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Behavior of Sulfhydryl Groups of Yeast Alcohol Dehydrogenase for 5,5'-Dithiobis(2-nitrobenzoic Acid)

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1. Four to six moles of sulfhydryl groups of yeast alcohol dehydrogenase were found to be high sensitivity for 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB).

2. There were two types of sulfhydryl groups in the active site of yeast alcohol dehydrogenase. Sulfhydryl groups of two of four active sites were easily modified by DTNB at 0° and the other were not.

3. Yeast alcohol dehydrogenase which was modified sulfhydryl groups of two of four active sites by DTNB, was fully restored in its enzyme activity by cysteine and was found to be the same conformation as the native enzyme. On the other hand, the enzyme which was modified more than two sulfhydryl groups in the active sites, was partially restored by cysteine, probably due to irreversibly conformational change.

4. DTNB competed with NAD for sulfhydryl group in the active site of yeast alcohol dehydrogenase.

Introduction

Yeast alcohol dehydrogenase (EC 1.1.1.1, alcohol: NAD oxidoreductase (yeast)) has been considered to be as a thiol enzyme and has four active sites[2] and composes of four subunits per molecule.[3] The enzyme molecule contains thirty eight half-cystine residue, based on amino acid analysis.[4]

However, the number of detectable sulfhydryl groups varied depending upon the experimental condition used.[5] Reagents for sulfhydryl group titration, such as *p*-chloromercuribenzoic acid (PCMB) or Ag^+, bring about the denaturation of protein and they react not only with sulfhydryl groups situated on the surface of the enzyme, but also those in folding protein conformation. Snodgrass, *et al.*[6] reported that PCMB, Ag^+ and Hg^+ instantaneously inactivated yeast alcohol dehydrogenase with the appearance of more slowly sedimenting species than native enzyme upon ultracentrifugal analysis. The properties of sulfhydryl groups in native state of protein molecule cannot be investigated by these reagents.

In the previous papers,[7] it has been reported that 2-methyl-1,4-naphthoquinone (K_3) reacted with sulfhydryl group of low molecular compounds and proteins, such as bovine serum albumin or papain, to form the thioether linkage at 3-position of K_3 with absorption maximum at 420—430 mμ and papain was irreversibly inactivated.

In this present report, the authors studied on the chemical reactivities of sulfhydryl groups in the molecule of yeast alcohol dehydrogenase, using 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) as a disulfide-exchange reagent which was developed by Ellman,[8] for the examination of the reaction of the enzyme with K_3.

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Experimental

Materials and Methods——Yeast alcohol dehydrogenase was prepared from baker's yeast according to the method of Racker.[9] It was recrystallized two or three times with ammonium sulfate.

DTNB was synthesized from *m*-chlorotoluene as a starting material. It was oxidized with potassium permanganate[10] and then was nitrated with fuming nitric acid to 2-nitro-5-chlorobenzoic acid,[11] from which DTNB was obtained by the method of Ellman.[8]

NAD was purchased from Sigma Chemical Co. Other chemicals were purchased commercial preparations available of extra grade. Ammonium sulfate used in the enzyme preparation was recrystallized from 0.001M EDTA solution and then from distilled water with the treatment of charcoal.

Enzyme activity was assayed by the method of Racker.[9]

Titration of sulfhydryl groups by PCMB was carried out by the method of Boyer.[12]

The interaction of sulfhydryl groups of yeast alcohol dehydrogenase with DTNB was measured spectrophotometrically at 412 mμ and at the same time the enzyme activity was assayed. The reaction mixture contained 2×10^{-6}M the enzyme, 6.6×10^{-5}M DTNB and 0.033M phosphate buffer (pH 8.0) in a total volume of 3.0 ml. The modified enzyme by DTNB was reactivated by the addition of cysteine (0.04M) in 0.05M phosphate buffer (pH 8.0). In the case of urea denaturation, 5M urea in a final concentration was added to the reaction mixture mentioned above.

Number of sulfhydryl groups was calculated based on absorption coefficient of 1.36×10^4 cm^2 mole^{-1} at 412 mμ for reduced DTNB at pH 8.0.[8]

For the study of ultracentrifugal analysis, a mixture consisting of 1.3×10^{-4}M the enzyme, 4.0×10^{-3}M DTNB and 0.033M phosphate buffer (pH 8.0) in a total volume of 3.0 ml, was incubated for three hours at 0° and then was passed through Sephadex G–25 column (2.5×50 cm). Protein fraction which was eluated with 0.1M phosphate buffer (pH 8.0), was diluted with the same buffer and then analysed by Spinco model E ultracentrifuge.

