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The Reaction of 2-Methyl-1,4-naphthoquinone with Yeast Alcohol Dehydrogenase

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1. 2-Methyl-1,4-naphthoquinone (K_3) reacted with sulfhydryl groups of yeast alcohol dehydrogenase to form a thioehter linkage at 3-position of the quinone with absorption maximum at 430 mμ, as well as with sulfhydryl groups of low molecular compounds, bovine serum albumin and papain.

2. Under the reaction conditions at 0°, K_3 preferentially combined with essential sulfhydryl groups of the enzyme without any change in its conformation such as dissociation into subunits.

3. From the tryptic digest of the ^{14}C labeled K_3 bound yeast alcohol dehydrogenase, a radioactive peptide consisted of 37 amino acid residues was isolated.

Introduction

It has been previously reported that 2-methyl-1,4-naphthoquinone (K_3) reacted with sulfhydryl compounds such as thioglycolic acid, cysteine and glutathione to form a thioether linkage at 3-position of the quinone with a characteristic absorption at 420—430 mμ.[2] This reaction gradually proceeded with consumption of oxygen under the physiological conditions, and the resulting products were generally stable except that with cysteine, in which case dehydration, cyclization and then oxidative polymerization occurred.

It has been also reported that sulfhydryl groups of proteins such as bovine serum albumin and papain reacted with K_3 to form a thioether linkage, and papain was irreversibly inactivated.[3]

It is interesting to study on the rate of this reaction with essential sulfhydryl groups and the others in such enzyme as yeast alcohol dehydrogenase (EC 1.1.1.1, alcohol: NAD oxidoreductase (yeast)) which has many sulfhydryl groups. Yeast alcohol dehydrogenase has been considered to be a thiol enzyme and to be consisted of four subunits.[4] It has been reported that each of these subunits has a single coenzyme binding site[5] where an essential sulfhydryl group exists.[6] In the previous report,[7] twenty sulfhydryl groups in the enzyme were titrated by 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) or *p*-chloromercuribenzoic acid.

In the present study, we report the reactivity of sulfhydryl groups in the active site of yeast alcohol dehydrogenase through the study of the reaction of K_3 and present the evidence of binding of K_3 with cysteine residues in the active site.

Experimental

Materials——Crystalline yeast alcohol dehydrogenase was prepared from baker's yeast according to the method of Racker.[8] It was recrystallized twice with ammonium sulfate. The specific activity of the preparation used in this study was 110000 to 180000 units/mg when assayed with the method of Racker,[8] and the titrated sulfhydryl groups in this enzyme were 20.4 to 22.1 moles per mole of the enzyme.[7]

1) Location: *3190 Gofuku, Toyama.*
2) N. Nakai and J. Hase, *Chem. Pharm. Bull.* (Tokyo), **16**, 2334 (1968).
3) N. Nakai and J. Hase, *Chem. Pharm. Bull.* (Tokyo), **16**, 2339 (1968).
4) J.H.R. Kägi and B.L. Vallee, *J. Biol. Chem.*, **235**, 3188 (1960).
5) J.E. Häyes and S.F. Velick, *J. Biol. Chem.*, **207**, 225 (1952).
6) E.L. Whitehead and B.R. Rabin, *Biochem. J.*, **90**, 532 (1964).
7) N. Nakai and J. Hase, *Chem. Pharm. Bull.* (Tokyo), **17**, 722 (1969).
8) E. Racker, *J. Biol. Chem.*, **184**, 313 (1950).

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K_3 was prepared by the chromic acid oxidation of β-methylnaphthalene according to the method of Fieser[9] and was recrystallized twice from methanol. 2-Methyl-^{14}C-1,4-naphthoquinone (^{14}C-K_3, 2.29 mCi/mM) obtained from the Radio Chemical Centre was diluted five times with unlabeled K_3 and was recrystallized from methanol.

DTNB was prepared according to the method of Ellman.[10] NAD was purchased from Sigma Chemical Co. Trypsin (twice recrystallized preparation) was kindly provided by Mochida Pharm.Co., Ltd., and prior to use, it was incubated in 0.01 M HCl at 37° for 24 hours.[11] Bovine insulin and yeast cytochrom c were furnished by Shimizu Pharm. Co.,and Sankyo Co., respectively. K_3 bound gultathione (2-methyl-3-gultathionyl-1,4-naphthoquinone) was synthesized according to the method of Nickerson, *et al.*[12] Other reagents used were purchased commercial preparation available of extra grade.

