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# ACTIVATION OF ASPARTASE BY SITE-DIRECTED MUTAGENESIS[1]

Sachiko Murase, Jun S. Takagi[2], Yasuyuki Higashi, Hiromasa Imaishi, Noboru Yumoto[3], and Masanobu Tokushige[4]

*Department of Chemistry, Faculty of Science, Kyoto University, Kyoto 606, Japan*

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**To elucidate the role of sulfhydryl groups in the enzymatic reaction of the aspartase from *Escherichia coli*, we used site-directed mutagenesis which showed that the enzyme was activated by replacement of Cys-430 with a tryptophan. This mutation produced functional alterations without appreciable structural change: The $k_{cat}$ values became 3-fold at pH 6.0; the Hill coefficient values became higher under both pH conditions; the dependence of enzyme activity on divalent metal ions increased; and hydroxylamine, a good substrate for the wild-type enzyme, proved a poor substrate for the mutant.** 

Aspartase (L-aspartate ammonia-lyase, EC 4.3.1.1) catalyzes the reversible conversion of L-aspartate to fumarate and $NH_4^+$. The aspartase purified from *Escherichia coli* W cells is composed of four identical subunits and has a molecular weight of 208,896 (1,2). Previously, we showed that aspartase lost its enzyme activity following modification by *N*-ethylmaleimide of one or two sulfhydryl groups per subunit (3). The use of *N*-(7-dimethylamino-4-methylcoumarynyl)maleimide (DACM), a fluorescent derivative of *N*-ethylmaleimide, showed the Cys-140 and Cys-430 of the 11 cysteine residues to be modified selectively (4). We could not determine which residue actually is associated with the enzyme activity because specific modification of one of these two

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[2]Died November 17, 1986.

[3]To whom correspondence should be addressed.

[4]Died October 4, 1990.

Abbreviations used: DACM, *N*-(7-Dimethylamino-4-methylcoumarynyl)maleimide; MES, 2-(*N*-morpholino)ethane sulfonic acid; TAPS, *N*-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid.

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residues was impossible. Therefore we changed Cys-430 to tryptophan by site-directed mutagenesis in order to determine its role. Unexpectedly, the enzyme was markedly activated by mutation. We here describe the effects of this mutation on the catalytic and structural properties of the *E. coli* aspartase.

## MATERIALS AND METHODS

***Materials.*** Monosodium L-aspartate was purchased from Kyowa Hakko (Tokyo, Japan), and 2-(*N*-morpholino)ethanesulfonic acid (MES) and *N*-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid (TAPS) from DOJINDO (Kumamoto, Japan). All the other chemicals used were of reagent grade.

***Site-directed mutagenesis.*** The Trp-430 mutant was constructed by the method described by Straus *et al.* (5): The pAB1 plasmid containing the aspartase gene of *E. coli* (1) was used as the target of site-directed mutagenesis with an oligonucleotide 17 bases long that was purchased from Takara Shuzo (Kyoto, Japan). The *Bam*H1-*Nar*1 fragment of the plasmid having the mutant aspartase gene was inserted into pSU1. The changed plasmid was named pSUW430. The pSU1 used was derived from pBR322 and carries the promoter from the *SupB.E* tRNA operon and the *ori* from pUC19 (6). *E. coli* TK237 strain cells that have no aspartase activity (7) were transformed with the plasmid pSUW430. Mutation was confirmed by DNA sequence analysis.

***Enzyme preparations.*** Wild-type aspartase was purified as described elsewhere (8). The purification procedures for the mutant enzyme were essentially the same as those for the wild-type, the details of which will be reported elsewhere. Polyacrylamide gel electrophoresis showed the enzyme preparations used were homogeneous.

***Activity measurements.*** The catalytic activity of the aspartase was determined by routine spectrophotometry by measuring the increase in absorbance at 240 nm following formation of the fumarate. The standard assay mixture (total 1ml) contained 0.1 M sodium L-aspartate, 2 mM $MgCl_2$, 50 mM TAPS-NaOH buffer (pH 8.5) and the enzyme. In the fumarate hydroxylamination reaction assay, the activity was determined spectrophotometrically by measuring the decrease in absorbance at 293 nm following the disappearance of fumarate in a 1 ml assay mixture that contained 10 mM sodium fumarate, 0.1 M $NH_2OH$-HCl, 2 mM $MgCl_2$, 50 mM TAPS-NaOH buffer (pH 8.5) and the enzyme. All measurements were made at 30°C.

