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Chemical Modification of Amino Groups in Alanine Dehydrogenase from *Bacillus natto* KMD 1126[1]

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Amino groups of alanine dehydrogenase from *Bacillus natto* KMD 1126 were modified by treatment with 2,4,6-trinitrobenzenesulfonic acid (TNBS); this resulted in loss of the enzyme activity with pseudo first order kinetics. However, sulfhydryl groups were not modified by TNBS, and the circular dichroism spectrum of the TNBS-modified enzyme was identical with that of the native enzyme. Complete inactivation was obtained upon modification of 5 amino groups per subunit of the enzyme. However, a plot of log [reciprocal of the half-time of inactivation] versus log [concentration of TNBS] suggested that one amino group has an essential role in the catalysis. Protection against inactivation by TNBS was not observed on the addition of nicotinamide adenine dinucleotide or its reduced form.

Keywords——alanine dehydrogenase; *Bacillus natto*; chemical modification; amino groups; trinitrobenzenesulfonic acid; active site

In a previous paper,[2] the authors reported that alanine dehydrogenase [L-alanine: NAD^+ oxidoreductase (deaminating), EC 1.4.1.1.] of *Bacillus natto* is composed of 6 identical subunits, each with a molecular weight of 48000 daltons, and that one subunit of the enzyme contains one catalytic site and also one sulfhydryl group, which plays an essential role in the catalysis. However, the contributions of other amino acid residues to the active site of the enzyme have not been studied. In this paper, the authors report the results of chemical modification of amino groups in alanine dehydrogenase with 2,4,6-trinitrobenzenesulfonic acid (TNBS), a compound known to attack amino and sulfhydryl groups in peptides.[3] Although TNBS will react with sulfhydryl groups, Kotaki *et al.*[4] found it possible to modify amino groups selectively with TNBS even in the presence of free reactive sulfhydryl groups.

Materials and Methods

Chemicals——Nicotinamide adenine dinucleotide (NAD^+) and its reduced form (NADH) were obtained from Sigma Chemical Company. TNBS and 2-mercaptoethanol were obtained from Wako Chemical Company. All other chemicals were of reagent grade and were used without further purification.

Alanine Dehydrogenase——Alanine dehydrogenase was purified to homogeneity from *Bacillus natto* KMD 1126 as described previously.[5] Before use, the enzyme preparation was passed through a column of Sephadex G 25 to remove 2-mercaptoethanol. The concentration of protein was determined by Lowry's method.[6]

Assay of Alanine Dehydrogenase Activity——The enzyme activity was determined at 30° by following the appearance of absorbance of NADH at 340 nm within 60 sec after initiation of the reaction, as described previously,[5] using a Hitachi spectrophotometer, model 100-10.

Circular Dichroism (CD) Spectrum——CD spectra were measured in a 10 mm light-path cell with a JASCO J-20A spectropolarimeter at room temperature. In the calculation of residue molar concentration, an average residue weight of 105 was used.

Chemical Modification of the Enzyme with TNBS——A mixture of 2 ml of enzyme solution (1.5 mg/ml), 2 ml of 0.6 M borate buffer (pH 9.5), and 0.5 ml of 0.2 N NaOH was incubated with 20 μl of freshly prepared TNBS solution (31.5 μmol/ml) at 25° in the dark, and the activity was measured at appropriate interval. The amount of modified amino groups was determined by measuring the absorbance at 367 nm, using an extinction coefficient of 11000 $mol^{-1} \cdot cm^{-1}$ for the trinitrophenylated amine.[7] For CD measurement, the reaction was stopped by gel filtration of the incubation mixture on a Sephadex G 25 column (1.6 × 35 cm) which had previously been equilibrated with 0.02 M phosphate buffer (pH 7.2).

Determination of Free Sulfhydryl Groups——Chemically modified enzyme solution was passed through a column of Sephadex G 25 to remove excess reagents, and the reactive sulfhydryl groups were titrated with *p*-chloromercuribenzoic acid (PCMB) by the method of Boyer.[8]

Gel Filtration Studies——The binding of the enzyme with NADH was studied at 25° by the gel filtration method of Hummel and Dreyer.[9] The samples (3 ml), consisting of 2.5 ml of the native enzyme solution (1.7 mg/ml) or TNBS-modified enzyme solution (1.7 mg/ml) which showed about 60% reduction in specific activity and 0.5 ml of NADH solution (150 μmol/l), were applied to a Sephadex G 25 column (1.6×35 cm) equilibrated with 0.05 M phosphate buffer (pH 7.2) containing the same concentration of NADH (25 μmol/l). Elution was also carried out with the same buffer containing NADH. The eluate was collected in 3 ml fractions. The concentration of NADH was determined spectrophotometrically at 340 nm, using a molar absorption coefficient of 6220.[10]

