Biochem. J. (1975) **145**, 581–590
Printed in Great Britain

Evidence for a Histidine and a Cysteine Residue in the Substrate-Binding Site of Yeast Alcohol Dehydrogenase

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(*Received* 29 *July* 1974)

1. Yeast alcohol dehydrogenase (EC 1.1.1.1) is inhibited by stoicheiometric concentrations of diethyl pyrocarbonate. The inhibition is due to the acylation of a single histidine residue/monomer (mol.wt. 36000). 2. Alcohol dehydrogenase is also inhibited by stoicheiometric amounts of 5,5′-dithiobis-(2-nitrobenzoate), owing to the modification of a single cysteine residue/monomer. 3. Native alcohol dehydrogenase binds two molecules of reduced coenzyme/molecule of enzyme (mol.wt. 144000). 4. Modification of a single histidine residue/monomer by treatment with diethyl pyrocarbonate prevents the binding of acetamide in the ternary complex, enzyme–NADH–acetamide, but does not prevent the binding of NADH to the enzyme. 5. Modification of a single cysteine residue/monomer does not prevent the binding of acetamide to the ternary complex. After the modification of two thiol groups/monomer by treatment with 5,5′-dithiobis-(2-nitrobenzoate), the capacity of enzyme to bind coenzyme in the ternary complex was virtually abolished. 6. From the results presented in this paper we conclude that at least one histidine and one cysteine residue are closely associated in the substrate-binding site of alcohol dehydrogenase.

In NAD^+-specific dehydrogenases hydride-transfer steps between NAD^+ and NADH and specific substrates proceed by hydride ion transfer and liberation or uptake of a proton to or from the solvent, probably via an ionizing buffering group on the enzyme (Gutfreund, 1971). Rossmann *et al.* (1971) and Adams *et al.* (1973) in their X-ray structure of dogfish lactate dehydrogenase have suggested that the imidazole nitrogen of histidine-195 of the enzyme is sufficiently close to the hydroxyl/carbonyl group of the substrate in the productive ternary complex to perform the role of an ionizing buffering group. It has been shown by chemical methods that histidine is involved in catalytic centres of several dehydrogenases (Woenckhaus *et al.*, 1969; Leskovac, 1969, 1973; Hucho *et al.*, 1973). Further, Woenckhaus *et al.* (1969) and K. Mella & G. Pfleiderer (unpublished work) have isolated peptides of similar composition, by equivalent modification procedures on essential histidine residues, from H_4 and M_4 isoenzymes of pig lactate dehydrogenase (EC 1.1.1.27). If the sequence is homologous in pig H_4 and M_4 isoenzymes, a homology might also be expected for lactate dehydrogenases from different species. If the proposed mechanism of proton transfer in dogfish lactate dehydrogenase is essentially correct, the same mechanism may be operative in many other NAD^+-linked dehydrogenases.

Motivated by this possibility, we decided to search for a specific histidine residue in the active site of yeast alcohol dehydrogenase. We also investigated the role of specific cysteine residues in the catalytic mechanism of the enzyme. All calculations presented in this paper are based on the assumption that the molecule of yeast alcohol dehydrogenase consists of four very similar subunits with a molecular weight of 144000 for the tetramer. This assumed value is almost the mean of the values obtained by Bühner & Sund (1969) (141000) and Dickinson (1972) (149000).

Experimental

Materials

Water. All solutions were prepared with glass-distilled water.

Coenzyme. NADH (grade I for fluorimetry) was purchased from Boehringer G.m.b.H., Mannheim, Germany, and was used without further purification. Its concentration was determined by using the molar extinction coefficient of 6200 litre·mol^{-1}·cm^{-1} at 340nm (Bergmeyer, 1970).

