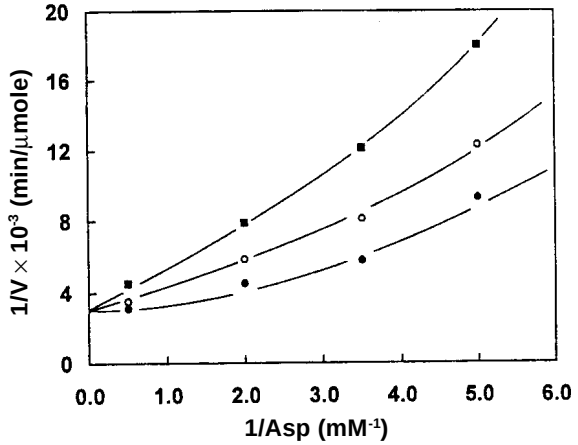


Figure 1. Initial velocity pattern of Aspartase by Mg^{2+} at pH 9.0. The magnesium concentration used was as follows: 0.1 mM (■); 1 mM (○); 10.0 mM (●). All substrate concentrations were corrected for the amount of metal chelate complex formation.

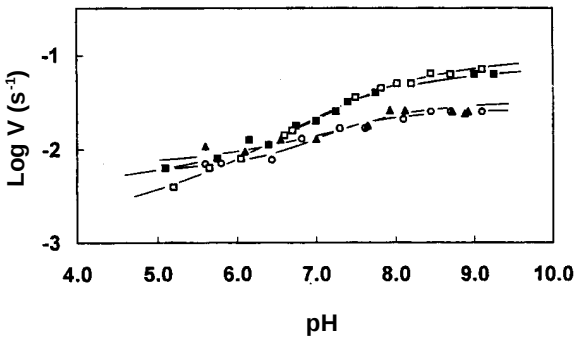


Figure 2. pH dependence of V_{max} for aspartase. (■) Enzyme activity in cationic acid buffers in water. (□) Enzyme activity in neutral acid buffers in water. (▲) Enzyme activity in neutral acid buffers in 25% ethanol. (○) Enzyme activity in cationic acid buffers in 25% ethanol

pK values in organic solvents at the active site and no denaturation in the presence of organic solvents.

Figure 2 shows the logV_{max} versus pH plots for the enzyme in cationic acid buffers in water (top curve), in neutral acid buffers in water (second curve), in neutral acid buffer in 25% ethanol (third curve), in cationic acid buffer in 25% ethanol (bottom curve). The pK values in cationic acid buffer in water, in neutral acid buffer in water, in the cationic acid buffer in 25% ethanol and in the neutral acid buffer in 25% ethanol were 7.5±0.1, 7.3±0.2, 7.5±0.4 and 7.2±0.3, respectively in Table 1. The logV_{max} plots imply the effect of these solvents on the pK values of the ionizing groups on the enzyme-substrate complex responsible for its catalytic activity. The pK values were changed in the neutral acid and in the cationic acid on the 25% ethanol. Two groups were observed in V_{max}/K_m profile (*vide infra*). On the other hand, neither of the two groups are observed in V profiles. The two catalytic groups are environmentally perturbed when aspartate and Mg^{2+} are bound, or a combination of both. There is very little shift in the pK of the enzyme in either neutral acid buffer or cationic acid buffer in 25% ethanol within errors.

Figure 3 shows the logV_{max}/K_m versus pH plot of this enzyme in cationic acid buffers in water (top curve), in neutral acid buffers in water (second curve), in neural acid buffer in

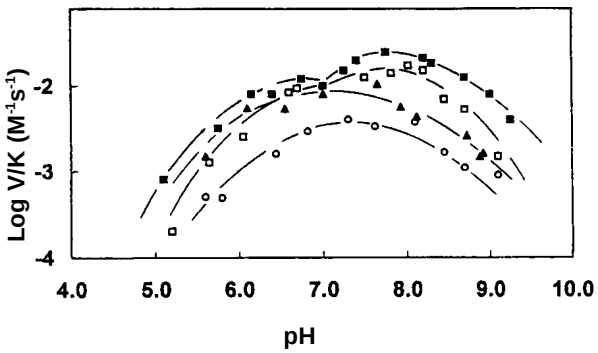


Figure 3. pH dependence of V_{max}/K_m for aspartase. (■) Kinetic parameters in cationic acid buffers in water. (□) Kinetic parameters in neutral acid buffers in water. (▲) Kinetic parameters in neutral acid buffers in 25% ethanol. (○) Kinetic parameters in cationic acid buffers in 25% ethanol.