Methods——The enzyme activity was assayed by the method of Racker[8] and titration of sulfhydryl groups by DTNB was performed by the method of Ellman[10] in the presence of 5 M urea.[7]

The reaction of K_3 with yeast alcohol dehydrogenase was performed as follows. A mixture consiting of K_3, the enzyme and 0.1 M phosphate buffer (pH 7.5) was incubated at 30° or 0°. K_3 used was dissolved in ethanol of which final concentration was less than 10% in the reaction mixture. Each tests were always compared with the control omitting K_3. In order to obtain the evidence on the binding of ^{14}C-K_3 to the enzyme, the reaction mixture was passed through Sephadex G-25 column and the radioactivity of the protein fractions were measured.

Sedimentation study was carried out in a Spinco model E ultracentrifuge. A mixture consiting of 1.0×10^{-4} M the enzyme, 1.0×10^{-3} M K_3 and 0.1 M phosphate buffer (pH 7.5) in a total volume of 3.0 ml, was incubated at 0° for 24 hours and then was passed through a Sephadex G-25 column (2.5×35 cm) to separate the protein fraction from the excess of K_3. The protein fraction was analyzed at 59720 rpm and at 12°.

^{14}C-K_3 bound yeast alcohol dehydrogenase was prepared and was digested as follows. The enzyme (360 mg) was incubated with ten fold molar excess of ^{14}C-K_3 in 24.0 ml of 0.1 M phosphate buffer (pH 7.5) at 0° for 24 hours. The labeled protein was precipitated by the addition of 30 ml of cold acetone and the precipitate was washed repeatedly with acetone.

The protein (150 mg) was suspended in 5.0 ml of 0.1 M ammonium bicarbonate buffer (pH 8.5) and was digested with 3.0 mg of acid-treated trypsin at 37° for 24 hours. After the digestion for 12 hours, 1.5 mg of tryspin was added further. The digest was centrifuged to remove the small amount of precipitate formed and then the supernatant was lyophilyzed.

Ion-exchange chromatography was performed as follow. The dried digest of the labeled protein was dissolved in 6.0 ml of 0.1 M pyridine formate buffer (pH 3.2) and was applied to Dowex 50-X2 column (200 to 400 mesh, H^+type, 1.5×65 cm) in equilibrium with the same buffer. Elution was initiated with 0.1 M pyridine formate buffer (pH 3.2), followed succesively by 0.2 M pyridine formate (pH 3.2), 0.4 M pyridine acetate (pH 4.6), 1.5 M pyridine acetate (pH 5.0) and finally 2.0 M pyridine (pH 8.0). Eluate was fractionated in 5.0 ml per tube at a flow rate of 60 ml per hour. The peptides eluated in each fraction were determined by the ninhydrin method.[13] Fractions were concentrated below 40° by rotary evaporator under the reduced pressure and were pooled in 2.0% acetic acid.

Paper chromatography was carried out on Toyo No. 51 paper by the ascending method using *n*-butanol-pyridine-acetic acid-water (15:10:3:12) as the developing solvent. For the determination of radioactivity, after color reaction with ninhydrin, the paper strip was clipped by each 1.0 cm and each segment was placed in counting vial containing 5.0 ml of Bray's solution.[14]

The peptide for amino acid analysis was hydrolyzed in a sealed evacuated glass tube with constant boiling HCl at 110° for 24 hours. The acid was removed under vacuum and the residue was dissolved in water and then was subjected to the analysis.

The radioacivity of materials was determined by a liquid scintillation spectrometer (Packerd Tricarb Model 3003) in the Bray's solution.

For the comparison of molecular weight of radioactive materials, gel filtration was carried as follow. The sample was dissolved in 1.0 ml of 2.0% of acetic acid and was applied to a Sephadex G-50 column (1.5×40 cm), and was eluated with the same solvent at flow rate of 16 ml/hour.