Diethyl pyrocarbonate. Diethyl pyrocarbonate (pure) was purchased from Serva Feinbiochemica, Heidelberg, Germany, as a single batch; the actual purity of the commercial preparation was 50%. The purity of diethyl pyrocarbonate solution was calculated from the increase in extinction at 240nm after reaction of 0.2mM-diethyl pyrocarbonate with 15mM-

histidine in 0.1M-sodium phosphate buffer, pH6.0, by using a molar extinction coefficient for free histidine of 3200 litre·mol^{-1}·cm^{-1} (Ovadi *et al.*, 1967).

Chemicals. All other chemicals were of analytical grade, obtained from commercial sources. Acetamide was recrystallized from water.

Enzyme. Yeast alcohol dehydrogenase (EC 1.1.1.1) was purchased from Boehringer G.m.b.H., and 50mg of freeze-dried enzyme contained 30mg of enzyme, 15mg of sucrose and 5mg of phosphate. Before each experiment the commercial preparation was dialysed against 0.1M-sodium phosphate buffer, pH6.0, 7.0 or 7.5, with 0.3mM-EDTA, or desalted on a Sephadex G-10 column.

Methods

All spectrophotometric measurements were performed in a double-beam spectrophotometer (Carl Zeiss, Jena, model Specord UV VIS), at room temperature in cells with a 1cm light-path. Difference spectra were recorded with two pairs of matched cells, arranged in the blank and sample compartments of the spectrophotometer in the following way: (*a*) blank: cell 1, inhibitor in buffer; cell 2, enzyme in buffer; (*b*) sample: cell 3, buffer; cell 4, enzyme plus inhibitor in buffer. Concentrations of enzyme, inhibitor and buffer were identical in their respective cells.

Enzyme assay. Alcohol dehydrogenase activity was measured spectrophotometrically by the method given in the Boehringer catalogue (1970 edition). The assay mixture contained 20–100μl of enzyme solution, 0.1ml of GSH (9mg/ml), 0.2ml of NAD$^+$ (20mg/ml), 0.1ml of 96% ethanol, 0.1ml of neutralized semicarbazide hydrochloride (25mg/ml) and 2.40–2.48ml of 0.1M-sodium pyrophosphate buffer, pH9.0 (1.67mg of glycine/ml). The initial rate of NADH formation was measured at 340nm. One unit of enzyme activity is defined as the amount of enzyme catalysing the formation of 1μmol of product/min.

Protein determination. The concentration of alcohol dehydrogenase was determined spectrophotometrically, by using $E^{1\%}_{1cm} = 12.6$ at 280nm (Hayes & Velick, 1954).

Reactions with 5,5′-dithiobis-(2-nitrobenzoate). The number and reactivity of thiol groups in native and chemically modified enzyme preparations were determined by the method of Ellman (1959). Total cysteine content of the enzyme was determined by Ellman's (1959) method, in 8M-urea, as described in Table 1. A molar extinction coefficient of 13600 litre·mol^{-1}·cm^{-1} at 412nm was used to calculate extents of reaction with 5,5′-dithiobis-(2-nitrobenzoate) at pH7.5.

Reactions with diethyl pyrocarbonate. The number and reactivity of histidine residues in alcohol dehydrogenase was determined by the spectrophotometric method of Ovadi *et al.* (1967). A molar extinction coefficient of 3200 litre·mol^{-1}·cm^{-1} at 240nm for free histidine was used to calculate the concentration of modified histidines in the enzyme.

Fluorescence titrations. These were performed in an Aminco–Bowman spectrophotofluorimeter, without any modification in the original design of the instrument. All titrations were performed at room temperature and the instrument settings were kept constant in all fluorescence titrations, except for an experiment shown in Fig. 7. Electrical signals from the photomultiplier were recorded on a Rikadenki Kogyo Co. Ltd. recorder. The excitation wavelength was kept constant (340nm) and emission spectra were recorded continuously from 400 to 500nm; fluorescence readings were determined from chart records of emission spectra at 433nm. It was difficult to obtain a reproducible and constant speed for the motor-driven grating in the emission monochromator of the Aminco–Bowman instrument. Therefore we had difficulties in synchronizing chart speed with the speed of the motor-driven grating in the monochromator.