***Protein determinations.*** The protein concentrations of the purified wild-type and mutant aspartases were calculated from the $A^{1\%}$ values at 278 nm (wild-type: 4.3, mutant: 5.7) which had been determined from measurements of the absorbance and protein concentration of each preparation by quantitative amino acid analysis in a Waters Pico-Tag system.

## RESULTS

The spectra of both enzymes were measured in order to compare the structure of the mutant aspartase with that of the wild-type enzyme. The mutant had almost the same spectrum as the wild-type enzyme (Fig. 1), and gel filtration

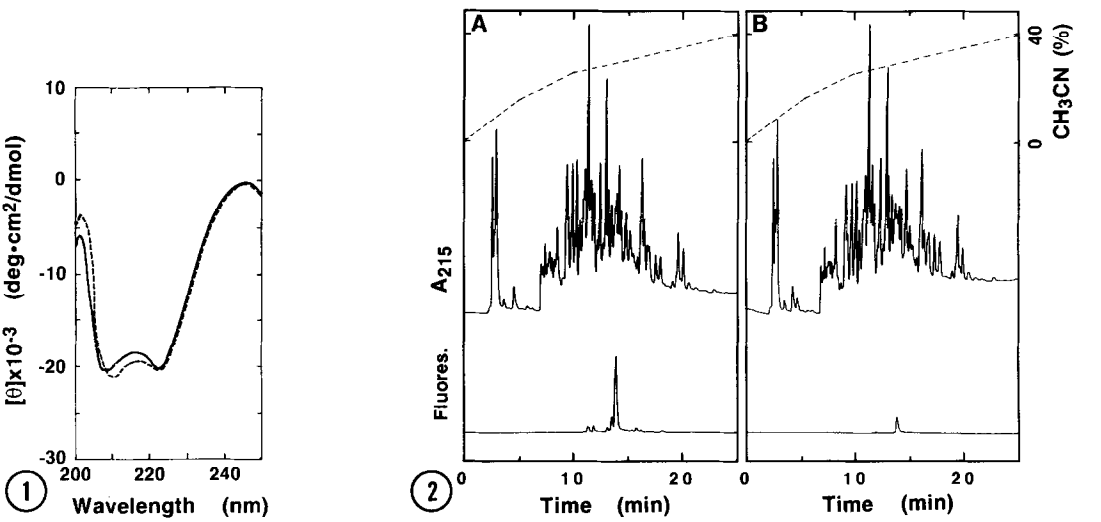


**Fig. 1. CD spectra of the wild-type (solid line) and mutant (broken line) *E. coli* aspartases.** CD spectra were measured at 25°C in a Jasco J600 spectropolarimeter with a 0.1 cm light path. The wild-type (65.9 μg/ml) and mutant (79.8 μg/ml) enzyme were placed in 21 mM potassium phosphate buffer (pH 6.8) containing 43 mM KCl, 0.43 mM EDTA and 2.1 mM 2-mercaptoethanol.

**Fig. 2. HPLC patterns of the tryptic peptides obtained from the DACM-modified *E. coli* aspartase.** DACM modification was done in the absence (A) and presence (B) of 10 mM sodium L-aspartate and 2 mM $MgCl_2$ as described elsewhere (4). The respective residual activities of the DACM-modified enzyme in the absence and presence of L-aspartate and $MgCl_2$ were 25% and 96%. After trypsin digestion, the sample obtained was injected to a CAPCELL PAK C18 reverse-phase column (Shiseido, 4.6 x 250 mm). The gradient system was composed of solvents A (10 mM potassium phosphate buffer, pH 6.0) and B (acetonitrile). Peptides were eluted by increasing the concentration of solvent B from 0% to 40% in 25 min at 40°C. HPLC was done in a Hitachi HPLC system connected to a Hitachi 655A UV monitor and a Shimadzu fluorescence spectromonitor RF-530 at a flow rate of 1.0 ml/min. The absorbance at 215 nm and fluorescence (excitation at 380 nm, emission at 480 nm) were monitored simultaneously. Broken lines show the acetonitrile concentration.

showed that it had the same molecular weight as the wild-type enzyme (data not shown). These results indicate that there are no major differences in the secondary and quaternary structures of the wild-type and mutant *E. coli* enzymes.