Protein concentration was determined by the method of Lowry, *et al.*[15] on the basis of the absorption coefficient of 1.89×10^5 cm^2 mole^{-1} for yeast alcohol dehydrogenase at 280 mμ.[5]

9) L.F. Fieser, *J. Biol. Chem.*, **133**, 391 (1940).
10) G.L. Ellman, *Arch. Biochem. Biophys.*, **82**, 70 (1959).
11) R.R. Redfield and C.B. Anfinsen, *J. Biol. Chem.*, **221**, 385 (1956).
12) W.J. Nickerson, F. Falcone and G. Strauss, *Biochemistry*, **2**, 537 (1963).
13) H. Rosen, *Arch. Biochem. Biophys.*, **67**, 10 (1957).
14) G.A. Bray, *Anal. Biochem.*, **1**, 279 (1960).
15) O.H. Lowry, N.J. Rosebrough, A.L. Farr, and R.J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

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Result

The Reaction of K_3 with Yeast Alcohol Dehydrogenase

In the present study, yeast alcohol dehydrogenase was confirmed to be rapidly inactivated by low concentration of K_3 under the incubation conditions at 30°. After the incubation of the enzyme with different concentration of K_3 at 30° for 30 minutes, the enzyme activity was assayed (Fig. 1). From the figure, an apparent I_{50} value was estimated to be 3.0×10^{-5}M under the present reaction conditions.

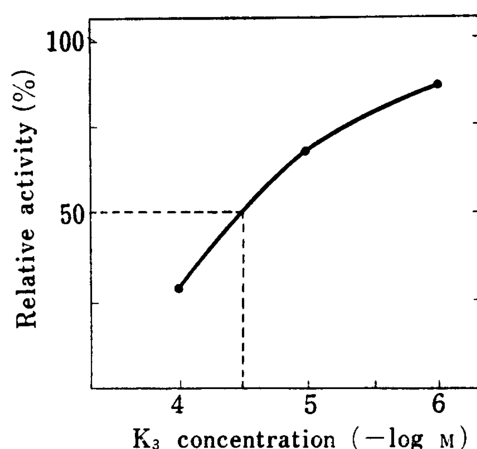

Fig. 1. Effect of K_3 Concentration on Yeast Alcohol Dehydrogenase Activity

The enzyme (4.3×10^{-7}M) was preincubated with K_3 (1, 10 and 100 μM) in 0.1M phosphate buffer (pH 7.5) at 30° for 30 min.

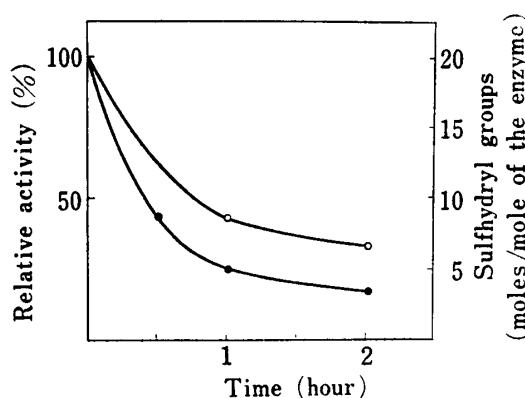

Fig. 2. The Reaction of Yeast Alcohol Dehydrogenase with K_3 at 30°

5.7×10^{-5}M the enzyme was incubated with 5.0×10^{-4}M K_3 in 0.1M phosphate buffffer (pH 7.5) at 30°.

—○—: the enzyme activity
—●—: sulfhydryl groups

Approximately twenty sulfhydryl groups per mole of the enzyme were determined by the titration using DTNB or *p*-chloromercuribenzoic acid.[7] When the enzyme was incubated with nine molar excess of K_3 at 30°, the progressive loss of the enzyme activity and titratable sulfhydryl groups were observed (Fig. 2). After the incubation for one hour, 55% of the original enzyme activity and fifteen sulfhydryl groups (75% of the control) were lost. If K_3 in the reaction mixture binds stoichiometrically to sulfhydryl groups of the enzyme by the same manner as it reacts to sulfhydryl compounds such as thioglycolate, loss of sulfhydryl groups of the enzyme by binding with K_3 must be only nine. Therefore, it is suggested that loss of fifteen sulfhydryl groups per mole of the enzyme might be caused not only by the binding of K_3 but also by other reactions such as oxidation.