The blank reaction of alcohol dehydrogenase with NADH was not accounted for in our fluorescence measurements for the reasons which are described in the text. Titrations were performed as quickly as possible: usually a titration with 10–15 additions of coenzyme was terminated in 15–20min. The usual procedure was to titrate 2ml of enzyme solution (0.7–5μM) with 10–15 samples (2, 5, 10 and/or 20μl) of NADH (0.1–0.4mM). Enzyme was dissolved in 0.1M-sodium phosphate buffer, pH7.0, with 0.3mM-EDTA, in the absence or presence of 0.3M-acetamide. Fluorescence readings were corrected for small dilutions caused by the addition of coenzyme. No corrections were performed for inner filter effects. Fluorescence readings are given in arbitrary units.

Preparations of native enzyme were titrated after dialysis or immediately after gel filtration. 5,5′-Dithiobis-(2-nitrobenzoate)-modified enzyme preparations were titrated immediately after the removal of excess of 5,5′-dithiobis-(2-nitrobenzoate) by gel filtration. Alcohol dehydrogenase treated with stoicheiometric or substoicheiometric amounts of diethyl pyrocarbonate was titrated directly after completion of the diethyl pyrocarbonate reaction. After each titration of an enzyme sample with NADH, an identical blank without enzyme was titrated in the same range of NADH concentrations. Fluorescence readings for coenzyme were simply subtracted from readings for enzyme, along the titration curve (Fig. 5). From calculated curves obtained in this manner, maximum (final) fluorescence readings on the ordinate scale were measured.

Repetition of experiments and statistical treatment. Unless otherwise stated, each experiment presented

in the Figures, Tables and text was repeated. Maximum differences between experiments never exceeded 10%. For each experiment reported in this paper a result of a single determination only is given. Whenever a linear relationship for a set of experimental points was expected on theoretical grounds, the least-squares method was applied to draw straight lines for graphical representation.

Results

Stability of alcohol dehydrogenase at neutral pH values

Yeast alcohol dehydrogenase is spontaneously and rapidly inactivated in 0.1M-sodium phosphate buffer, pH7.0, in the absence of protecting agents. In the presence of 0.3mM-EDTA, enzyme activity is stable at room temperature for at least several hours. Spontaneous inactivation is also retarded in the presence of 0.3mM-thioglycollic acid in the same buffer (Fig. 1).

Spontaneous inactivation can be stopped at any time by the addition of EDTA to the enzyme (up to a final concentration of 0.3mM) in the same buffer, but no significant reactivation was observed after the addition of EDTA to partially inactivated enzyme preparations. Total cysteine content of our enzyme preparations was determined by the method of Ellman (1959). In three separate determinations the average value of 24 (22–26) thiol groups/molecule of enzyme (mol.wt. 144000) was found, with an average specific activity of 400 (370–430) units/mg for the same preparations.

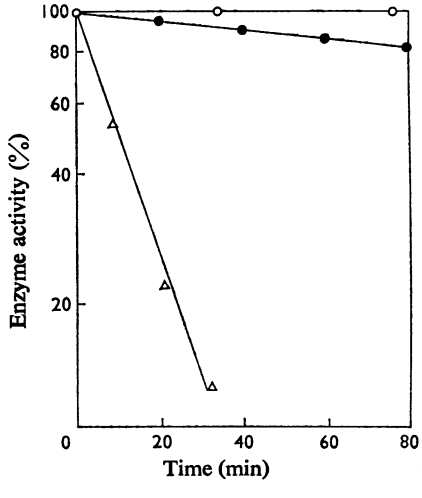

Fig. 1. *Spontaneous inactivation of alcohol dehydrogenase at neutral pH values*

Yeast alcohol dehydrogenase (0.21μM) was incubated at room temperature in three separate tubes, in 0.1M-sodium phosphate buffer, pH7.0, without protecting agent (△), with 0.3mM-EDTA (○) or with 0.3mM-thioglycollic acid (●). Enzyme activities were assayed at the times indicated.

