[Chem. Pharm. Bull. 28(5)1503—1508(1980)]

Reactivity and Function of Sulfhydryl Groups in Alanine Dehydrogenase of *Bacillus natto* KMD 1126[1]

KATSUHIKO MATSUI, KIYOMI KIKUNO, and YUKIO KAMEDA

School of Pharmacy, Hokuriku University[2]

(Received October 29, 1979)

Alanine dehydrogenase of *Bacillus natto* KMD 1126 is composed of 6 identical subunites each with a molecular weight of 48000 daltons. One cysteine residue per subunit of the enzyme was detected by amino acid analysis, and by titration with *p*-chloromercuribenzoic acid (PCMB) or 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) in the presence of 8 M urea. Titration of the sulfhydryl group with PCMB resulted in a linear decrease in activity with a concomitant increase in the absorbance at 250 nm. The loss of activity was restored by the addition of mercaptoethanol. N-Ethylmaleimide (NEM) inhibited the alanine dehydrogenase with pseudo first order kinetics. One mole of S-succinylcysteine per subunit was detected by amino acid analysis of the NEM inactivated enzyme. Some protection against inactivation by NEM was observed by the addition of nicotinamide adenine dinucleotide (NAD^+) or its reduced form (NADH). Alanine dehydrogenase was also inhibited by iodoacetamide, and no free sulfhydryl group was detected on titration of the inactivated enzyme with PCMB or DTNB. Gel filtration studies on the binding of NADH to the enzyme revealed that the enzyme contains one coenzyme binding site per subunit.

Keywords——alanine dehydrogenase; *Bacillus natto*; subunit molecular weight; chemical modification; sulfhydryl group; coenzyme binding site

In a previous paper,[3] the authors reported the purification and some properties of alanine dehydrogenase [L-alanine: NAD^+ oxidoreductase (deaminating), EC 1.4.1.1] from vegetative cells of *Bacillus natto* KMD 1126. There are several reports regarding the presence of alanine dehydrogenase in microorgnisms,[4] but there is little structural information concerning the active site of alanine dehydrogenase. Determination of the nature of amino acid residues involved in substrate binding or in catalysis is fundamental for an understanding of the mechanism of the enzymatic reaction. Thus, the authors studied the function of sulfhydryl groups as a part of chemical modification studies on alanine dehydrogenase. The present paper describes the requirement for sulfhydryl groups for enzyme activity, and the number of binding sites for the coenzyme on the basis of studies on the subunit structure.

Materials and Methods

Chemicals——Nicotinamide adenine dinucleotide (NAD^+) and its reduced form (NADH) were obtained from Sigma Chemical Company. *p*-Chloromercuribenzoic acid (PCMB), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), N-ethylmaleimide (NEM), iodoacetic acid, iodoacetamide, and urea were obtained from Wako Junyaku Company. All other chemicals were of reagent grade and were used without further purification.

1) A part of this work was reported at the 47th Meeting of the Hokuriku Branch of the Pharmaceutical Society of Japan, Toyama, November 1978.
2) Location: *3 Ho, Kanagawa-machi, Kanazawa, 920, Japan.*
3) K. Matsui, Y. Tamegai, A. Miyano, and Y. Kameda, *Chem. Pharm. Bull.*, **25**, 2061 (1977).
4) J.M. Wiame and A. Pierad, *Nature* (London), **176**, 1073 (1955); D.S. Goldman, *Biochim. Biophys. Acta*, **34**, 527 (1959); M.M. Hong, S.C. Shen, and A.E. Braunstein, *Biochim. Biophys. Acta*, **36**, 288 (1959); R.J. O'Connor and H.O. Halvorson, *Arch. Biochem. Biophys.*, **91**, 290 (1960); A. Yoshida and E. Freese, *Biochim. Biophys. Acta*, **92**, 33 (1964); S. Sasaki, K. Otsuka, N. Fukunaga, and S. Usami, *J. Fac. Sci., Hokkaido Univ., Ser. V*, **8**, 230 (1965); G.J. Germano and K.E. Anderson, *J. Bacteriol.*, **96**, 55 (1968); S.M. McCowen and P.V. Phibbs, Jr., *J. Bacteriol.*, **118**, 590 (1974).

Alanine Dehydrogenase——Alanine dehydrogenase was purified to homogeneity from *Bacillus natto* KMD 1126 as described previously.[3] Before use, the enzyme preparation was passed through a column of Sephadex G 25 to remove mercaptoethanol. The concentration of protein was determined by Lowry's method.[5]

Assay of Alanine Dehydrogenase Activity——The enzyme activity was assayed in terms of the increase in absorbance at 340 nm as described previously[3] using a Hitachi spectrophotometer, model 101.

