Behavior of Acetyl Groups Combined with Sulfhydryl Groups in Protein

II. Behavior of Acetyl Groups Combined with Sulfhydryl Groups in Yeast Alcohol Dehydrogenase

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It was found that acetyl groups combined with SH groups in yeast alcohol dehydrogenase (YADH) were easily released and transferred to SH group of coenzyme A (CoA) in contrast with acetyl group bound to SH group of glutathione. Stabilization of thioester bonds in S-acetyl-YADH was observed upon denaturation of the protein in the presence of urea.

These findings appeared to open new approach to a problem about mechanism of storage and supply of active acetyl groups in living cells.

It seems possible to assume that, when protein molecule is strained stereochemically by modification within certain limits, the modified protein is able to recover itself from strained structure to the native, particular structure leaving out the modifier which interact through changes in protein structure.

Our attention has devoted to the study of the nature of acetyl groups combined with protein SH* groups, because the behavior of acetyl groups of S-acetyl-protein is expected to be different from that of acetyl groups combined with SH groups of substances of low molecular weight due to self-restorable character of protein.

In order to investigate the behavior of acetyl groups bound to SH groups in protein, acetylation of SH groups in YADH (EC 1.1.1.1), which contains essential SH groups for

activity, has been attempted. S-Acetyl-YADH resulting from the reaction with DAT as an acetylating reagent was isolated, which was found to contain two acetylated SH groups in a molecule and to show about 50 % of its original activity (1). Interestingly, in the subsequent studies it was found that acetyl groups combined with SH groups in YADH differ from those bound to SH compounds of low molecular weight. In the present paper the properties of acetyl groups combined with SH groups in YADH are described.

EXPERIMENTAL

1. Materials

DAT was prepared by the method of Matsukawa *et al.* (2) from thiamine hydrochloride. NAD and NADH were obtained from Sigma,

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* The abbreviations used are: SH, sulfhydryl; YADH, yeast alcohol dehydrogenase; DAT, O, S-diacetylthiamine; NAD, nicotinamide-adenine dinucleotide; NADH, reduced form of nicotinamide-adenine dinucleotide; acetyl-SG, acetyl glutathione; GSH, glutathione; CoA, coenzyme A; DEAE, diethylaminoethyl; PCMB, p-chloromercuribenzoate; TCA, trichloroacetic acid.

and determined by the method of Ciotti *et al.* (3). Acetyl-SG was prepared by acetylation of GSH according to the method of Kielly *et al.* (4). CoA was a product of Sigma and a gift from Dainihon Seiyaku, which was prepared by Pabst Laboratory. CoA was dissolved in 0.05 M phosphate buffer, pH 6.0, and adjusted the pH to 6.0 with 0.1 N sodium hydroxide. The solution was diluted to an appropriate concentration with the same buffer. DEAE-cellulose was obtained from Serva. Phosphate acetyltransferase (EC 2.3.1.8) was a product of Boehringer and Soehne (specific activity 1,200 I.U./mg (5), 1 mg/ml) and diluted 10-fold before use. Arylamine acetyltransferase (EC 2.3.1.5) was prepared from pigeon liver and partially purified according to the method of Chou and Lipmann (6).

2. Preparation and Assay of YADH

YADH was prepared by the method of Ohta *et al.* (7) with slight modifications as described in the preceding paper (1), and purified by DEAE-cellulose column chromatography after recrystallization several times according to the method of Hayes *et al.* (8). Enzyme assay was carried out by the method of Racker (9) and the activity was represented by Racker units. The enzyme solution was diluted, if necessary, with 0.05 M phosphate buffer, pH 6.0, for assay.

Protein was determined by measuring absorbance at 280 m μ . Molecular weight for the enzyme of 150,000 (8) and the molecular extinction coefficient at 280 m μ of 1.89×10^5 cm² mole⁻¹ (8) were used in the calculation.

Specific activity of YADH preparation used in the present experiments was 150,000 units/mg.

3. Determination of SH Groups

Determination of SH group of GSH was performed by the method of Boyer (10). Number of SH groups in YADH was determined as described in the preceding paper (1) essentially according to the method of Boyer (10).

4. Acetylation of YADH with DAT

Unless otherwise stated, acetylation was carried out at pH 6.0 and 30° by incubating

1.0 ml of 33 μ M YADH solution in 0.05 M phosphate buffer, pH 6.0, with 1.0 ml of 136.4 mM DAT solution in the same buffer for 30 minutes. The protein was separated from reagent by passage through a Sephadex G-25 column (1.0 $\times$ 20 cm) in the cold. Aliquots of the protein effluent were taken up for determination of YADH activity and contents of SH groups. The recovery of the protein, as measured by ultraviolet absorption, was about 94 %. *S*-Acetyl-YADH thus obtained could be stored by lyophilization of the effluent to dryness immediately after the gel filtration.

