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D-Aminoacylase from *Alcaligenes faecalis* Possesses Novel Activities on D-Methionine†

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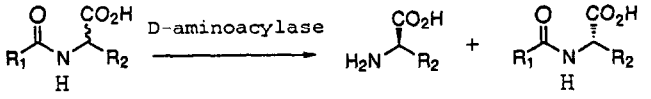
Abstract—D-Aminoacylase isolated from *Alcaligenes faecalis* DA1 has a great potential for future application in D-amino acids production. This paper reports for the first time that D-aminoacylase can reverse the catalysis direction on D-Met and deacylate *N*-Ac-D-Met-OMe and *N*-Ac-D-Met-Gly. The results provide important insights regarding the binding and affinity of substrates to the active site of this enzyme. Based on a systematic study of kinetic properties and relative reactivities for a broad range of substrates, a model to elucidate the reaction mechanism is proposed.

Introduction

D-Aminoacylase (*N*-acetyl-D-amino acid amidohydrolase), which exists in only a few species of bacteria such as *Pseudomonas*,[1-3] *Streptomyces*[4,5] and *Alcaligenes*,[6-9] was shown to catalyze the enantioselective hydrolysis of *N*-acyl-D-amino acids.



Using L-aminoacylase for the production of L-amino acids has been one of the most successful industrial applications in biotechnology.[10-13] Like L-aminoacylase, D-aminoacylase with strict stereospecificity should be a promising tool for the manufacture of D-amino acids. Despite the importance of D-aminoacylase in enzyme technology, its physiological role in bacteria is still not clearly established.

D-Aminoacylase isolated from *Alcaligenes faecalis* DA1 with high stereospecificity was reported previously.[9,14] The molecular weight and isoelectric point of this enzyme were determined to be 55,000 and 5.4, respectively. Also, its active site was previously investigated using a broad range of amino-acid derivatives as substrates.[15] The results indicated that it possessed high activity for deacylation of *N*-Ac(acetyl)-D-Met, *N*-Ac-D-Leu and *N*-Ac-D-Phe. In this study we report for the first time that in addition to the activity of deacetylation, the enzyme can acetylate D-Met. A model is proposed to elucidate the reaction mechanism of this novel catalytic reaction.

*To whom correspondence should be addressed.
†Dedicated to Professor Charles J. Sih on the occasion of his 60th birthday.
Keywords—D-aminoacylase, enantioselectivity, acylation and deacylation, D-methionine, amino acids.

Results and Discussion

Acylation of D-amino acids by D-aminoacylase

The most interesting observation in Table 1 is that among the substrates of D-Met and its analogues, the enzyme only acylates D-Met and D-ethionine (D-Eth), but not *S*-methyl-D-Cys. Because the length difference between the side chains of D-Met and *S*-methyl-D-Cys is a methylene group, it implies that the location of a sulfur atom on the side chains of D-Met may play a crucial role in triggering the acylation reaction. As might be expected, the binding site (*r*-site in Figure 1) in the enzyme possesses a specially high affinity to a sulfur atom locating at the side chain of D-Met. However, the shape of the *r*-site might be somewhat distorted in 50% DMSO-buffer solution to lose enzymatic activity.

Deacylation of N,C-protected amino acids and N-protected dipeptides by D-aminoacylase

From Table 2 and previous results,[9,14,15] it was revealed that the substrates of the enzyme were restricted to C-terminal free amino-acid derivatives only, with the exception of *N*-Ac-D-Met-OMe. In the deacylation of dipeptide derivatives, only *N*-Ac-D-Met-Gly could be

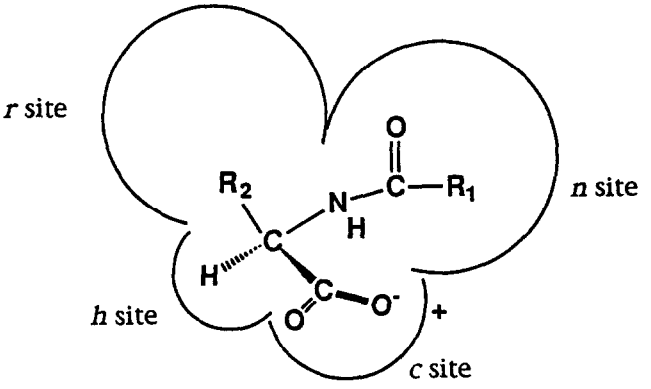


Figure 1. The proposed active site of D-aminoacylase. R_1: N-protected group, R_2: side chain of amino acid