Amino Acid Analysis——The purified enzyme solution was passed through a column of Sephadex G 25 equilibrated with H_2O and the protein fraction was lyophilized. A sample of dried protein was hydrolyzed with 6 N HCl at 110° for 24 hr in an evacuated, sealed tube. The hydrolysate was evaporated to dryness *in vacuo*. Amino acid analysis was carried out using a Hitachi KLA-5 amino acid analyser. Cysteine was measured as cysteic acid following performic acid oxidation. NEM-modified protein was hydrolyzed for 72 hr to assure conversion of the imido form to the free diacid.

Subunit Molecular Weight——The molecular weight of alanine dehydrogenase was measured by the method of Weber and Osborn.[6] Electrophoresis was carried out in sodium dodecyl sulfate (SDS)-containing gels at 7.5% and 10% polyacrylamide concentration. Marker proteins used for the molecular weight determination were cytochrome c (12400), chymotrypsinogen (25000), ovalbumin (45000), and bovine albumin (67000).

Determination of Free Sulfhydryl Groups——The reactive sulfhydryl groups were titrated with PCMB by the method of Boyer.[7] The reaction was started by addition of freshly prepared PCMB solution. The absorbance of the mixture was followed at 250 nm, and the activity was determined. Free sulfhydryl groups were also titrated with DTNB in the presence of 8 M urea by measuring the increase in the absorbance at 412 nm.[8]

Chemical Modification of Sulfhydryl Groups——The enzyme solution was incubated with a 1000-fold molar excess of NEM at 25° in 0.05 M phosphate buffer (pH 7.2) and the activity was measured at appropriate intervals. The reaction was terminated by passage of the solution through a Sephadex G 25 column. The effect of coenzyme on the inactivation rate was assayed by the addition of NAD^+ or NADH to the incubation mixture. Controls incubated under conditions identical with those of the test experiments, but without the modifying reagent, were assayed in parallel experiments. In the case of iodoacetamide and iodoacetic acid, the enzyme solution was incubated with a 200-fold molar excess of iodoacetamide or iodoacetic acid at 25° in 0.05 M phosphate buffer (pH 7.2) for 20 min and the activity was measured. Next, a 300-fold molar excess of iodoacetamide or iodoacetic acid was added and the mixture was incubated for 20 min.

Optical Rotatory Dispersion (ORD)——ORD was measured over the range of 200—600 nm at room temperature using a JASCO automatic recording spectropolarimeter, model J-20A.

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 containing the enzyme and NADH 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 as the sample. 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

Subunit Molecular Weight

The molecular weight of the intact alanine dehydrogenase was estimated to about 280000 daltons by calibrated Sephadex G 200 gel filtration as described previously.[3] Under denaturing conditions (Fig. 1), a single protein band of 48000 daltons was observed by SDS electrophoresis. Thus, the alanine dehydrogenase is composed of 6 identical Subunits with a molecular weight of 48000.

Amino Acid Composition

Amino acid analysis of the native enzyme was carried out after acid hydrolysis. In order to estimate the cysteine content, a sample of performic acid-oxidized enzyme was subjected

5) O.H. Lowry, N.J. Rosenbrough, A.L. Farr, and R.J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
6) K. Weber and M. Osborn, *J. Biol. Chem.*, **244**, 4406 (1969).
7) P.D. Boyer, *J. Am. Chem. Soc.*, **76**, 4331 (1954).
8) G.L. Ellman, *Arch. Biochem. Biophys.*, **82**, 70 (1959).
9) J.P. Hummel and W.J. Dreyer, *Biochim. Biophys. Acta*, **63**, 530 (1962).
10) B.L. Horecker and A. Kornberg, *J. Biol. Chem.*, **175**, 385 (1948).

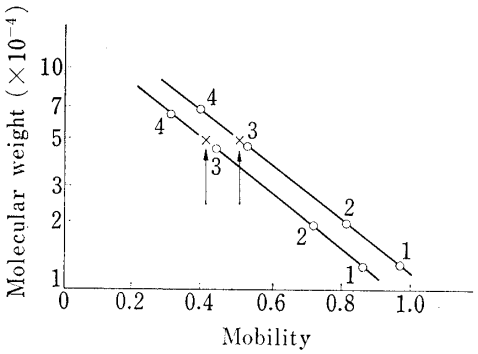

Fig. 1. Determination of the Subunit Molecular Weight of Alanine Dehydrogenase by SDS-Polyacrylamide Gel Electrophoresis

The concentration of polyacrylamide for the upper plot was 7.5% and that for the lower plot was 10%. The numbers over the points denote: 1, cytochrome c (12400); 2, chymotrypsinogen(25000); 3, ovalbumin (45000); 4, bovine albumin (67000). The arrows indicate the mobility of alanine dehydrogenase.