Since the reactivity of sulfhydryl groups of yeast alcohol dehydrogenase was demonstrated to be affected by the reaction temperature in the case of the reaction with DTNB,[7] the effect of temperature on this reaction was investigated.

As is shown in Fig. 3, under the incubation conditions at 0°, the reaction was much slower than that at 30°. The enzyme was inactivated by 50% of the control after 24 hours and by 85% of that after 72 hours. On the other hand, approximately four sulfhydryl groups in the enzyme disappeared within 24 hours, and the additional only one group disappeared after 72 hours. From the results described in Fig. 3, Table II, it is considered that loss of sulfhydryl groups might be solely due to the interaction of the enzyme with K_3 at 0°, and that the other reaction, such as oxidation, observed at 30° might be excluded at 0°. Therefore, all the following investigations were performed under the incubation conditions at 0°.

When yeast alcohol dehydrogenase was incubated with K_3, a change in the K_3 spectrum is shown as in Fig. 4. A characteristic absorption of a thioether linkage of naphthoquinone

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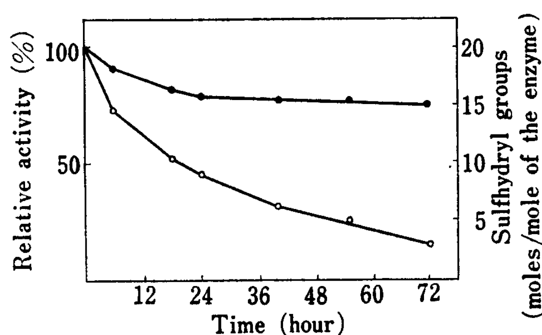

Fig. 3. The Reaction of Yeast Alcohol Dehydrogenase with K_3 at 0°

5.7×10^{-5}M the enzyme was reacted with 5.0×10^{-4}M K_3 in 0.1M phosphate buffer (pH 7.5) at 0°.
—○—: the enzyme activity
—●—: sulfhydryl groups

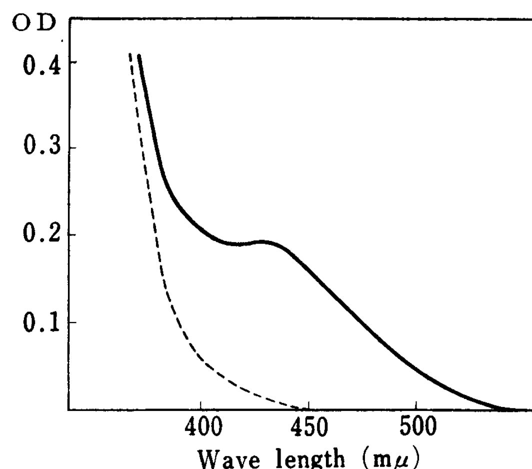

Fig. 4. Absorption Spectrum of the Reaction Product of Yeast Alcohol Dehydrogenase with K_3

A mixture consisting of 2.4×10^{-4}M yeast alcohol dehydrogenase, 4.0×10^{-4}M K_3 and 0.1M phosphate buffer (pH 7.5) was allowed to stand at 0° for 24 hours.
——: the enzyme and K_3
-----: K_3 alone

was observed. Thus sulfhydryl groups of this enzyme also reacted with K_3 to form the same linkage as well as those of bovine serum albumin, papain and low molecular sulfhydryl compounds did.

From these results, however, it is not decided that whether or not the inactivation of the enzyme was caused by the binding of K_3 to essential sulfhydryl groups in the enzyme.

Molar Ratio of Bound K_3 to Yeast Alcohol Dehydrogenase

In order to determine the correlation between molar ratio of bound K_3 to the enzyme and loss of its catalytic activity, experiments using 2-methyl-^{14}C-1,4-naphthoquinone (^{14}C-K_3) were carried out.