Table 1. Acylation of amino acids by D-aminoacylase

Acyl Donor	Amino Acids	Conversion
sodium acetate	D-Met	40.1%
	D-Ethionine	22.9%
	D-S-methyl-Cys	No reaction
	D-Met-OMe	No reaction
	D-Met (50% DMSO)	No reaction
	L-Met	No reaction
	D,L-Ile	No reaction
	D,L-Phe	No reaction
	D,L-Trp	No reaction
	D,L-Cys	No reaction
	D,L-Leu	No reaction
sodium formate	D-Met	45.1%
sodium propionate	D-Met	No reaction
sodium butyrate	D-Met	No reaction
sodium benzoate	D-Met	No reaction
	D-Phe	No reaction

Table 2. Deacylation of N,C-protected amino acids and N-protected dipeptides by D-aminoacylase

substrate	conversion	substrate	conversion
N-Ac-D-Met-OMe*	28.4%	N-Bz-D-Met-OMe#	no reaction
N-Ac-L-Met-OMe*	no reaction	N-Bz-D-Val-OMe#	no reaction
N-Ac-D-Leu-OMe*	no reaction		
N-Ac-D-Phe-OMe*	no reaction		
N-Ac-D-Trp-OMe*	no reaction	N-Ac-D-Met-Gly	21.2%
N-Ac-D-Val-OMe	no reaction	N-Ac-D-Leu-Gly	no reaction
N-Bz-D-Leu-OMe#	no reaction	N-Ac-D-Phe-Gly	no reaction

*Reaction in 10% DMSO.
#Reaction in 50% DMSO.

Table 3. Comparison of relative reactivities of different substrates in deacylation reaction by D-aminoacylase*

Relative Reactivity (%)	**Substrates**					
No Reaction	N-Ac-D-Asp	N-Bz-D-Asp	N-Z-D-Ser	N-Ac-D-Trp-OMe	N-Bz-D-Val-OMe	N-Ac-D-Phe-Gly
	N-Ac-D-Glu	N-Bz-Gly	N-Z-Gly	N-Ac-D-Val-OMe	N-Bz-D-Leu-OMe	N-Ac-D-Leu-Gly
		N-Bz-D-Trp	N-Z-D-Ala	N-Ac-D-Phe-OMe	N-Bz-D-Met-OMe	
		N-Bz-D-Thr	N-Z-D-Phe	N-Ac-D-Leu-OMe		
Fair (0.01-10)	N-Ac-Gly		N-Z-D-Leu	N-Ac-D-Met-OMe		N-Ac-D-Met-Gly
	N-Ac-D-Asn		N-Z-D-Met			
Good (11-50)	N-Ac-D-Ala	N-Bz-D-Phe				
	N-Ac-D-Trp	N-Bz-D-Leu				
		N-Bz-D-Met				
Very Good (51-100)	N-Ac-D-Phe					
	N-Ac-D-Leu					
	N-Ac-D-Met					

*The activity obtained with N-Ac-D-Met was assigned a value of 100%.