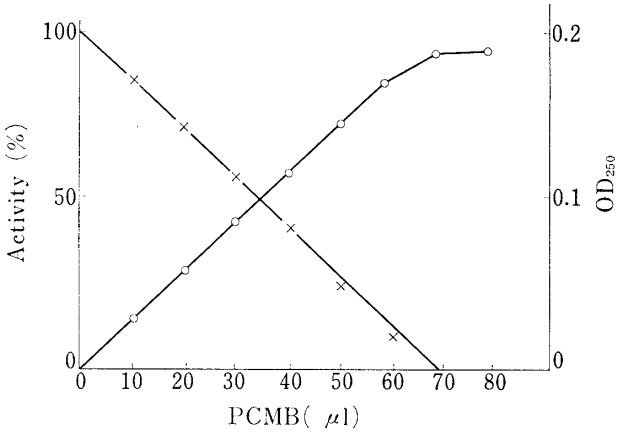

Fig. 2. Titration of Sulfhydryl Groups of Alanine Dehydrogenase with PCMB

Enzyme solution (72 nmol of subunit) was titrated with 1 mmol PCMB, and the activity and absorbance at 250 nm were followed.

Table I. Amino Acid Composition of Alanine Dehydrogenase

Amino acid	Found (μmol)	Nearest integer
Lysine	0.204	20
Histidine	0.068	7
Arginine	0.113	11
Cysteine	0.0098	1
Aspartic acid	0.425	43
Threonine	0.267	28
Serine	0.164	18
Glutamic acid	0.522	52
Proline	0.246	25
Glycine	0.509	51
Alanine	0.648	65
Valine	0.370	37
Methionine	0.114	11
Isoleucine	0.277	28
Leucine	0.401	40
Tyrosine	0.124	12
Phenylalanine	0.061	6
Tryptophan		2

The values are the numbers of residues per molecule calculated based on a molecular weight of 48000. Cysteine was measured as cysteic acid following performic acid oxidation. The values for threonine and serine were extrapolated to zero time of hydrolysis. Tryptophan was estimated spectrophotometrically.

to amino acid analysis. Tryptophan was estimated by measuring the extinction at 280 and 288 nm.[11] The amino acid composition of alanine dehydrogenase calculated based on a subunit molecular weight of 48000 is shown in Table I. One cysteine residue was detected in the subunit of alanine dehydrogenase.

Titration of Sulfhydryl Groups of Alanine Dehydrogenase with PCMB and DTNB

Alanine dehydrogenase is inhibited by PCMB, as described previously.[3] suggesting an important role of a sulfhydryl groups of the enzyme in its catalytic action. Fig. 2 shows that

11) H. Edelhoch, *Biochemistry*, **7**, 1948 (1967).

titration of sulfhydryl groups with PCMB resulted in a linear decrease in activity with a concomitant increase in the absorbance at 250 nm. Thus, the loss of activity is attributable to the modification of sulfhydryl groups. One sulfhydryl group per subunit of the enzyme was titrated with concomitant complete loss of the activity. The loss of activity caused by PCMB was restored by the addition of a 2- to 3-fold molar excess of mercaptoethanol to 90% of the initial activity. DTNB did not affect the enzyme activity or the absorbance at 412 nm. However, sulfhydryl groups of the enzyme were titrated with DTNB in the presence of 8 M urea. The results showed that one sulfhydryl group per subunit reacted with DTNB. Thus, only one sulfhydryl group is present per subunit of the enzyme and this plays an essential role in the catalysis.

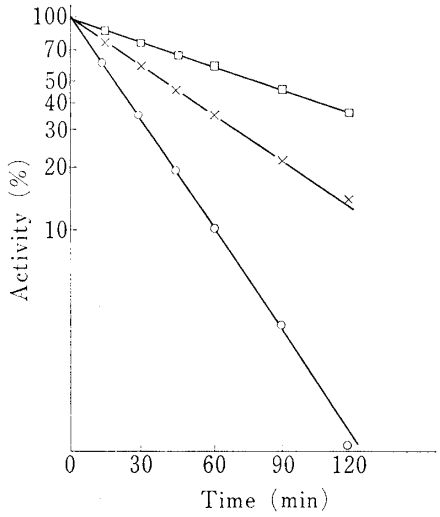

Fig. 3. Effect of NEM on the Activity of Alanine Dehydrogenase

Enzyme solution was incubated with a 1000-fold molar excess of NEM at 25° in 0.05 M phosphate buffer (pH 7.2) and the activity was measured at 15 min intervals in the absence of coenzyme (—○—), in the presence of a 100-fold molar excess of NAD^+ (—×—), and in the presence of a 10-fold molar excess of NADH (—□—).