Protein concentrations were determined by the method of Lowry, *et al.*[13] on the basis of absorption coefficient of 1.89×10^5 cm^2 mole^{-1} for yeast alcohol dehydrogenase at 280 mμ.

Results

Number of Sulfhydryl Groups of Yeast Alcohol Dehydrogenase

Ohta and Ogura[14] reported that yeast alcohol dehydrogenase was irreversibly denatured by urea of higher concentration than two molar and was dissociated into four subunits.

All the sulfhydryl groups in yeast alcohol dehydrogenase which was denatured by 5M urea, were modified instantaneously with DTNB within ten minutes, as is shown in Fig. 1. Number of all the sulfhydryl groups, socalled "total" sulfhydryl group, of the enzyme preparations were shown in Table I and were twenty moles per mole enzyme on the average. "Total" sulfhydryl group was equal to the value measured by the PCMB titration method as is shown in Fig. 2 and in Table I (c).

On the other hand, the reaction curve in the absence of urea, seemed to be biphasic and be linear after ten minutes, as is shown in Fig. 1. This result suggests that sensitive sulfhydryl groups for DTNB exist in yeast alcohol dehydrogenase.

It was necessary to incubate the reaction mixture for three hours in order to modify all the sulfhydryl groups of the enzyme with DTNB at 20° in the absence of urea.

These results suggest that sulfhydryl groups on the surface of the protein were sensitive and reacted rapidly with DTNB and then initial modification caused gradual denaturation of protein which allowed to react all the sulfhydryl groups of yeast alcohol dehydrogenase molecule.

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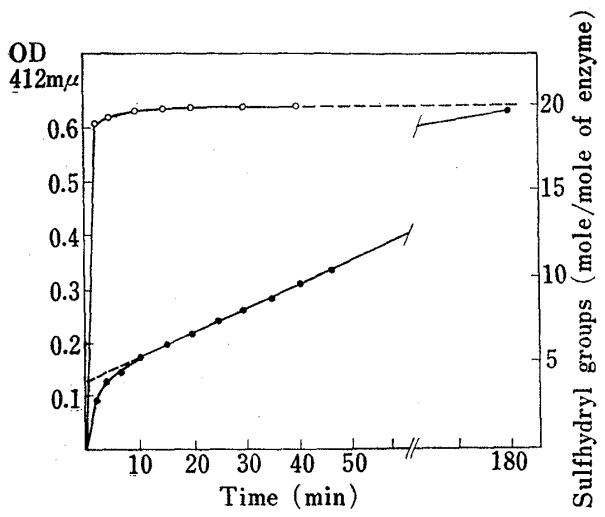

Fig. 1. Reaction of Yeast Alcohol Dehydrogenase with DTNB

The enzyme (2.2×10^{-6} M) was incubated with DTNB (6.6×10^{-5} M) in 3.0 ml of 0.033 M phosphate buffer (pH 8.0) at 20°.
—○—, in the presence of 5M urea
—●—, in the absence of urea

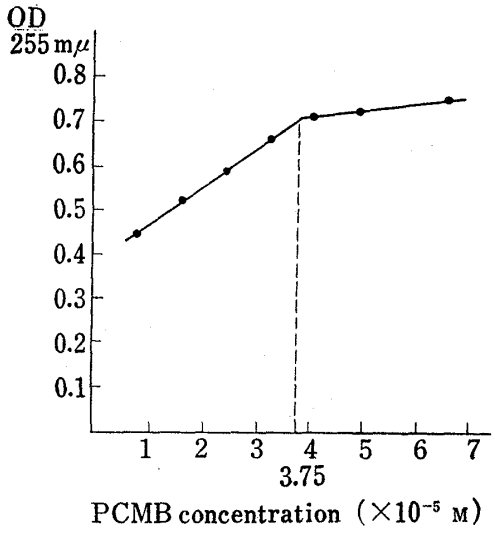

Fig. 2. Titration of Sulfhydryl Groups of Yeast Alcohol Dehydrogenase with PCMB

The enzyme (1.8×10^{-6} M) was reacted with PCMB (0.83 to 6.67×10^{-5} M) in 0.067 M phosphate buffer (pH 7.5).