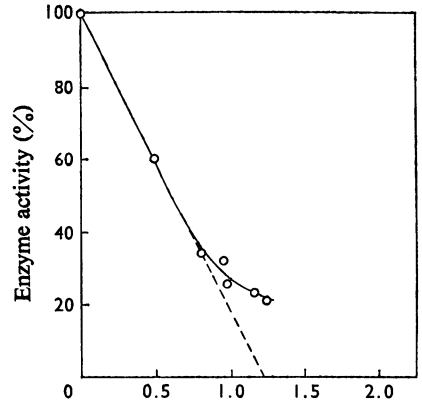

Fig. 2. *Stoicheiometric inhibition of alcohol dehydrogenase with diethyl pyrocarbonate*

Acylation procedure: diethyl pyrocarbonate was added to enzyme samples in stoicheiometric concentrations as indicated. Each sample (2–3ml) of alcohol dehydrogenase (10–20μM) was mixed with a 100μl portion of freshly diluted diethyl pyrocarbonate at room temperature in 0.1M-sodium phosphate buffer, pH6.0, with 0.3mM-EDTA. Enzyme activity was assayed immediately after the completion of the acylation reaction.

Reaction of alcohol dehydrogenase with diethyl pyrocarbonate

Native yeast alcohol dehydrogenase is inhibited in the presence of low concentrations of diethyl pyrocarbonate and 0.3mM-EDTA in 0.1M-sodium phosphate buffer, pH6.0. Reaction of stoicheiometric amounts of diethyl pyrocarbonate with the enzyme brings about almost complete inhibition of enzymic activity (Fig. 2).

Diethyl pyrocarbonate in low concentrations reacts stoicheiometrically and specifically with histidine residues of the enzyme, estimated by the spectrophotometric method of Ovadi *et al.* (1967). Low concentrations of diethyl pyrocarbonate (0.5–1.25mol of diethyl pyrocarbonate/alcohol dehydrogenase subunit, mol.wt. 36000) produce a specific modification of approximately the same amount (0.5–1.25mol) of histidine/subunit, with a concomitant loss of enzymic activity (Fig. 2). This acylation reaction is specific, since all added diethyl pyrocarbonate is accounted for in the formation of ethoxycarbonylhistidine. If the concentrations of added diethyl pyrocarbonate were below 2 equiv. of inhibitor/subunit,

the reaction proceeded at pH 6 with histidine residues only. Under these conditions u.v.-difference spectra of native and diethyl pyrocarbonate-modified enzyme show a characteristic difference spectrum of carbethoxyhistidine only, with a sharp maximum between 238 and 245 nm and no significant difference spectrum in the 260–280 nm region (Ovadi *et al.*, 1967; Mühlrad *et al.*, 1967). Enzyme derivative with one or less modified histidine residue/subunit is stable in 0.1 M-sodium phosphate buffer, pH 6.0, for at least 24 h at +2°C, at the enzyme concentrations indicated in Fig. 2, on the basis of spectrophotometric measurements. If the enzyme is allowed to react with inhibitor concentrations higher than 2 equiv. of diethyl pyrocarbonate/subunit (2–5 equiv./subunit), reactions with both histidine and tyrosine (negative trough between 260 and 290 nm) residues can be observed in the difference spectra. With these higher diethyl pyrocarbonate concentrations an initial faster reaction with histidine residues and subsequent slower migration of carbethoxy groups from histidine to tyrosine residues can be observed by difference spectrophotometry.

It appears from Fig. 2 that the modification of a single histidine residue/subunit is sufficient to abolish completely catalytic activity of the enzyme. Low concentrations of diethyl pyrocarbonate (below 2 equiv./subunit) do not react with thiol groups of native enzyme (Table 1).