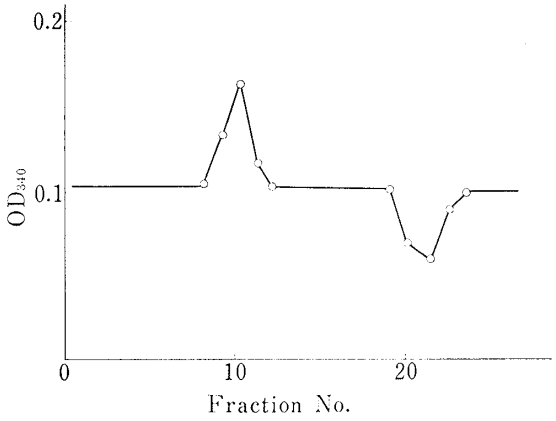

Fig. 4. Elution Profile obtained on Gel Filtration of Alanine Dehydrogenase Bound with NADH at pH 7.2

Two ml of enzyme solution (8 nmol) was applied to a Sephadex G 25 column (1.6×35 cm) equilibrated with NADH-containing buffer. Fractions of 3 ml were collected and their absorbances at 340 nm were measured.

TABLE II. Effects of Iodoacetamide and Iodoacetic Acid on the Activity of Alanine Dehydrogenase

Added reagent	Concentration of NADH (μmol)	Remaining activity (%)
Iodoacetamide 10 μM	0	52
+15 μM	0	10
+15 μM	0	0
Iodoacetamide 10 μM	0.5	85
+15 μM	0.5	72
+15 μM	0.5	55
Iodoacetic acid		
10 μM	0	95
+15 μM	0	90
+15 μM	0	90

Enzyme solution (50 nmole of subunit) was incubated with a 200-fold molar excess of iodoacetamide or iodoacetic acid at 25° in 0.05 M phosphate buffer (pH 7.2) for 20 min and the activity was measured. Next, a 300-fold molar excess of reagent was added to the enzyme solution and the mixture was incubated for 20 min. A further 300-fold molar excess of reagent was then added to the enzyme solution and the mixture was incubated for 20 min. Chemical modification with iodoactamide was also carried out in the presence of NADH (0.5 μmol) and the remaining activity was measured.

Chemical Modification of Sulfhydryl Groups with NEM

NEM inhibited alanine dehydrogenase activity when the enzyme was assayed for its ability to reduce NAD^+. The reaction with NEM displayed pseudo-first order kinetics. As shown in Fig. 3, a 1000-fold molar excess of NEM caused 100% loss of catalytic activity in 2 hr. The amount of NEM bound covalently to the enzyme was determined by amino acid analysis. The NEM-inhibited sample was passed through a column of Sephadex G 25 to remove excess NEM, hydrolyzed at 110° for 72 hr in 6 N HCl, and the amino acid composition was measured. One mole of S-succinyl cysteine was found, which was identical with standard S-succinyl cysteine. The ORD of this inactivated enzyme solution was identical with that of the native enzyme. Inactivation studies using a 1000-fold molar excess of NEM were repeated in the presence of NAD^+ or NADH. As shown in Fig. 3, the rate of activity loss with NEM in the presence of a 100-fold molar excess of NAD^+ was slower than that in the absence of NAD^+. The reduced coenzyme, NADH, was a much better protectant. Addition of a 10-fold molar excess of NADH to the incubation mixture caused a 65% loss of initial activity during the 2 hr incubation.

Chemical Modification of Sulfhydryl Groups with Iodoacetamide or Iodoacetic Acid

As shown in Table II, a 200-fold molar excess of iodoacetamide caused a 50% loss of activity in 20 min. No significant change in the activity was detected on further incubation. Next, a 300-fold molar excess of iodoacetamide was added to the enzyme solution. After 20 min, it caused a 90% loss of catalytic activity. A 300-fold molar excess of iodoacetamide was then added to the enzyme solution. After 20 min, the activity was lost completely. The ORD of the inactivated enzyme was identical with that of the native enzyme. The rate of activity loss with iodoacetamide in the presence of a 10-fold molar excess of NADH was slower than that in the absence of NADH. In order to identify the amino acid residue modified with iodoacetamide, the inactivated sample was passed through a column of Sephadex G 25 to remove excess reagents, and titration of the sulfhydryl group was carried out with PCMB or DTNB in the presence of 8 M urea. However, no free sulfhydryl group was observed by this procedure. That is, iodoacetamide modified at least one sulfhydryl group per subunit of alanine dehydrogenase. As shown in Table II, iodoacetic acid did not inhibit the enzyme activity under the same conditions.