RESULTS

1. Instability of Thiolester Bonds in *S*-Acetyl-YADH

Since it was fundamentally important for subsequent studies to obtain some information about stability of thiolester bonds in *S*-acetyl-YADH, which were produced by acetylation of YADH with DAT, following experiments were designed to take views of reactivation of *S*-acetyl-YADH and restoration of SH groups lost due to the acetylation.

A 2.64 mg sample of *S*-acetyl-YADH, in which the activity was reduced to 50 % of that of the native enzyme, was dissolved in 2.0 ml of 0.05 M phosphate buffer, pH 6.0, and incubated

FIG. 1 Spontaneous reactivation of *S*-acetyl-YADH

A 2.64 mg sample of *S*-acetyl-YADH was dissolved in 2.0 ml of 0.05 M phosphate buffer, pH 6.0, and incubated at 30°. Aliquots of the solution were taken out for determination of YADH activity. The activity was expressed as percentage relative to the specific activity of native YADH.

TABLE 1
Spontaneous restoration of SH groups in S-acetyl-YADH

Acetylation of YADH with DAT		Restoration of SH groups in <i>S</i> -acetyl-YADH	
Incubation time	<i>S</i> -Acetyl groups formed	Incubation time	<i>S</i> -Acetyl groups remaining
minutes	residues/molecule	minutes	residues/molecule
15	0.8	30	0.3
30	1.5	30	0.2
60	1.9	30	0.2
90	1.9	30	0.2

Acetylation of YADH with DAT was carried out as described in the text, and incubation of *S*-acetyl-YADH solution was performed at 30° for 30 minutes as described in the legend to Fig. 1. Aliquots of the solution were taken out for determination of SH contents.

at 30° to determine the changes in catalytic activity during the incubation. As shown in Fig. 1, the activity was spontaneously restored to the level of the native enzyme within about 30 minutes of incubation.

In Table 1 are presented the results of a study of restoration of SH groups. As shown in the left column of the Table, the number of SH groups acetylated during the reaction with DAT increased as the incubation time was prolonged. In all of samples of YADH acetylated in various degree as presented in the left column of Table 1, almost complete restoration of SH groups was observed upon incubation of each of them in 0.05 M phosphate buffer, pH 6.0, at 30°, as shown in the right column of the Table.

The reactivation and restoration of SH groups were also observed in the case where acetate buffer or citrate buffer, pH 6.0, was used in place of phosphate buffer.

These results give evidence that thiolester bonds in *S*-acetyl-YADH are by no means stable. In general, it is known that thiolester bonds of substances of low molecular weight are stable in weakly acidic pH regions. Upon incubation of a solution of acetyl-SG in 0.05 M phosphate buffer, pH 6.0, (0.3 mg/ml) at 30° for 30 minutes, no increase in SH content resulted from liberation of acetyl groups was observed at all.

2. Effect of Urea on the Instability of Thiolester Bonds in *S*-Acetyl-YADH

In consideration of the effect of urea on denaturation of protein structure, effect of this reagent on the instability of thiolester bonds in *S*-acetyl-YADH was examined.

In an experiment, 1.6 ml of solution of *S*-acetyl-YADH in 0.05 M phosphate buffer, pH 6.0, (1.3 mg of protein) was incubated at 30° for 7 minutes, and then 0.4 ml of 8 M urea solution was added. In this case, as shown in Fig. 2, no further reactivation occurred upon addition of urea. Upon dilution of the reactivation mixture to lower the final concentration of urea to 0.4 M after 28 minutes of incubation, the catalytic activity was progressively restored again.

It was observed in the following experiment that restoration of SH groups was also prevented in the presence of 1.6 M urea. A 0.45 mg sample of *S*-acetyl-YADH, in which 2.0 acetyl groups were acetylated per mole of the protein, was dissolved in 0.8 ml of 0.05 M phosphate buffer, pH 6.0, and 0.2 ml of 8 M

FIG. 2 *Effect of urea on spontaneous reactivation of S-acetyl-YADH*

A 1.6 ml solution of *S*-acetyl-YADH in 0.05 M phosphate buffer, pH 6.0 (1.3 mg of protein) was incubated at 30°. Addition of urea to a final concentration of 1.6 M and subsequent dilution of the incubation mixture to lower the concentration of urea to 0.4 M were carried out at the time indicated by the arrows. The activity was expressed as percentage relative to the specific activity of native YADH.