Results

Chemical Modification of Alanine Dehydrogenase with TNBS

Alanine dehydrogenase was rapidly and irreversibly inactivated by incubation with TNBS. The time dependence of the enzyme inactivation by TNBS is shown in Fig. 1. As shown in Fig. 1, the inactivation rate obeys pseudo first order kinetics. Approximately 85%-inactivated enzyme solution was passed through a column of Sephadex G 25 to remove the excess reagents, then sulfhydryl groups were measured by PCMB titration. One free sulfhydryl group per subunit was observed in the inactivated enzyme solution. That is, trinitrophenylation occurred only at amino groups of the alanine dehydrogenase. The CD spectrum of this inactivated enzyme solution was also measured. As shown in Fig. 2, the CD spectrum of the TNBS-modified enzyme was identical with that of the native enzyme. That is, TNBS modification of amino groups has a marked effect on the enzymatic activity, but not on the conformation of the enzyme.

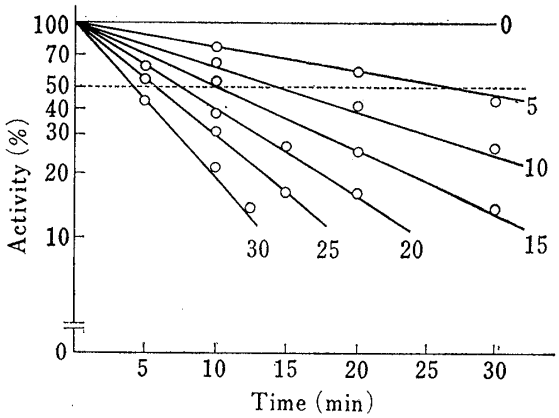

Fig. 1. Inactivation of Alanine Dehydrogenase by TNBS

A mixture of 2 ml of enzyme solution (1.5 mg/ml), 2 ml of 0.6 M borate buffer (pH 9.5), and 0.5 ml of 0.2 N NaOH was incubated with 10, 20, 30, 40, 50, or 60 μl of TNBS solution (31.5 μmol/ml) at 25° in the dark, and the activity was measured at appropriate intervals. The numbers in the figure denote the molar ratios of TNBS/subunit of the enzyme.

The extent of modification by TNBS was followed spectrophotometrically to determine its correlation with catalytic activity. As shown in Fig. 3, modification by TNBS of an average of one residue per subunit (48000 daltons) resulted in a 20% loss in catalytic activity. Extrapolation of the data to zero activity indicated that the reaction of 5 amino groups per subunit occurred during complete inactivation. In order to determine the number of reactive amino groups at the active site of the enzyme, a plot of log [reciprocal of the half-time of inactivation] versus log [concentration of TNBS] was used. Such a plot should be a straight line of slope n where n is the number of inhibitor molecules reacting with each active unit of enzyme to form an enzyme-inhibitor complex.[11] As shown in Fig. 4, the plot of log $[1/t_{0.5}]$ versus log [concentration of TNBS] for alanine dehydrogenase was a straight line having a slope of 0.98. This suggests strongly that inactivation is the result of the reaction of a single amino group per subunit.

Effect of NAD^+ or NADH on the Inactivation with TNBS

To demonstrate whether or not this amino group is essential for the interaction of alanine dehydrogenase with its coenzyme, a series of protection experiments was performed. As shown in Fig. 5, the rate of activity loss with TNBS in the presence of a 100-fold molar excess of NAD^+ or NADH was identical with that in the absence of NAD^+ or NADH. That is, NAD^+

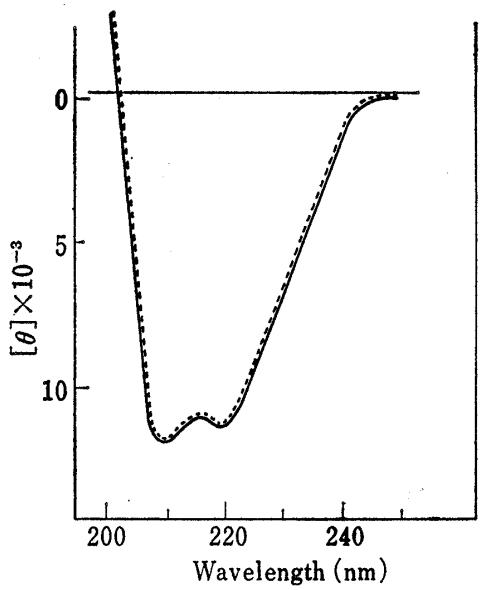

Fig. 2. Circular Dichroism Spectra of the Native and TNBS-Modified Alanine Dehydrogenase

A solid line indicates native enzyme and a dotted line indicates approximately 85%-inactivated enzyme (TNBS-modified).