After incubation of the enzyme with excess of ^{14}C-K_3 in 0.1M phosphate buffer (pH 7.5) at 0° for 24 hours, the enzyme activity was determined and at the same time, gel filtration on Sephadex G-25 column was performed to remove excess of ^{14}C-K_3 from the protein. The radioactivity incorporated and the number of sulfhydryl groups of isolated protein were determined. As is shown in Table I, approximately two moles of K_3 combined to one mole of the

Table I. Analysis of ^{14}C-K_3 Bound Yeast Alcohol Dehydrogenase

Exp.	Concentration in reaction mixture (10^{-5}M)			^{14}C-K_3 bound enzyme		
	^{14}C-K_3	YADH	K_3/YADH[a]	Molar ratio (^{14}C-K_3/YADH)	ΔSH[b]	Activity (I%)[c]
1	100	11.5	9	1.8	—	—
2	50	5.0	10	1.9	1.6	56
3	50	4.8	10	2.0	2.2	—
4	100	8.7	11	2.4	2.2	—
5	80	5.0	16	2.0	—	50
6	50	1.7	30	2.0	4.3	66
7	50	0.5	100	2.5	3.8	76

a) molar ratio of ^{14}C-K_3 to yeast alcohol dehydrogenase in the reaction mixture
b) Sulfhydryl groups were measured by the reaction with DTNB in the presence of 5M urea.[7] The number of decreased sulfhydryl groups was compared with that of these groups of the control omitting of K_3.
c) inhibition of the enzyme activity
The enzyme was reacted with ^{14}C-K_3 in 0.05M phosphate buffer (pH7.5) at 0° for 24 hours and then the reaction mixture was passed through Sephadex G-25 column.

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enzyme without any relation to the molar ratio of the reactans in the reaction mixture. When the molar ratios of K_3 to the enzyme in the reaction mixture were 10: 1 and 16: 1, two sulfhydryl groups disappeared and the enzyme activity was decreased by about 50% of the control omitting K_3. When molar ratios of K_3 to the enzyme were 30: 1 and 100: 1, however, approximately four sulfhydryl groups disappeared and losses of the enzyme activity were 66 and 76% respectively of the control, while 2.0 and 2.5 moles of K_3 respectively were determined.

From the results that two moles of K_3 bound to one moles of the enzyme when 50% loss of its catalytic activity were observed, it is suggested that K_3 combined to two of the essential sulfhydryl groups among four active sites of the enzyme.

When the enzyme in low concentration was incubated with 30 to 100 molar excess of K_3 (experiments 6 and 7 in Table I), the enzyme activity seemed to be inhibited not only by the binding of K_3, but by unknown reaction, because more sulfhydryl groups than those combined to K_3 disappeared.

Table II. The Binding of K_3 to Yeast Alcohol Dehydrogenase[a]

Experiment	Reaction time (hour)		
	24	48	72
1	1.8	2.3	2.8
2	2.0 (50)[b]	2.9 (62)	3.2 (77)

a) moles of ^{14}C-K_3 per mole of the enzyme
b) Aparenthesis is inhibition % of the enzyme activity.
Reaction mixture consisting of the enzyme, K_3 and 0.05M phosphate buffer (pH 7.5) in a final volume of 3.0 ml were allowed to stand at 0°. At the time indicated, an aliquot of a reaction mixture was passed through Sephadex G-25 column and protein fraction obtained was analyzed.
1, 1.15×10^{-4}M the enzyme and 1.0×10^{-3}M ^{14}C-K_3
2, 5.0×10^{-5}M the enzyme and 8.0×10^{-4} M^{14}C-K_3

The results obtained after the incubation for 24 to 72 hours are shown in Table II. After 72 hours, approximately three moles bound K_3 to the enzyme and 77% inactivation were observed. These results also suggest that K_3 preferentially combined to essential sulfhydryl groups in four active sites of yeast alcohol dehydrogenase resulting in the inactivation.

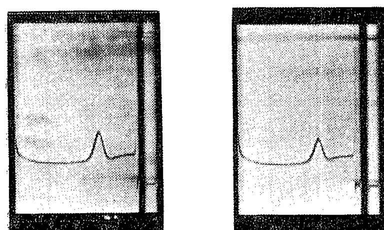

Fig. 5. Sedimentation Patterns of Yeast Alcohol Dehydrogenase and K_3 Bound Enzyme

1.3×10^{-4}M the enzyme was reacted with 1.0×10^{-3}M K_3 in 0.1M phosphate buffer (pH 7.5) in final volume of 3.0 ml at 0° for 24 hours and then was passed through a Sephadex G-25 column (2.5×35 cm). Protein fraction obtained was diluted with the same buffer. Photographs were taken at 32 minutes after reaching 59740 rpm.