Table 1. *Reactivity of thiol groups in native alcohol dehydrogenase towards diethyl pyrocarbonate*

Alcohol dehydrogenase (4 ml; 6 μM), dissolved in 0.1 M-sodium phosphate buffer, pH 6.0, with 0.3 mM-EDTA, was mixed with 50 μl of diethyl pyrocarbonate (6 mM). At indicated times concentrations of diethyl pyrocarbonate-modified histidines in the enzyme were determined spectrophotometrically from the increase in E_{240}. Portions (0.2 ml) of the reaction mixture were removed at indicated times and mixed immediately with 1.8 ml portions of 8 M-urea solution in 0.2 M-sodium phosphate buffer, pH 7.5, containing 0.3 mM-EDTA and 0.5 mM-5,5′-dithiobis-(2-nitrobenzoate). Total concentrations of thiol groups in the enzyme were determined from the increase in E_{412}.

Time after addition of diethyl pyrocarbonate to enzyme (min)	Number of modified histidine residues/subunit (mol.wt. 36000)	Total number of thiol groups/molecule (mol.wt. 144000)
0	—	24.0
0.5	0.4	21.8
5	1.1	23.0
9	1.3	24.0
14	1.5	24.0
20	1.6	23.5

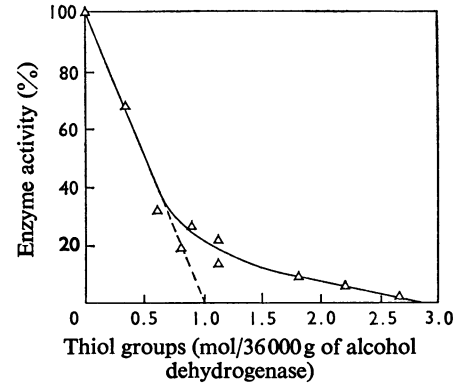

Fig. 3. *Stoicheiometric inhibition of alcohol dehydrogenase with 5,5′-dithiobis-(2-nitrobenzoate)*

Procedure for the calculation of experimental points (△) was as follows. To obtain each experimental point a sample (3–7 μM) of alcohol dehydrogenase in 2.38–3.30 ml was mixed with a small portion of 5,5′-dithiobis-(2-nitrobenzoate) (0.2–1 mM) in 0.12–0.20 ml. 5,5′-Dithiobis-(2-nitrobenzoate) was added in the stoicheiometric concentrations indicated. Enzyme activity was assayed immediately after the completion of the reaction in each sample. Concentrations of 5,5′-dithiobis-(2-nitrobenzoate)-modified thiol groups were determined from the difference spectra between native and 5,5′-dithiobis-(2-nitrobenzoate)-modified alcohol dehydrogenase on chart records of the spectrophotometer at 412 nm. The difference spectrum for each point was obtained with two pairs of cells (1 cm light-path). For each experimental point concentrations of enzyme, 5,5′-dithiobis-(2-nitrobenzoate) and buffer were identical in the respective cells. Blank: cell 1, 5,5′-dithiobis-(2-nitrobenzoate) in buffer; cell 2, alcohol dehydrogenase in buffer. Sample: cell 3, buffer; cell 4, alcohol dehydrogenase + 5,5′-dithiobis-(2-nitrobenzoate) in buffer. The buffer in each case was 0.1 M-sodium phosphate (pH 7.5)–0.3 mM-EDTA.

We conclude, from the experimental data in Table 1, that the essential histidine residue is more reactive towards diethyl pyrocarbonate at pH 6 than free thiol groups in the native enzyme. However, we cannot entirely exclude the possibility that some *S*-ethoxycarbonylcysteine, which may have been formed in the reaction of native alcohol dehydrogenase with diethyl pyrocarbonate at pH 6, was transformed back into free cysteine after the mixing of modified enzyme with 5,5′-dithiobis-(2-nitrobenzoate) at pH 7.5.