Table I. Specific Activity and Titrated Sulfhydryl Groups of Yeast Alcohol Dehydrogenase

Preparations	Specific activity[a]	Number of SH groups[b] (moles/mole of enzyme) "Native"	"Total"
1[c]	180000	6.0	20.4
2	105000	3.6	22.1
3	103000	4.5	21.1
4	95000	4.4	22.2
5	93000	4.5	21.0

a) Specific activity (units/mg of protein) was measured according to the method of Racker.[9]
b) Sulfhydryl groups were measured by the reaction with DTNB as described in the text. "Native" corresponds to a sensitive type of sulfhydryl group for DTNB and "Total" does to all the sulfhydryl group of denatured enzyme, reacted with DTNB in the presence of 5 M urea.
c) This crystalline preparation contained 20.8 moles of sulfhydryl groups per mole of the enzyme by PCMB titration method (Fig. 2).

Sensitive sulfhydryl groups for DTNB was measured by extraporating the linear portion to zero time (Fig. 1 dotted line). This type of sulfhydryl groups is socalled "native" sulfhydryl groups.

As is shown in Table I, crystalline preparations of yeast alcohol dehydrogenase contained four to six moles of "native" sulfhydryl groups. However, we could not observed any correlation between specific activity of crystalline enzyme and number of "native" sulfhydryl groups or that of "total" sulfhydryl groups.

Modification of Sulfhydryl Groups and Enzyme Activity

Fig. 3 shows the time courses of modification of sulfhydryl groups and of enzyme inhibition in the reaction of yeast alcohol dehydrogenase with DTNB at 20°.

The rate of inhibition was proportional to that of modification of sulfhydryl groups. Half of "total" sulfhydryl groups, that is, ten of twenty moles in the enzyme, were modified after thirty minutes incubation and at that time enzyme activity was also 50% of the control.

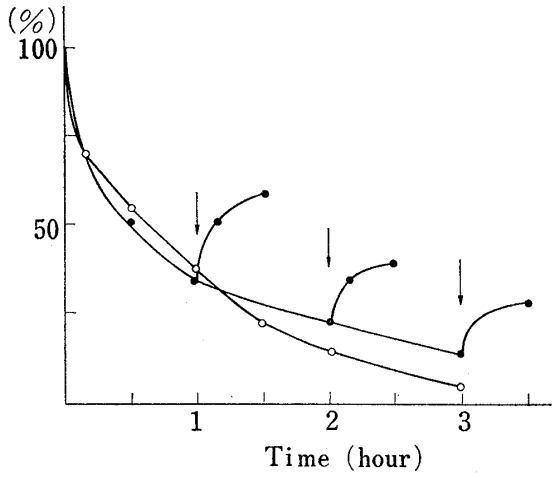

Fig. 3. Decrease in Enzyme Activity and Number of Modified Sulfhydryl Groups in the Reaction of Yeast Alcohol Dehydrogenase with DTNB and the Reactivation of Enzyme Activity by Cysteine

A reaction mixture consisting of 2.2×10^{-6}M the enzyme, 6.6×10^{-5}M DTNB and 0.033 M phosphate buffer (pH 8.0) was incubated at 20°. At the time indicated by arrows, an aliquot of a reaction mixture was added to 0.05 M phosphate buffer (pH 8.0) containing 0.04 M cysteine. Number of modified sulfhydryl groups was calculated from an absorbance at 412 mμ.

—●—, enzyme activity
—○—, sulfhydryl groups

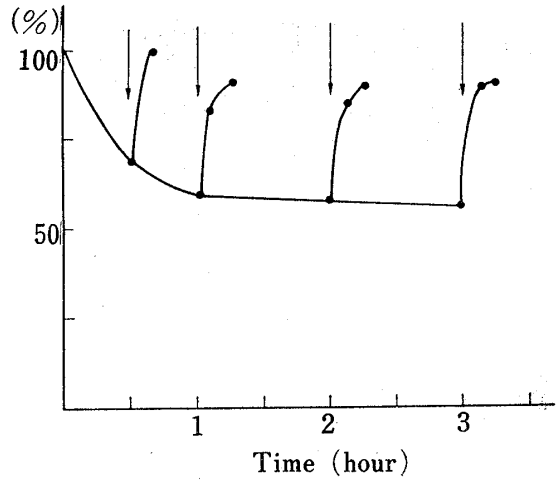

Fig. 4. Decrease in Enzyme Activity and Reactivation by Cysteine in the Reaction of Yeast Alcohol Dehydrogenase with DTNB

1.8×10^{-6}M the enzyme in 0.033 M phosphate buffer (pH 8.0) was incubated with 6.6×10^{-5}M DTNB at 0°. At the time indicated by arrows, an aliquot of reaction mixture was added to 0.05M phosphate buffer (pH 8.0) containing 0.04 M cysteine.