Left; the native enzyme, right; K_3 bound enzyme Protein concentrations were 7.1 and 7.5 mg/ml respectively.

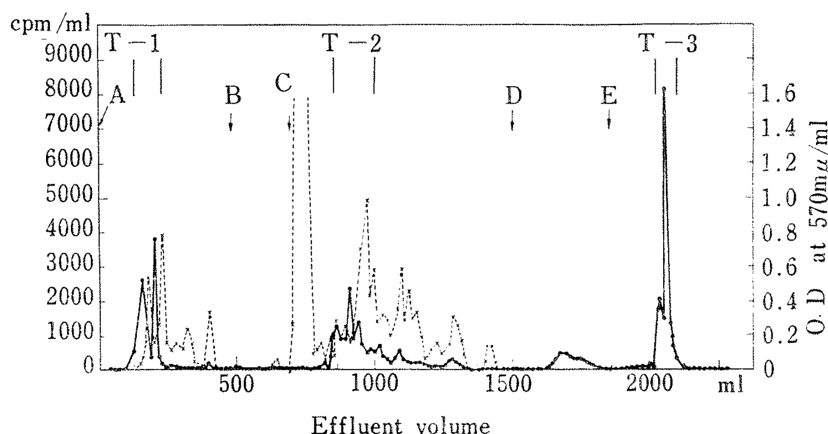

Fig. 6. Chromatographic Pattern of Tryptic Digest of ^{14}C-K_3 Bound Yeast Alcohol Dehydrogenase on Dowex 50-X2 Column

Chromatogram shows the radioactivity (—●—) and OD at 570 mμ (---×---) after reaction with ninhydrin (described in text).
column: Dowex 50-X2 1.5×65 cm, solvent; A: 0.1M pyridine formate (pH 3.2); B: 0.2M pyridine formate (pH 3.2); C: 0.4M pyridine acetate (pH 4.6); D: 1.5M pyridine acetate (pH 5.0); E: 2M pyridine (pH 8.6), flow rate: 60 ml/hr

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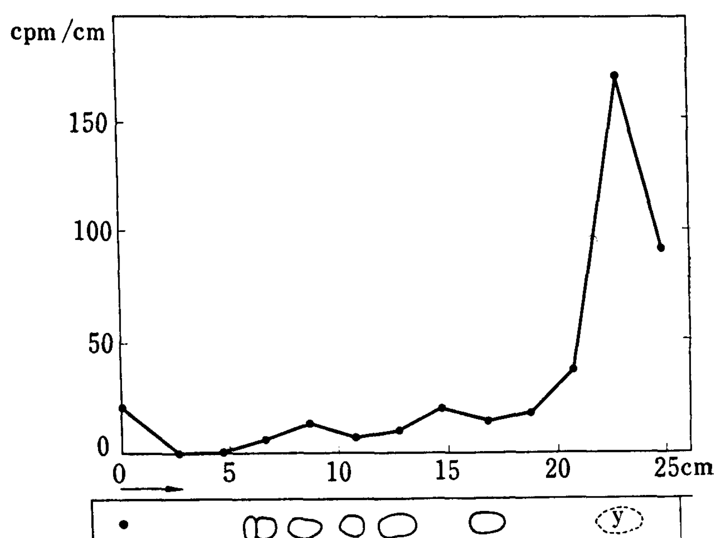

Fig. 7. Paper Chromatogram of the Acid Hydrolysate of T-3 Peptide

The hydrolysate was developed by ascending paper chromatography for 16 hr with the solvent system of *n*-butyl alcohol-pyridine-acetic acid-water (15:10:3:12). After color reaction by ninhydrin, the paper was cut and each segments were placed in counting vials and the radioactivities were counted.
y: yellow color

It must be considered whether the modification of sulfhydryl groups by K_3 is accompanied with any change in the enzyme conformation or not. As is shown in Fig. 5, however, the sedimentation patterns of the enzyme which was incubated with ten fold molar excess of K_3 at 0° for 24 hours was the same as that of the control omitting K_3. This observation supports that the modification of sulfhydryl groups in two of four active sites of the enzyme with K_3 did not cause the conformational change of this protein such as dissociation into subunits.