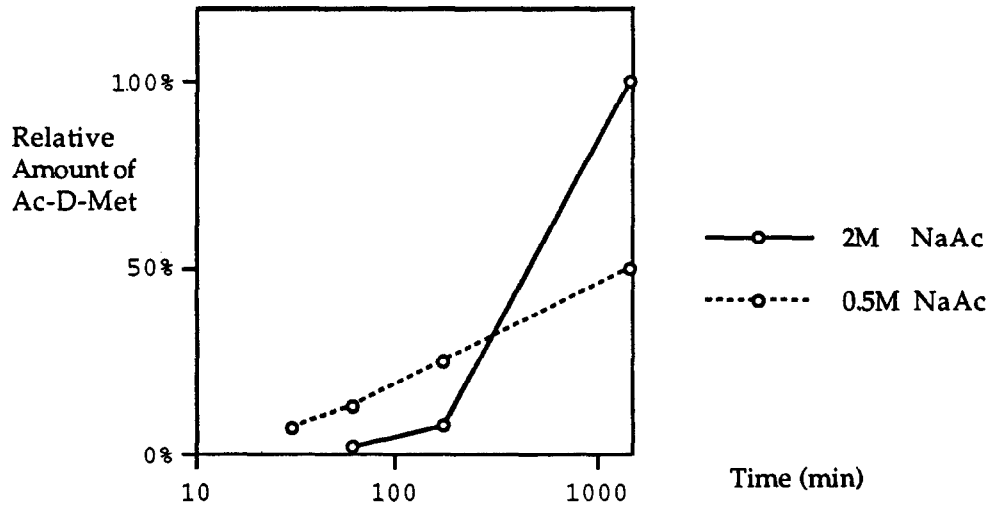


Figure 2. The kinetic measurement of acylation by D-aminoacylase

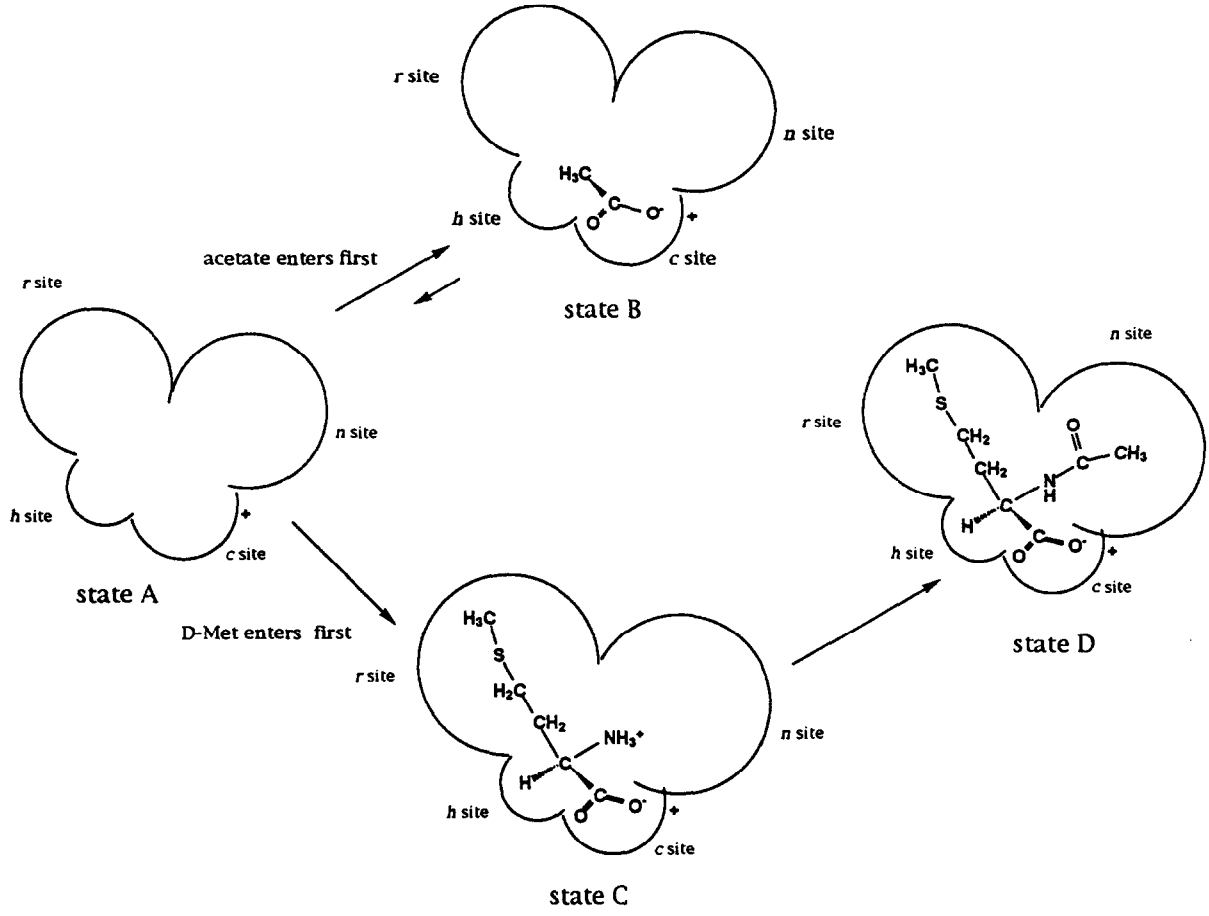


Figure 3. Model of acylation by D-aminoacylase

hydrolyzed. These phenomena might be explained by the specific interaction between the sulfur atom in the side chain of D-Met and the *r*-site of the enzyme, which would overcome the disadvantage of *N*-Ac-D-Met-OMe and *N*-Ac-D-Met-Gly as substrates in binding to the enzyme.

Substrate specificity

From the consideration of different molecular sizes of side chains and the relative affinity, as shown in Tables 2 and 3 plus previous reports,[9,14,15] it can be deduced that D-aminoacylase possesses some stereochemical restraints regarding the substrate binding and affinity to the active site of the enzyme (Figure 1). Due to the steric hindrance of various substrates, the order for optimal binding of the *n*-site is acetyl (Ac) > benzoyl (Bz) > benzyloxycarbonyl (Z) group. The *r*-site, as depicted in Figure 1, is expected to accommodate a hydrophobic side chain of amino acids for a high-affinity binding to the enzyme. On the other

hand, the charged and polar side chains of amino acids such as Asp and Glu might be repulsive to the *r*-site and the side chain of Gly is too small to have productive binding. It is also expected that the *c*-site may have positive charges so forming a salt bridge with the carboxyl group of the substrates.