TABLE 2
Effect of urea on spontaneous restoration of SH groups in *S*-acetyl-YADH

Incubation system	Time	<i>S</i> -Acetyl groups remaining
	minutes	residues/molecule
<i>S</i> -Acetyl-YADH	0	2.0
<i>S</i> -Acetyl-YADH	30	0.8
<i>S</i> -Acetyl-YADH + urea	30	1.6

A 0.45 mg sample of *S*-acetyl-YADH was dissolved in 0.8 ml of 0.05 M phosphate buffer, pH 6.0, and 0.2 ml of 8 M urea solution was added, and the mixture was incubated at 30°. In the control experiment, 0.2 ml of water was added in place of 8 M urea solution.

urea solution was added, and the mixture was incubated at 30°. As shown in Table 2, 1.6 acetylated SH groups out of 2.0 remained unchanged after 30 minutes of incubation, whereas in the control mixture, to which was added 0.2 ml of water in place of 8 M urea solution, acetylated SH groups were restored to the level of only 0.8 free SH residue less than that of the native enzyme.

From these results, it was apparent that thiolester bonds in *S*-acetyl-YADH were stabilized in the presence of urea.

It has been reported that, in the presence of 2 M urea, YADH is reversibly dissociated into four subunits in 0.1 M phosphate buffer, pH 8.5 (7). It was also observed in the following experiment that YADH activity was completely inhibited in the presence of 1.6 M urea, in 0.05 M phosphate buffer, pH 6.0, and the inhibition was reduced partially in the presence of 0.4 M urea. The reaction mixture contained 0.1 ml of 1.32 μ M NADH, 0.02 ml of 0.5 M acetaldehyde, 5 μ g of YADH, 0.05 M phosphate buffer, pH 6.0, and urea solution in an appropriate dilution in a final volume of 3.0 ml. Decrease in absorbance at 340 m μ due to oxidation of NADH was followed in the presence or in the absence of urea. In the absence of urea, about 50 % of added NADH was oxidized after 1 minute, and in the presence of urea in a final concentration of 1.6 M, oxidation of NADH was completely inhibited, whereas in the presence of 0.4 M urea about 30 % of added NADH was oxidized.

Considering these facts, it is suggested that a cause of instability of thiolester bonds in *S*-acetyl-YADH lies in the structure of YADH, and their stabilization in the presence of urea is a consequence of a structural change of the protein molecule by urea.

3. Transfer of Acetyl Group to CoA and GSH

The data of Fig. 3 showed that CoA accelerated the reactivation of *S*-acetyl-YADH. In this case, 1.28 mg of *S*-acetyl-YADH was dissolved in 0.3 ml of 0.05 M phosphate buffer, pH 6.0, and incubated at 30°, and subsequently 0.3 ml of 2 mM CoA solution was added.

Similar result was obtained when 0.05 M GSH solution was added in place of 2 mM CoA solution. In the control system it was confirmed that neither CoA nor GSH showed any effect on the native YADH activity under the experimental conditions.

In the subsequent experiment, effect of these compounds on the restoration of SH groups was studied, since the accelerative effect of CoA on the reactivation of *S*-acetyl-

FIG. 3 Effect of CoA on spontaneous reactivation of *S*-acetyl-YADH

A 1.28 mg sample of *S*-acetyl-YADH was dissolved in 0.3 ml of 0.05 M phosphate buffer, pH 6.0, and incubated at 30°, and 0.3 ml of 2 mM CoA solution was added subsequently at the time indicated by the arrow. The activity was expressed as percentage relative to the specific activity of native YADH.

—Δ—, *S*-Acetyl-YADH; —▲—, *S*-Acetyl-YADH + CoA.

TABLE 3
Effect of CoA on spontaneous restoration of SH groups in S-acetyl-YADH

Incubation system	Time	Relative specific activity		S-Acetyl groups remaining
		minutes	per cent	
S-Acetyl-YADH	0		56	2.2
S-Acetyl-YADH	6		64	1.7
S-Acetyl-YADH + CoA	6		85	0.9

A 8.68 mg sample of S-acetyl-YADH was dissolved in 0.5 ml of 0.05 M phosphate buffer, pH 6.0, and 0.5 ml of 4 mM CoA solution was added. After incubation of the mixture for 6 minutes at 30°, the protein was separated by passage through a Sephadex G-25 column in the cold with the use of the above buffer for elution. Aliquots of the effluent were taken out for determination of SH contents and YADH activity.