Digestion of ^{14}C-K_3 Bound Yeast Alcohol Dehydrogenase

As mentioned above, the activity of yeast alcohol dehydrogenase was reduced by half of the original activity by the incubation at 0° for 24 hours with ten fold molar excess of K_3 and was combined with two moles of the quinone. It is assumed from the results that K_3 preferentially binds to two of twenty sulfhydryl groups and these two sulfhydryl groups situate in two of four active sites of the enzyme.

In order to obtain the evidence of the binding of K_3 to yeast alcohol dehydrogenase, the labeled peptide was isolated from the tryptic digest of the ^{14}C-K_3 bound enzyme. The enzyme which was incubated with ^{14}C-K_3 at 0° for 24 hours, was isolated from excess of K_3 and was digested by trypsin for 24 hours. The resulting digest was fractionated by ion-exchange chromatography using a column of Dowex 50-X2. The elution pattern is shown in Fig. 6. The major fraction (T-3) and other two fractions (T-1 and T-2) containing radioactive materials were obtained in which the recoveries of radioactivity were 60, 15 and 10% respectively. As is seen in the figure, two peaks were observed in T-3 fraction on chromatographic pattern. However, both of these peaks gave only a single yellow spot by ninhydrin reaction, possessing the same *Rf* value (*Rf*, 0.308 and 0.304) on paper chromatogram. Thus, T-3 fraction contained a single radioactive peptide.

To confirm that ^{14}C-K_3 bound cysteine residue was contained in T-3 peptide, this peptide was hydrolyzed by 6N HCl at 110° for 24 hours, and then the hydrolysate was subjected to

Table III. Amino Acid Composition of T-3 Peptide

Amino acid	Molar equivalent per mole of Lys	Amino acid	Molar equivalent per mole of Lys
Asp	5.0	Val	3.2
Thr	2.9	Ile	0.84
Ser	1.7	Leu	4.4
Glu	1.1	Tyr	1.8
Pro	1.9	Lys	1.0
Gly	5.7	His	4.2
Ala	0.4		

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paper chromatography. As is shown in Fig. 7, several ninhydrin-positive spots were observed and the radioactivity was localized on a single yellow spot having a *Rf* value of 0.93. This *Rf* value of the yellow spot was identical with that of the reaction product of K_3 with cysteine,[6] and also with that of a yellow spot of the acid hydrolysate of K_3 bound glutathione,[12] on paper chromatogram under the same condition. Therefore, it is assumed that the peptide in T-3 fraction contained a ^{14}C-K_3 bound cysteine residue.

The result of amino acid analysis of T-3 peptide is shown in Table III. The peptide contained 37 amino acid residues, Asp 5, Thr 3, Ser 2, Glu 1, Pro 2, Gly 6, Ala 2, Val 3, Ile 1, Leu 4, His 4 and Lys 1, and K_3 bound cysteine which could not be detected by the amino acid analysis. From the composition of amino acids, molecular weight of T-3 peptide was calculated to be approximately 3900.

Table IV. Gel Filtration on Sephadex G-50 Column

Sample	Effluent Volume (ml)	Sample	Effluent volume (ml)
Cytochrome c (yeast)	24	T-1	72
Insulin (bovine)	44	T-2	64
Glutathione	72	T-3	60

column: 1.5×40 cm, sample: 1.0 ml in 2% acetic acid, eluennt: 2% accetic acid, flow rate: 16 ml/hr
Cytochrome c was determined by OD at 412 mμ, insulin by OD at 280 mμ, glutathione by the DTNB method, and T-1, T-2 and T-3 by the counting of radioactivity, respectively.