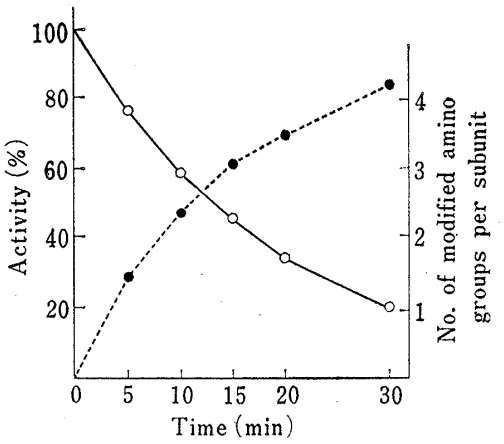

Fig. 3. Activity Loss on TNBS Modification and the Number of Modified Amino Groups per Subunit of the Enzyme

A mixture of 2 ml of enzyme solution (1.0 mg/ml), 2 ml of 0.6 M borate buffer (pH 9.5) and 0.5 ml of 0.2 N NaOH was incubated with 20 μl of TNBS solution (20 μmol/ml) and the activity (——) and absorbance at 367 nm (------) were followed. The number of modified amino groups was calculated from the increase of absorbance at 367 nm using an extinction coefficient of 11000 $mol^{-1}\cdot cm^{-1}$.

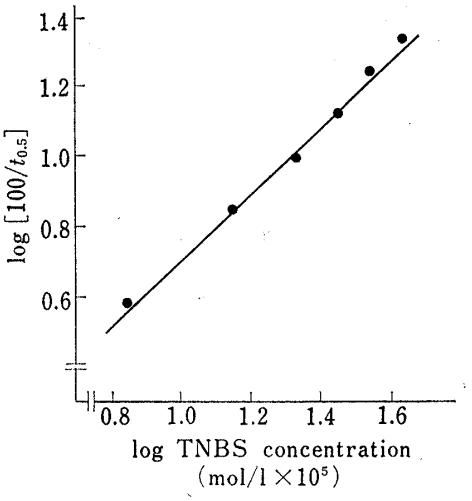

Fig. 4. Order of the Inactivation Process with Respect to Concentration of TNBS

Enzyme solution was incubated with various con centrations of TNBS, and the activities were measured. The half-time for inactivation was obtained from the results in Fig. 1.

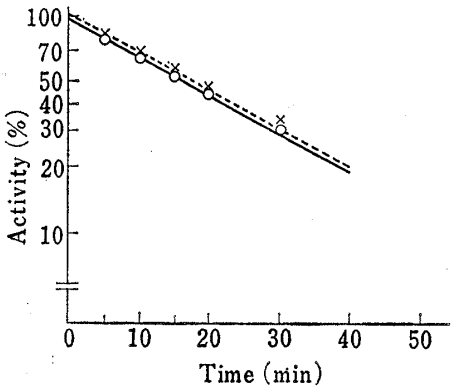

Fig. 5. Effects of NAD^+ and NADH on the Inactivation with TNBS

A mixture of 2 ml of enzyme solutfon (1.0 mg/ml), 2 ml of 0.6 M borate buffer (pH 9.5), and 0.5 ml of 0.2 N NaOH was incubated with 20 μl of TNBS solution (20 μmol/ml), and the activities were measured at appropriate intervals in the absence of coenzyme (——), or in the presence of a 100-fold molar excess of NAD^+ or NADH (------).

or NADH did not provide protection. TNBS, as is well known ,reacts with alanine, so protection experiments with alanine were not performed.

NADH Binding Activity of TNBS-Modified Enzyme

The binding of NADH was examined by gel filtration on a Sephadex G 25 column. Modified enzyme which showed about 60% reduction in catalytic activity was compared with the native enzyme as regards the binding of NADH. As shown in Fig. 6, TNBS-modified enzyme has strong absorbance at 340 nm, so the binding activity could not determined exactly from the increase of absorbance at 340 nm of fractions 9—11. However, it was observed that

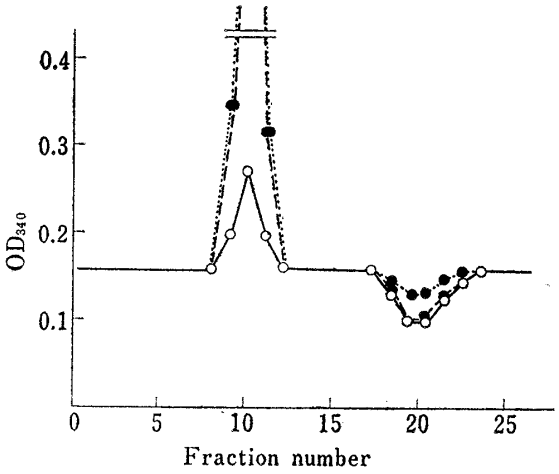

Fig. 6. Elution Profile Obtained on Gel Filtration of Native and TNBS-Modified Alanine Dehydrogenase Bound with NADH at pH 7.2

Three ml of enzyme solution (4.25 mg of protein) containing NADH (25 μmol/l) was applied to a Sephadex G 25 column (1.6×35 cm) equilibrated with NADH (25 μmol/l)-containing buffer. Fractions of 3 ml were collected and their absorbances at 340 nm were measured. A solid line indicates the native enzyme, a dotted line indicates TNBS-modified enzyme in the absence of NADH, and a broken line indicates TNBS-modified enzyme in the presence of NADH.