YADH was expected to be correlated with the restoration of SH groups lost by the acetylation. In Table 3, 8.68 mg of S-acetyl-YADH was dissolved in 0.5 ml of 0.05 M phosphate buffer, pH 6.0, and 0.5 ml of 4 mM CoA solution was added. After incubation of the mixture for 6 minutes at 30°, the protein was separated by passage through a Sephadex G-25 column in the cold with the use of the above buffer for elution, and aliquots of the effluent were taken out for determination of SH contents and YADH activity. The results indicated that in the presence of CoA, 1.3 SH groups were restored, whereas in the absence of CoA only 0.5 SH group was restored. Therefore it was apparent that the restoration of SH groups was also accelerated by CoA.

These accelerative effects of CoA on the reactivation of S-acetyl-YADH and restoration of SH groups acetylated, strongly support the possibility that acetyl groups combined with SH groups in YADH are transferred to CoA or GSH. Therefore an attempt to verify the formation of acetyl-CoA or acetyl-SG as the result of the above reaction was made.

Arylamine acetyltransferase catalyzes the transfer of acetyl group from acetyl-CoA to sulfanilamide quantitatively (11). If it is true that acetyl groups bound to SH groups of YADH are transferred to CoA, then acetylation of sulfanilamide is expected to result

from the reaction between S-acetyl-YADH and sulfanilamide in the presence of CoA and arylamine acetyltransferase as shown in Scheme 1.

SCHEME 1 Possible sequence of acetyl transfer

In Table 4, 15 mg of S-acetyl-YADH was dissolved in a mixture of 0.25 ml of 2 mM CoA, 0.05 ml of 10 mM sulfanilamide and 0.1 ml of 1 M Tris-hydrochloric acid buffer, pH 8.3. To the mixture was added 0.1 ml of arylamine acetyltransferase solution and the reaction mixture was incubated for 60 minutes at 30°, and then 0.5 ml of 10 % TCA was added to terminate the reaction. The precipitated protein was removed by filtration and 1.0 ml of the filtrate was diluted 100-fold with water. Aliquots of this solution was taken out for determination of free sulfanilamide by the method of Bratton and Marshall (12). In the control experiment, 10 % TCA was added prior to the addition of sulfanilamide solution. Amounts of acetylsulfanilamide formed were calculated from the difference between the values of the main and the control experiments.

The results showed that in the complete system, formation of 0.1 μ mole of acetylsulfanilamide from S-acetyl-YADH was observed,

TABLE 4
Transfer of acetyl groups from S-acetyl-YADH to CoA

Incubation system	Acetylsulfanilamide formed
	μ mole
Complete	0.10
Arylamine acetyltransferase omitted	0.01
CoA omitted	0.00
S-Acetyl-YADH omitted*	0.01

* Equivalent amount of YADH was added.

A 15 mg sample of S-acetyl-YADH was dissolved in a mixture of 0.25 ml of 2 mM CoA, 0.05 ml of 10 mM sulfanilamide and 0.1 ml of Tris-hydrochloric acid buffer, pH 8.3. Incubation of the mixture with arylamine acetyltransferase and determination of acetylsulfanilamide formed were carried out as described in the text.

whereas in the absence of either *S*-acetyl-YADH, CoA or arylamine acetyltransferase, the amount of acetylsulfanilamide formed was less than 0.01 μ mole. These facts give evidence that acetyl groups combined with SH groups in YADH are transferred to CoA.

In the case where sodium acetate was added in place of *S*-acetyl-YADH, no acetylation of sulfanilamide was observed. Therefore this finding is strong evidence that acetyl-CoA formed by treatment of *S*-acetyl-YADH with CoA is a product of direct transfer of acetyl groups from *S*-acetyl-YADH to CoA, rather than that of indirect transfer, in which acetyl groups bound to SH groups in YADH are once liberated by hydrolysis to form acetic acid which is in turn activated to result in acetyl-CoA formation.

Meanwhile, direct support for the formation of acetyl-SG in the reaction of *S*-acetyl-YADH with GSH was provided by thin layer chromatography.

A 15 mg sample of *S*-acetyl-YADH was dissolved in 1.0 ml of 0.1 M GSH solution in 0.05 M phosphate buffer, pH 6.0, and kept for 3 minutes at 30°. Acetic acid was added to the mixture to bring the pH to 2.6, and then the precipitated protein was removed by centrifugation. The supernatant was applied to a column of Dowex 50 $\times$ 4 (pyridinium form) (1 $\times$ 55 cm), and elution was performed with 0.2 M pyridine-acetic acid buffer, pH 3.1. The first fractions giving a positive ninhydrin test, which corresponded exactly to the fractions containing acetyl-SG when the column was calibrated with the authentic compound, were concentrated under reduced pressure, and the residue was examined by thin layer chromatography on cellulose powder with butanol-pyridine-acetic acid-water (15:10:14:12) as the solvent. The spot was visualized by spraying with ninhydrin solution.