The activity of the mutant was 134% that of the wild-type enzyme under standard assay conditions and when this was modified with DACM, rapid inactivation took place. Only one large peak of fluorescence was present in the HPLC elution patterns for the tryptic digest of the DACM-modified mutant enzyme (Fig. 2A). This peak was collected and further purified by reverse-phase HPLC with a linear concentration gradient of acetonitrile in trifluoroacetic acid. The amino acid composition of the purified peptide showed good correspondence to the sequence from Cys-140 to Arg-152, evidence that Cys-140 was selectively modified by DACM. When the enzyme was incubated with DACM in the presence of $Mg^{2+}$

TABLE I. KINETIC PROPERTIES OF THE WILD-TYPE AND MUTANT *E. COLI* ASPARTASES

| | wild-type enzyme | mutant enzyme |
|---|---|---|
| pH 6.0 | | |
| $k_{cat}$ ($sec^{-1}$) | $3.5 \times 10^{-9}$ | $1.1 \times 10^{-8}$ |
| $S_{0.5}$ (mM) | 3.5 | 19.0 |
| Hill coefficient | 0.42 | 0.78 |
| $Mg^{2+}$ dependence (%) | 0 | 53 |
| pH 8.5 | | |
| $k_{cat}$ ($sec^{-1}$) | $5.3 \times 10^{-8}$ | $6.5 \times 10^{-8}$ |
| $S_{0.5}$ (mM) | 2.8 | 22.5 |
| Hill coefficient | 1.4 | 2.3 |
| $Mg^{2+}$ dependence (%) | 50 | 96 |

An assay mixture containing MES instead of TAPS buffer was used to determine the catalytic activity at pH 6.0. The aspartases (5.0 μg) were incubated at 30°C. $Mg^{2+}$ dependence is defined as:

$$\left(1 - \frac{\text{activity in an assay mixture containing no } Mg^{2+}}{\text{activity in an assay mixture containing 2 mM } Mg^{2+}}\right) \times 100 .$$

and L-aspartate, there was no activity loss and no prominent fluorescent peak in the elution patterns for the tryptic digest (Fig. 2B).

The kinetic properties of the wild-type and mutant enzymes are compared in Table I. At pH 6.0, the $k_{cat}$ value for the mutant was about 3 times that of the wild-type enzyme, and at pH8.5 its $k_{cat}$ value was slightly higher than that of the latter (123%). The value of $S_{0.5}$ for the L-Asp of the mutant was larger than that of the wild-type enzyme at both pH 6.0 and 8.5. At these pHs, the Hill coefficients (*n* value) for the mutant were higher than those for the wild-type enzyme. The addition of $Mg^{2+}$ ions did not affect the activity of the wild-type enzyme at pH 6.0; whereas, it enhanced the activity of the mutant enzyme about 2-fold. At pH 8.5, the activities of both enzymes were stimulated by the addition of $Mg^{2+}$ ions; but the $Mg^{2+}$-dependence of the mutant was much greater than that of the wild-type enzyme, the mutant enzyme having very little activity in the absence of $Mg^{2+}$ ions.

The activity of the mutant was 0.2% that of the wild-type enzyme when hydroxylamine, a good substrate for the wild-type enzyme (9), was the substrate in the backward reaction, evidence that it is a poor substrate for the mutant enzyme.

## DISCUSSION

In our previous study, Cys-140 and Cys-430 were selected as candidates for the catalytically essential residues (4). Our present results indicate that Cys-430 is

not an essential active site residue and that the modification of Cys-140 by sulfhydryl reagents causes its inactivation.

The substitution of Trp for Cys-430 produced three major functional alterations in the enzyme with no appreciable structural change: an increase in $k_{cat}$; stronger divalent metal ion dependence and higher substrate specificity. Attempts have been made to make enzymes more useful by means of site-directed mutagenesis (e.g., to alter the substrate (10) or coenzyme (11) specificity, to change the pH optimum of the activity (12) or to increase thermal stability (13)). Only a few enzymes, such as the malate dehydrogenase from *Thermus flavus* (14) and the chorismate mutase from yeast (15), have been reported to be activated by the mutation of one residue. These mutant enzymes do not respond to allosteric activators nor to inhibitors, which indicates that they are locked in the activated state by the mutation. As reported elsewhere (16-18), aspartase is activated by limited proteolysis. The properties of the mutant aspartase in our study are similar to those of the protease-activated enzyme. Moreover limited proteolysis of our mutant aspartase with trypsin inactivated the enzyme, evidence that activation of the wild-type by protease and enhancement of activity by the mutation are not additive phenomena. Our results suggest that similar mechanisms act in functional alteration by mutation and in protease-activation. There are, however, critical differences in the properties of the two types of activated enzyme; e.g., in susceptibility to inhibition by $Cl^-$ ions (data not shown). The molecular basis of the functional alterations is now being investigated in order to clarify the relation between the two enzyme types.

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