It is known that yeast alcohol dehydrogenase which composes of four subunits,[3] combines four moles of coenzyme[2] and containes four[15] gram atoms of zinc in a molecule.[17] Therefore, it is assumed that the enzyme has four active site in a molecule.

After thirty minutes incubation, the modified sulfhydryl groups were two in four active sites and eight of non-active sites. There was no difference in the reactivity for DTNB between sulfhydryl groups in active site and those in non-active site.

The enzyme activity of modified yeast alcohol dehydrogenase was restored by the addition of cysteine, but degree of reactivation decreased gradually with reaction time. Thus, the reaction of yeast alcohol dehydrogenase with DTNB at 20° not only modified the sulfhydryl group, but brought about the irreversible change of conformation of the enzyme.

On the contrary, the enzyme activity under the reaction condition at 0°, was inhibited by 50% of the control after one hour and then became constant, and was fully restored by the addition of cysteine, as is shown in Fig. 4. Thus, DTNB reacted easily at 0° with sulfhydryl groups of two of four active sites of yeast alcohol dehydrogenase molecule, but not reacted with the other of active site at least within three hours.

The sedimentation pattern of yeast alcohol dehydrogenase which reacted with DTNB for three hours at 0° showed the same as that of the control omitting DTNB, as is shown in Fig. 5.

These observations support that the modification of the enzyme with DTNB at low temperature did not cause the conformational change of this protein.

15) Wallenfels, *et al.*[16] reported that yeast alcohol dehydrogenase contained about five gram atoms of zinc.
16) K. Wallenfels, H. Sund, A. Faessler and W. Burchard, *Biochem. Z.*, **329**, 31 (1957).
17) B.L. Vallee and E.L. Hoch, *J. Am. Chem. Soc.*, **77**, 821 (1955).

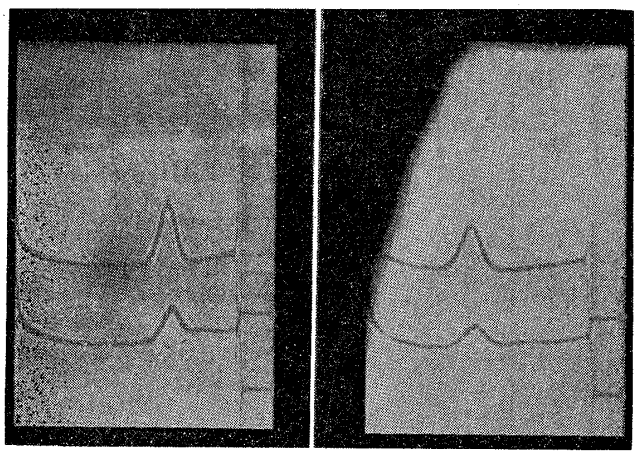

Fig. 5. Sedimentation Patterns of Modified Yeast Alcohol Dehydrogenase by DTNB

1.3×10^{-4}M the enzyme in 0.033M phosphate buffer (pH 8.0) was incubated with 4.0×10^{-3}M DTNB in a final volume of 3.0 ml at 0° for three hours and then was passed through Sephadex G-25 column (2.5×50 cm). Protein fraction obtained was diluted with 0.1M phosphate buffer (pH 8.0).
protein, 6.0 mg/ml
Photographs were taken at 32 min (left) and at 64 min (right) after reaching 59740 rpm.
above; native enzyme below; modified enzyme

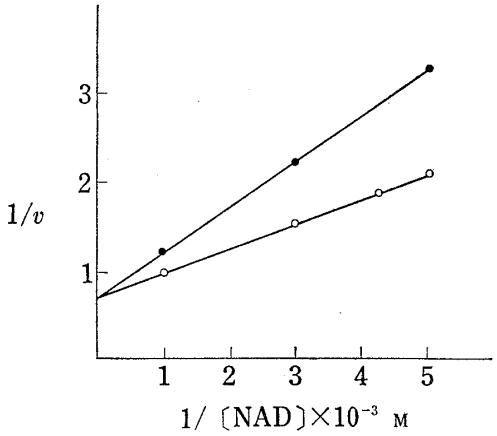

Fig. 6. Lineweaver-Burk Plots for NAD in the Presence and in the Absence of DTNB

1.2×10^{-5}M yeast alcohol dehydrogenase in 0.05 M phosphate buffer (pH 8.0) was preincubated with DTNB (3.3×10^{-4}M) at 0° for three hours and then was diluted with the same buffer. Assay mixture (3.0 ml) contained the enzyme (8.8×10^{-10}M), ethanol (0.01 M), NAD (0.2 to 1.0 mM), pyrophosphate buffer (0.01 M, pH 8.8) and DTNB (—●—, 2.8×10^{-8}M; —○—, none).
v=ΔOD at 340 mμ/min