Reaction of alcohol dehydrogenase with 5,5′-dithiobis-(2-nitrobenzoate)

Native yeast alcohol dehydrogenase is inhibited by 5,5′-dithiobis-(2-nitrobenzoate) in 0.1 M-sodium phosphate buffer, pH 7.5, in the presence of 0.3 mM-EDTA (Fig. 3).

Inhibition of alcohol dehydrogenase with 5,5'-dithiobis-(2-nitrobenzoate) is biphasic; if the number of 5,5'-dithiobis-(2-nitrobenzoate)-modified cysteine residues of the first, faster inhibition reaction (as a function of enzyme activity) is extrapolated to 100% inhibition, 1.0 modified cysteine residue per subunit is obtained (Fig. 3). This extrapolated value indicates that the modification of a single cysteine residue per subunit completely abolishes the catalytic activity of the enzyme. However, the first faster reaction with 5,5'-dithiobis-(2-nitrobenzoate) proceeds only to 75–80% inhibition, followed by a second slower and less specific inhibition reaction. Since 5,5'-dithiobis-(2-nitrobenzoate) is known to be absolutely specific for free thiol groups in proteins (Ellman, 1959), kinetic analysis of the pseudo-first-order reaction of 5,5'-dithiobis-(2-nitrobenzoate) with thiol groups of native alcohol dehydrogenase was performed by the method of Guggenheim (1926) (Fig. 4).

Kinetic analysis at pH 7.5 reveals that the reaction of thiol groups with 5,5'-dithiobis-(2-nitrobenzoate) was biphasic. The first thiol group/monomer reacts faster (bimolecular rate constant, $k_2 = 570 M^{-1} \cdot min^{-1}$) than the second ($k_2 = 170 M^{-1} \cdot min^{-1}$), under the same experimental conditions. Thus a single rather reactive thiol group/subunit is responsible for maintenance of the catalytic activity of enzyme. If we exclude this first fast-reacting thiol group, we have been unable so far to find any difference among the other slower reacting, 5,5'-dithiobis-(2-nitrobenzoate)-sensitive thiol groups, by the application of simple kinetic or enzymic methods [except for the difference in their reactivity towards 5,5'-dithiobis-(2-nitrobenzoate); V. Leskovac, unpublished work]. These less reactive thiol groups can be differentiated in an indirect fashion, by applying the methods for the determination of coenzyme-binding capacity of modified enzyme (see below).

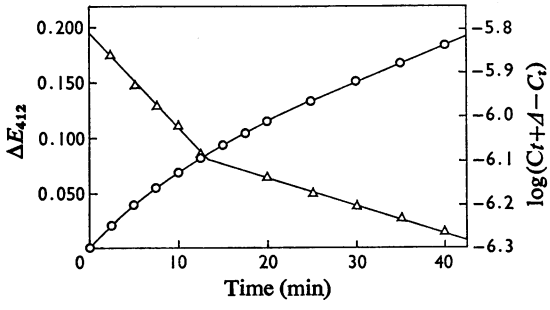

Fig. 4. *Guggenheim (1926) analysis of the pseudo-first-order reaction of 5,5'-dithiobis-(2-nitrobenzoate) with thiol groups in alcohol dehydrogenase*

Chart record at 412 nm of a difference spectrum between native alcohol dehydrogenase and alcohol dehydrogenase reacting with 5,5'-dithiobis-(2-nitrobenzoate), obtained with two pairs of cells (1 cm light-path). Blank: cell 1, 5,5'-dithiobis-(2-nitrobenzoate) (84 μM) in buffer; cell 2, alcohol dehydrogenase (1.86 μM) in buffer. Sample: cell 3, buffer; cell 4, alcohol dehydrogenase (1.86 μM) + 5,5'-dithiobis-(2-nitrobenzoate) (84 μM) in buffer. The buffer in each case was 0.1 M-sodium phosphate (pH 7.5)–0.3 mM-EDTA. Modification of a single thiol group/monomer corresponds to the change in extinction at 412 nm of 0.1 optical unit. Guggenheim (1926) analysis of the reaction is represented by a straight line (△). Bimolecular rate constants (k_2) for the reaction of first and second thiol groups/monomer were calculated from the slope of straight lines.