Number of NADH Binding Sites

The binding of NADH was examined by gel filtration with a Sephadex G 25 column. A typical elution profile is shown in Fig. 4. The results of a series of studies are summarized in Table III. There was one NADH binding site per subunit of the enzyme. Since no increase in the number of moles of NADH bound was seen at up to a 10: 1 NADH-to-enzyme

Table III. Binding of Alanine Dehydrogenase with NADH[a)]

Enzyme	Amount of enzyme (nmol)	Concentration of NADH (μmol)	Amount of NADH bound[b)] (nmol)	n[c)]
Native enzyme	8.1	10	49.5	6.1
	8.1	20	47.0	5.8
	8.1	100	47.5	5.9
NEM-modified enzyme	7.1	20	20.5	2.9
	7.1	20	22.8	3.2
Iodoacetamide-modified enzyme	9.0	20	49.8	5.5

a) The details are given in the legend to Fig. 4.
b) Calculated from the absorbance at 340 nm of protein fractions.
c) The amount of NADH bound (mol per mol of enzyme).

molar ratio, it appears that these levels of NADH completely saturated the NADH site and that there was no extraneous binding. The binding of NADH was also examined with chemically modified enzymes. As shown in Table III, the iodoacetamide-modified enzyme bound 5.5 mole of NADH, and this value was almost the same as that with the native enzyme. In the case of NEM-modified enzyme, the binding capacity of NADH decreased to half that of the native enzyme.

Discussion

Intact alanine dehydrogenase of *Bacillus natto* KMD 1126 has a molecular weight of 280000 daltons, and molecular weight measurements under denaturing conditions gave a single protein band of 48000 daltons. These results indicate that the enzyme is a hexamer composed of identical subunits. A similar hexameric subunit structure has been reported for alanine dehydrogenase of *Bacillus subtilis*,[12] leucine dehydrogenase of *Bacillus sphaericus*,[13] and glutamate dehydrogenases other than the *Neurospora* NAD^+-dependent enzyme.[14]

Sulfhydryl groups have been shown to be essential constituents of the active centers of several dehydrogenases. The loss of alanine dehydrogenase activity on treatment with PCMB was accompanied by the modification of sulfhydryl groups and was restored in the presence of excess mercaptoethanol. This indicates that the reactive sulfhydryl groups participate in the catalytic action. The results of amino acid analysis and PCMB titration indicate that one subunit contains one cysteine residue. In addition, alanine dehydrogenase binds 1 mole of NADH per subunit. Thus, it appears that one subunit of the enzyme contains one catalytic site and also one sulfhydryl group, which plays an essential role in the catalysis. Incubation of the enzyme with NEM resulted in the specific covalent modification of this sulfhydryl group and concomitant loss of the enzymatic activity. The ORD of this inactivated enzyme was identical with that of the native enzyme. These results indicate that under the conditions employed, NEM preferentially modified a cysteine residue, leading to complete inactivation of the enzyme, but without measurable change in its protein conformation. NAD^+ and NADH both protected the cysteinyl residue against modification by NEM, but the inactive enzyme still retained the capacity to bind NADH. Hence, the sulfhydryl group of this cysteinyl peptide is probably not required for coenzyme binding. The protection of the sulfhydryl group by NAD^+ and NADH must therefore be attributed to steric factors resulting from their proximity to the coenzyme binding site of the enzyme. A 200-fold molar excess of iodoacetamide inactivated the enzyme rapidly at pH 7.2, but the negatively charged iodoacetic acid was ineffective under these conditions. These data suggest the presence of a negatively charged group in close proximity to the reactive cysteine residue, hindering the approach of iodoacetic acid due to electrostatic repulsion.

12) A. Yoshida, *Biochim. Biophys. Acta*, **147**, 39 (1967).
13) T. Ohshima, T. Yamamoto, H. Misono, and K. Soda, *Agr. Biol. Chem.* (Tokyo), **42**, 1739 (1978).
14) E.L. Smith, B.M. Austen, K.M. Blumenthal, and J.F. Nyc, "The Enzymes," 3rd ed., Vol. 11, ed. by P.D. Boyer, Academic Press Inc., New York, N.Y., 1976, p. 293.