Competition of DTNB with NAD for Yeast Alcohol Dehydrogenase

To elucidate the mode of inhibition by DTNB, kinetical experiment was performed. The enzyme was preincubated with DTNB at 0° for three hours and then activity was assayed.

Fig. 6 shows the Lineweaver-Burk plots which were obtained in the presence of and in the absence of DTNB. As can be seen in the figure, DTNB competed with NAD for the same sulfhydryl group in the active site of yeast alcohol dehydrogenase.

Discussion

In the previous reports on the determination of sulfhydryl groups of yeast alcohol dehydrogenase by PCMB and a amperometric titration,[5] there was found that this enzyme was denatured by reagents used in these procedures and dissociated into subunits[6] so that the titrated sulfhydryl groups were not sulfhydryl groups of the native state of the enzyme but of a partially or completely denatured enzyme by reagents used for sulfhydryl groups titration.

This was ensured in this present study by the result that number of sulfhydryl groups of urea-denatured enzyme determined by DTNB method was equal to that obtained by PCMB titration method and was twenty moles per mole of the enzyme (Table I *c*).

Four to six moles of sulfhydryl groups per mole of yeast alcohol dehydrogenase existed as a sensitive type for DTNB modification and the other fourteen to sixteen residues reacted slowly at 20°. However, no correlation was found between number of "native" or "total" sulfhydryl groups per mole of the enzyme and specific activity (Table I).

The inhibition of enzyme activity by DTNB shows the modification of sulfhydryl group in the active site. Both the loss in the activity and the modification of sulfhydryl groups proceeded parallel with the reaction time at least within one hour. After three hours incubation at 20°, the enzyme was almost inactivated and all the sulfhydryl groups of the enzyme were modified (Fig. 3). Therefore, it is mentioned that the interaction of DTNB for sulf-

hydryl groups of the enzyme was non-selctive for active site and non-active site under these reaction condition.

On the other hand, under the reaction condition at 0°, it was found that half of enzyme activity was inhibited by DTNB after one hour and then the activity became constant.

This result shows that sulfhydryl groups of two of four active sites were easily modified by DTNB and the other resisted against modification.

It is assumed that the conformation of yeast alcohol dehydrogenase which was modified sulfhydryl groups of two of four active sites was stable. This assumption was proved by the sedimentation pattern of modified enzyme showing a single peak as well as that of the control and by the full reactivation of the enzyme activity by the addition of cysteine.

Under the reaction condition at 20°, the enzyme activity was partially restored by the addition of cysteine. Therefore, it was assumed that an irreversibly conformational change occured during the modification.

DTNB is competitive inhibitor with NAD for sulfhydryl group in the active site.

The enzyme possesses four[17] or five[16] gram atoms of zinc per molecule. They are not removed by dialysis against water or phosphate buffer at neutral pH and are comparatively stable for metal chelators such as EDTA and *o*-phenanthroline. For example, the addition of 0.001 M EDTA or *o*-phenanthroline[18] did not inhibit enzyme activity under the condition at 0° and pH 8.0. Therefore, it is assumed that zinc ions tightly bind to the enzyme molecule.

According to the proposed schema on the active site of yeast alcohol dehydrogenase by Wallenfels and Sund,[19] there are two sulfhydryl groups in one active site.

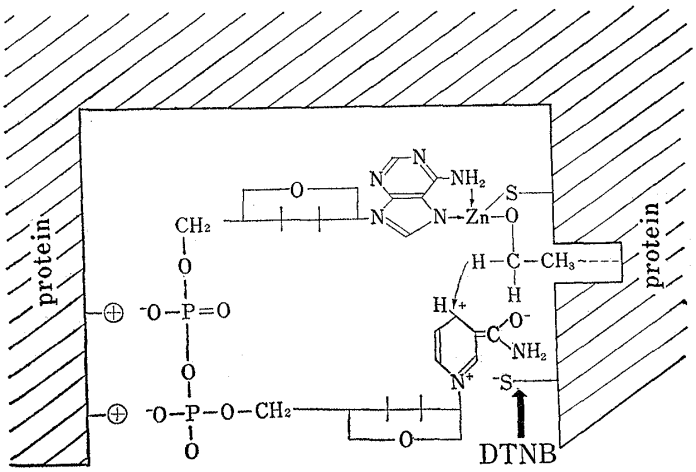

Fig. 7. Proposed Schema of Active Site of Yeast Alcohol Dehydrogenase According to Wallenfels and Sund[19]

The thick arrow represents the inhibition by DTNB.