It is also noteworthy that by comparing the relative reactivity for two pairs of compounds *N*-Bz-D-Phe/*N*-Z-D-Phe, and *N*-Ac-D-Trp/*N*-Bz-D-Trp with free carboxyl groups, the occurrence of deacylation reaction will depend on the interaction between *n*-site and *r*-site. Moreover, both the *n*-site and the *r*-site of the enzyme need to get optimal binding with the acetyl group and side chain of D-Met respectively, to trigger deacylation reaction in substrates with protected carboxyl termini.

Reaction model of acylation and kinetic measurements

Based on the results shown in Figure 2, a reaction model of acylation for D-Met is proposed and depicted in Figure 3. Due to the existence of positive charges in the *c*-site of the enzyme, acetate with negative charge binds faster with *c*-site than *n*-site, and results in reduced rate of the backward reaction. At a higher concentration of acetate, the reverse reaction was quite slow initially owing to kinetic equilibrium. Finally, by virtue of the thermodynamic equilibrium, the yield of the product would be substantial at high concentration of acetate for a longer period of reaction.

Free D-amino acids except D-Met cannot bind with free enzyme. Only D-Met possessing high affinity with the *r*-site and the *c*-site of the enzyme can be expected to bind tightly, followed by binding of acetate at the *n*-site and acylation occurs.

Although *N*-Bz-D-Met and *N*-butyl-D-Met[15] could be deacylated by D-aminoacylase, butyrate and benzoate might be too bulky to enter the *n*-site after D-Met has occupied the active site. Besides, D-aminoacylase can acylate D-Met as efficiently as sodium acetate by using sodium formate as acyl donor.

Conclusion and perspective

According to the studies of Sakai *et al.*,[8] D-aminoacylase from *Alcaligenes denitrificans xylosoxydans* MI-4 catalyzed the hydrolysis of *N*-Z-D-Met. Among the substrates tested in this study, *N*-Ac-D-Met also had the highest hydrolytic rate. It seemed that D-aminoacylase from the genus *Alcaligenes* possessed high affinity to D-Met. Further functional and structural characterization of this unique enzyme system should prove useful to shed some light on the biological significance and its possible application in enzyme technology.

Experimental Section

Chemicals

All D-form, L-form and DL racemic amino acids were obtained from Sigma Chemical Company (St Louis, MO, U.S.A.). The derivatives of amino acids were synthesized according to the previous report[16] and their structures were confirmed by ^{1}H-NMR spectroscopy which were recorded with a Brüker AM-200 using deuterium solvent peaks as references.

Microbe D-aminoacylase purification and enzyme assay

The microbial strain was kindly provided by Dr Ying-Chieh Tsai, National Yang-Ming Medical College, Taipei, Taiwan, R.O.C. The purification procedures and the method of enzyme assay for D-aminoacylase from *Alcaligenes faecalis* DA1 were the same as described previously.[9,14]

Acetylation of amino acids by D-aminoacylase

3 μg of the purified enzyme was added to a solution of 0.5 M acyl donor and 0.2 M amino acid in 10 ml of 50 mM Tris buffer (pH 7.8). The reaction mixture was incubated at 37 °C for 10 h and the reaction was stopped by adding 50 ml of 5% citric acid. The product was extracted with ethyl acetate three times, then evaporated and weighed.

Deacylation of N,C-protected amino acids and N-protected dipeptides

1 μg of the purified enzyme was added to a solution containing 20 mM substrate in 0.6 ml of 50 mM Tris buffer (pH 7.8). N,C-protected amino acids were dissolved by addition of DMSO. The reaction mixture was incubated at 37 °C for 10 h. The conversion rate was determined by the concentration of N-terminal free amino-acid derivatives with ninhydrin test,[17] which was measured at 570 nm in a Hitachi U-2000 spectrophotometer.

Kinetic measurements

The assay solution containing 0.2 M D-Met and various concentrations of sodium acetate plus 0.5 μg purified enzyme was dissolved in 1 ml of 50 mM Tris buffer (pH 7.8) at 37 °C. The reaction was stopped by freezing in liquid nitrogen, the mixture was lyophilized and then fractionated by reversed-phase HPLC (C_{18} column) with CH_3CN:H_2O:H_3PO_4 = 20:80:1 as eluent. The relative amount of Ac-D-Met was measured by UV absorbance at 200 nm.

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