Table 1. Summary of p*K* values obtained from the pH dependence of V_{max} and V_{max}/K_m in water and ethanol.

V_{max}/K_m	Solvent	pK_1±SE		pK_2±SE	
		Neutral acid buffer	Cationic acid buffer	Neutral acid buffer	Cationic acid buffer
	Water	6.5±0.2	6.3±0.1	8.0±0.2	8.4±0.3
	25% Ethanol	5.7±0.2	6.2±0.2	8.2±0.1	8.4±0.2

V_{max}	Solvent	p*K*±SE	
		Neutral acid buffer	Cationic acid buffer
	Water	7.3±0.2	7.5±0.1
	25% Ethanol	7.2±0.3	7.5±0.4

* pK_1 indicates that the group must be protonated for enzyme activity and pK_2 indicates that the group must be deprotonated for enzyme activity.

25% ethanol (third curve), in cationic acid buffer in 25% ethanol (bottom curve). The log V_{max}/K_m plots imply the effects of these solvents on the p*K* values of the ionizing groups on the free enzyme. The p*K* values were not changed between in the neutral acid buffer and in the cationic acid buffer in water within errors in Table 1. The p*K* values were changed between in the neutral acid buffer and in the cationic acid buffer on the 25% ethanol. Particularly, the p*K* in the acidic side was shifted 0.5 pH units from 6.2 to 5.7. The p*K* values on the acidic side and basic side is not shifted in the presence of 25% ethanol and cationic acid buffers. However, the p*K* values in the presence of 25% ethanol and neutral acid buffers appears to be shifted to lower pH. The p*K* in the acidic side is shifted 0.8 pH units from 6.5 down to 5.7 and the p*K* in the basic side is shifted from 8.0 to 8.2. The p*K* in the acidic region is considered a real shift (0.8 pH units) within errors. But the change in the basic p*K* value may be only an apparent shift caused by large shift in the acidic p*K* value in this profile. These results suggest that the enzyme group (pK_1) for the acidic side is of the cationic type and the enzyme group (pK_2) for the basic side is of the neutral type. The most likely candidate enzyme group for acidic side is a histidine residue at which 6.3 p*K* value occurs. This conclusion is supported by Yoon *et al.* who examined that a general base must be deprotonated for catalysis with a p*K* value near 6.3 and that a general acid must be protonated for substrate binding with a p*K* value near 8.4[7](*vide ante*). Another additional evidence is that the chemical modification study with diethylpyrocarbonate which is specific to histidine residue also supports that histidine residue is being in active site.[10] The possible candidate enzyme group for the basic side is a cysteine residue at which 8.4 p*K* value occurs. Another chemical modification study using NEM and DTNB which is specific to cysteine residue has supported the requirement of cysteine residue for catalytic activity.[11] In summary, the authors strongly suggest that a general base for the catalysis is a histidine residue and if any exist, a general acid for activity is a cysteine residue.

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Cleavage of N-O Bonds by Samarium Diiodide: A New Entry to Chemoselective Methods for the Conversion of Isoxazolines to β-Hydroxy Ketones

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Recently we reported a practical method for the selective cleavage of isoxazoline nuclei (N-O bonds) in the presence of double bonds by the use of Lindlar catalyst Eq. (1).[1] During these studies, we also observed that the reduction of isoxazoline rings containing the C-3 phenyl group Eq. (1), (R= Ph) requires much larger amount of Lindlar catalyst and accompanies the serious hydrogenation of the double bonds, resulting in poor chemoselectivity of the