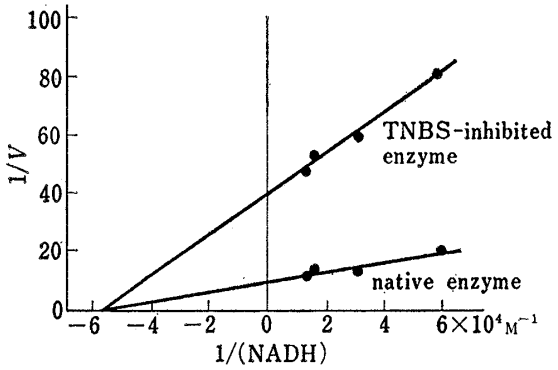

Fig. 7. Lineweaver–Burk Plots Indicating Km Values of NADH for Native and Partially Inactivated Alanine Dehydrogenase

Enzyme which had been inactivated approximately 70% by TNBS treatment was assayed in the usual reaction mixture[6] except that the NADH concentration was varied as indicated. The velocities are expressed as μmol/min.

the binding activity of NADH with TNBS-modified enzyme decreased to half that of the native enzyme from the decrease of absorbance at 340 nm of fractions 18—22. In the case of TNBS-modified enzyme (60% reduction in specific activity), in the presence of a 100-fold molar excess of NADH, the binding of NADH was almost same as that of the native enzyme. The apparent K_m value for NADH was determined for enzyme inhibited to the extent of about 70% by exposure to TNBS, as well as for the native enzyme. As shown in Fig. 7, the values obtained (2×10^{-5} M) were the same.

Discussion

Colman and Frieden demonstrated[12] that acetylation of one amino group per subunit of beef liver glutamate dehydrogenase with acetic anhydride resulted in 80% inactivation. Clark and Yielding also reported[13] that the modification of glutamate dehydrogenase from bovine liver with TNBS results in alteration of the catalytic, regulatory, and physical properties of the enzyme. Thus, the authors studied the function of amino groups of alanine dehydrogenase by means of chemical modifications. Alanine dehydrogenase is composed of 6 identical subunits, and one subunit of the enzyme contains 20 lysine residues. TNBS has been used for modification studies of several enzymes because of its specificity for the amino group, the ease with which modification can be quantitated,[14] and the mild conditions under which the experiments may be performed.

Alanine dehydrogenase was inactivated by incubation with TNBS, and the inactivation obeyed pseudo first order kinetics. Approximately 4 amino groups per subunit were substituted by TNBS and 85% inactivation was obtained after 40 min when the molar ratio of TNBS/subunit of the enzyme in the incubation was 10: 1. One free sulfhydryl group per subunit was observed by PCMB titration in the modified enzyme preparation, and no conformational change was observed. It was assumed that there was at least one amino group which

played an essential role in the catalysis. A plot of log [reciprocal of the half-time of inactivation] versus log [concentration of TNBS] was used to determine the number of reactive amino groups at the active site of the enzyme. It was a straight line having a slope of 0.98 for alanine dehydrogenase. This suggests strongly that the inactivation is a result of the reaction of a single amino group. To demonstrate whether or not this amino group is essential for the interaction of the enzyme with its coenzyme, a series of protection experiments was performed. No protection from inactivation by TNBS was observed in the presence of NAD^+ or NADH. This suggests that the inactivator does not act at the binding site of the coenzyme. However, the NADH-binding activity of the TNBS-modified enzyme (60%-inactivated) was decreased to half that of the native enzyme. In 60%-inactivated enzyme, about 3 amino groups per subunit were modified. Further, the NADH-binding activity of the TNBS-modified enzyme (60%-inactivated) in the presence of a 100-fold molar excess of NADH was almost the same as that of the native enzyme. From these results, it was assumed that a single amino group is essential for the catalysis, but that another amino group in the proximity of the NADH-binding site may be modified by TNBS in the absence of NADH. Further experiments are necessary for elucidate this point.

In addition to TNBS inactivation, alanine dehydrogenase was inactivated by modification with 5-dimethylaminonaphthalenesulfonyl chloride or acetic anhydride.

References and Notes

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