For the comparison of molecular weight of radioactive peptides in T-1 and T-2 fractions with T-3 peptide, Sephadex G-50 gel filtration was performed. As is shown in Table IV, the result supports the molecular weight of T-3 peptide calculated from the amino acid analysis, and suggests that radioactive peptides in T-1 and T-2 fractions were lower in molecular weight than T-3 peptide.

From this result, it is considered that radioactive peptides in T-1 and T-2 fractions were probably hydrolyzed products of T-3 peptide by trypsin.

Discussion

Sulfhydryl group has been considered to be essential for the catalytic activity of yeast alcohol dehydrogenase. In the previous report,[3] it was discussed that sulfhydryl groups of protein such as bovine serum albumin and papain react with K_3 to form a thioether linkage at 3-position of K_3 with a characteristic absorption at 420—430 mμ. In the case of papain, the modification of sulfhydryl groups was accompanied with the loss of activity.

Yeast alcohol dehydrogenase was also inactivated by K_3 and an apparent I_{50} value was estimated to be 3×10^{-5}M under the reaction conditions at 30° for 30 minutes. This inactivation was temperature dependent. Approximately half loss of the enzyme activity was observed within one hour at 30°, but after 24 hours at 0° using the same concentration of K_3.

The absorption spectrum of K_3 bound in the enzyme showed the characteristic absorption band of a thioether linkage of the quinone. From this result, it is suggested that sulfhydryl groups of yeast alcohol dehydrogenase reacted with K_3 by the same manner as those of low molecular sulfhydryl compounds, papain and bovine serum albumin.

When the enzyme was incubated with ten fold molar excess of K_3 at 0° for 24 hours, the binding of two moles of K_3 to the enzyme and 50% loss of the catalytic activity were observed, and further reaction slowly proceeded with time, and after 72 hours the number of bound K_3 increased to three moles and 77% of the enzyme activity was lost. Under these reaction conditions, therefore, loss of the enzyme activity was proportional with the number of binding of K_3. Yeast alcohol dehydrogenase has been shown to be composed of four subunits and

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four active sites per molecule. These results suggest that the enzyme was inactivated by K_3 through the preferential modification of sulfhydryl groups in active sites of the enzyme. Thus, sulfhydryl groups in two of four active sites were modified by K_3 resulting in 50% loss of the enzyme activity.

No effect of molar ratio of K_3 to the enzyme in the reaction mixture on the number of binding of K_3 was observed. When the enzyme in low concentration was incubated with 30 or 100 fold molar excess of K_3, the binding of K_3 were always two moles per mole of the enzyme. In these reactions, however, it was observed that approximately four sulfhydryl groups were lost and 66 or 76% of the enzyme was inactivated. These results suggest that disappearance of sulfhydryl groups and loss of the enzyme activity were caused not only by the binding of K_3 to that groups in active site of the enzyme but by unknown reaction with large excess of K_3.

From the comparison of sedimentation pattern between the enzyme in native state and that reacted with K_3 at 0° for 24 hours, it was observed that the modification caused no change in the conformation of the enzyme such as dissociation into its subunits.

The result of this reaction incubated at 30° quitely differed from that at 0°. In the reaction conditions the enzyme was rapidly inactivated and the number of disappeared sulfhydryl groups of the enzyme were more than that of added K_3 in the reaction mixture. It is suggested that the reaction at 30° was not only the binding reaction of K_3 to the enzyme but unknown ones. As was described in the previous report,[7] the reactivity of sulfhydryl groups of yeast alcohol dehydrogenase for DTNB was depended on the incubation temperature and the conformational change in the molecule occurred during modification. It is also considered that under the reaction conditions at 30° the conformational change in the enzyme molecule was caused by the binding of K_3 and then exposed sulfhydryl groups were oxidized probably by oxygen resulting in the additional inactivation of the enzyme.

From the result of analysis of an isolated peptide containing ^{14}C-K_3 (T-3) from tryptic digest of the reaction product of yeast alcohol dehydrogenase with ^{14}C-K_3 at 0° for 24 hours, it is confirmed that cysteine residue in the enzyme combined to K_3 forming a thioether linkage. From these results and the correlation between the catalytic activity and the number of binding of K_3 to the enzyme (Table I and Table II), it might be concluded that only sulfhydryl groups which situated in the active site of the enzyme combined to K_3.

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