As can be seen in Fig. 7, one of them combines directly to pyridine moiety of NAD and an other combines to adenine moiety of this coenzyme through zinc ion.

The sulfhydryl group for which DTNB and NAD competed with each other, is the group, which combined to pyridine moiety of NAD.

From the results obtained in this present study, we propose the model of yeast alcohol dehydrogenase molecule as is shown in Fig. 8.

Left figure represents a complex of the enzyme and NAD in the native state. The enzyme composes of four subunits, each of which has one binding site for coenzyme, and contains six moles of "native" sulfhydryl group. The model also shows that two of four active sites are characterized to be more reactive than the other two.

Right figure represents a modified complex of yeast alcohol dehydrogenase and NAD with DTNB. 1. Under the conditions for thirty minutes at 20° or for one to three hours at 0°, half of twenty moles, "total", ofsulfhydryl groups of the enzyme were modified and 50% of activity was lost. 2. Four sulfhydryl groups which exist in folding structure of the enzyme in the native state are exposed, because modification by DTNB causes a gradually conformational

18) Unpublished data.
19) K. Wallenfels and H. Sund, *Biochem. Z.*, 329, 59 (1957).

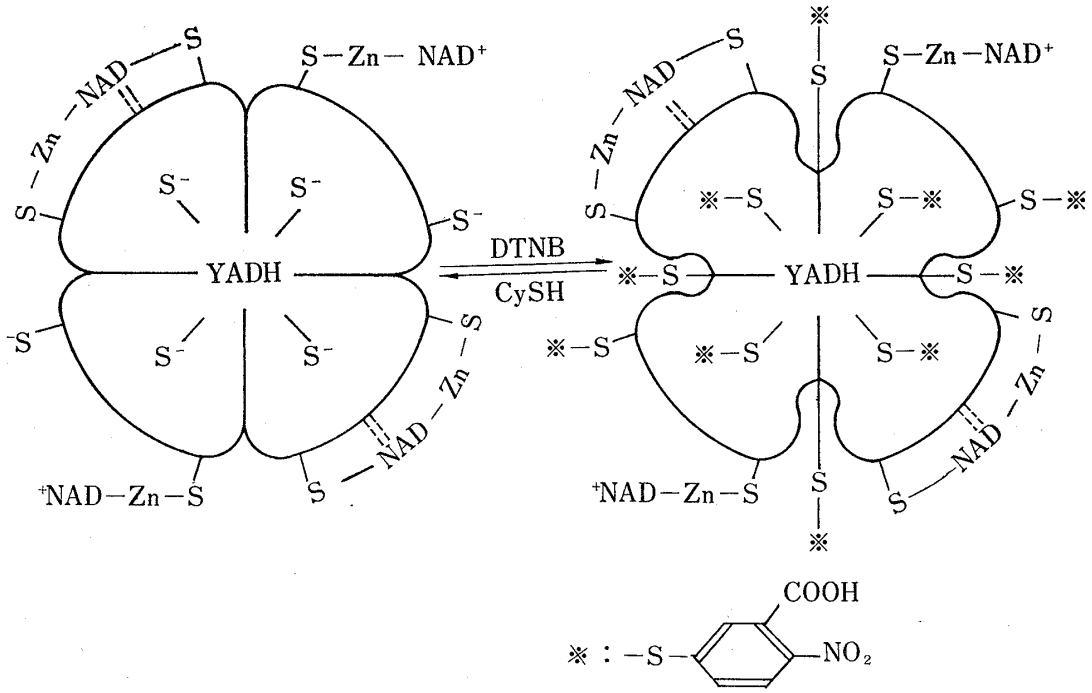

Fig. 8. The Proposed Model of Yeast Alcohol Dehydrogenase Molecule

change in yeast alcohol dehydrogenase molecule. The modified enzyme in this state is probably reversed to the native state by the addition of cysteine. However, more progress of modification lead the enzyme to an irreversibly changed conformation.

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