A substance having the R_F value of 0.63, which was identical with that of authentic acetyl-SG, was detected as a product of the above reaction.

4. Transfer of Acetyl Group from Acetyl-SG to CoA

In order to compare the reactivity of acetyl

groups combined with SH groups in YADH and that of acetyl group bound to SH group of GSH, a substance of low molecular weight, rate of acetyl transfer from each of those acetylated compound to CoA was examined.

Acetyl-CoA has absorption maximum at 232 m μ based upon thiolester bond, and it is hydrolyzed to form acetic acid and CoA in the presence of phosphate acetyltransferase and arsenate. Therefore, acetyl-CoA is determined by measurement of the decrease in optical density at 232 m μ that is associated with its hydrolysis by the action of phosphate acetyltransferase and arsenate (13).

A 20 mg sample of *S*-acetyl-YADH was dissolved in 0.5 ml of 2 mM CoA solution and incubated at 30°. The reaction was terminated by addition of 0.04 ml of 4 M perchloric acid, and after 3 minutes 4 M sodium hydroxide solution was added to the mixture to bring the pH to 6.0–7.0.

The mixture was centrifuged, and the precipitate was washed once with 1.0 ml of water. The supernatant and the washing were combined and water was added to make the final volume of 3.0 ml. In a quartz cuvette (1 cm light path) 0.5 ml of the test solution was mixed with 0.15 ml of 0.5 M arsenate buffer, pH 7.0, and water was added to make a total

FIG. 4 Comparative time course of acetyl transfer from *S*-acetyl-YADH and acetyl-SG to CoA

A 20 mg sample of *S*-acetyl-YADH or 0.05 ml of 0.01 M acetyl-SG was incubated with 0.5 ml of 2 mM CoA solution at 30°. Amount of acetyl-CoA formed was determined as described in the text.

—▲—, *S*-Acetyl-YADH + CoA; —△—, Acetyl-SG + CoA.

volume of 2.9 ml. The optical density at 232 m μ was read, and then 0.1 ml of phosphate acetyltransferase solution was added. The total decrease in optical density was measured and the amount of acetyl-CoA was calculated with the use of the molecular extinction coefficient at 232 m μ of 4.5×10^6 cm² mole⁻¹.

In the experiment to test the transfer of acetyl group from acetyl-SG to CoA, 0.05 ml of 0.01 M acetyl-SG, in place of S-acetyl-YADH, was incubated with CoA under the above conditions.

The results presented in Fig. 4 showed that acetyl groups combined with SH groups in YADH were rapidly transferred to CoA, whereas acetyl transfer from acetyl-SG to CoA was not observed.

DISCUSSION

Especially interesting is the fact that acetyl groups combined with SH groups in YADH are transferred to SH group of CoA contrary to acetyl group bound to SH group of GSH, which is inert in the acetyl transfer reaction. Although the reason why acetyl group of S-acetyl-YADH exhibit such an attractive property is obscure and remains to be investigated, one possible explanation would be that YADH molecule is strained stereochemically as a result of acetylation of its SH groups, and S-acetyl-YADH in strained state tends to the native structure leaving out acetyl groups introduced in the molecule.

In this paper, YADH was used as a convenient test object for acetylation of protein containing SH groups. One of important subjects in a subsequent study is to ascertain whether or not the phenomena observed in the work with YADH occur in common in the cases of all kinds of proteins, including enzyme protein, which have SH groups. If the phenomena observed here in fact occur in general in proteins containing SH groups, the above finding would appear to support our hypothesis that in living cells, active acetyl groups are stored as protein-bound acetyl groups, which are produced by acetylation of protein SH groups, and transferred to CoA to yield acetyl-

CoA when it is necessary to generate energy. Conception of storage of active acetyl group deserves special attention, since it seems to be of great significances not only in storage of energy but also in mechanism of detoxication reaction.

Ascertainment whether an enzyme which catalyzes the transfer of acetyl groups combined with protein SH groups to CoA exists or not, and elucidation of mechanism through which acetylation of protein SH groups is brought about in living cells also constitute important subjects and remain under investigation. There is a possibility that malonyl-CoA participates in acetylation of protein SH groups through concomitant decarboxylation. Work in progress may help to elucidate this point.

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