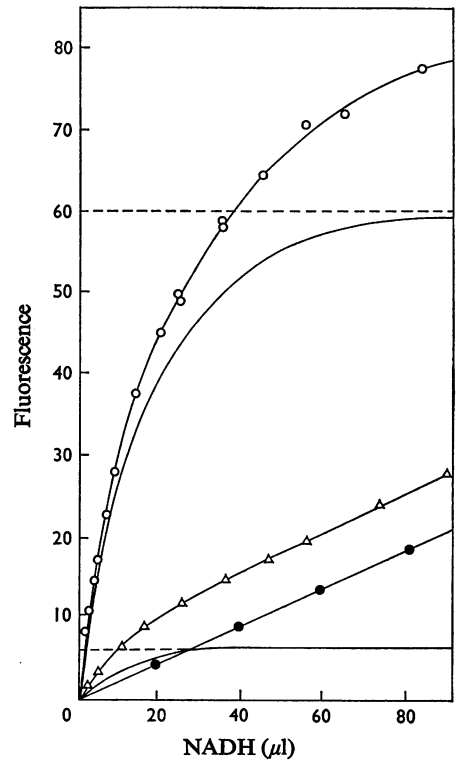

Fig. 5. *Fluorescence titration of native alcohol dehydrogenase with NADH, in the absence (△) and presence (○) of acetamide*

In each experiment the titration curve for NADH (●) has been subtracted from the titration curve for alcohol dehydrogenase (○, △). A calculated curve was obtained (——) which was used for graphical analysis by the method of Scatchard (1949) (Fig. 6). Enzyme concentration was 1.83 μM for both binary and ternary complexes. In each experiment 2 ml samples of alcohol dehydrogenase were titrated with 0.33 mM-NADH in portions that are indicated in the Figure. Acetamide concentration was 0.3 M and buffer was 0.1 M-sodium phosphate (pH 7.0)–0.3 mM-EDTA. Other experimental details are given in the Experimental section.

Binding of reduced coenzyme to the native and modified alchohol dehydrogenase

The model that seems to fit best the foregoing experimental data is one depicting an alcohol dehydrogenase molecule with four active sites, with one histidine and one cysteine residue within each active site. With this model in mind it is relevant to ask whether an active site with a covalently modified cysteine residue has similar properties to an active site with a covalently modified histidine residue. Since both derivatives are inactive, this question was investigated by determining the facility with which binary and ternary complexes between enzyme derivatives and coenzyme and substrate analogue can be formed. Binding of reduced coenzyme to native and modified alcohol dehydrogenase was studied by fluorescence titration, in the absence and presence of the substrate analogue, acetamide. The fluorescence of NADH is enhanced in the presence of the enzyme; it is much more enhanced in the ternary non-productive complex with the enzyme and acetamide (Dickinson, 1970). Fig. 5 shows the results of the fluorescence titration of native enzyme with reduced coenzyme in the presence and absence of acetamide.

From the fluorescence titration curve presented in Fig. 5, applying the graphical method of Scatchard (1949), we have calculated the dissociation constant of NADH from the ternary complex (enzyme–NADH–acetamide) and the coenzyme-binding capacity of alcohol dehydrogenase (Fig. 6).

Native alcohol dehydrogenase binds in 0.1M-sodium phosphate buffer, pH7.0, approx. 2 (1.75) coenzyme molecules/tetramer (mol.wt. 144000).

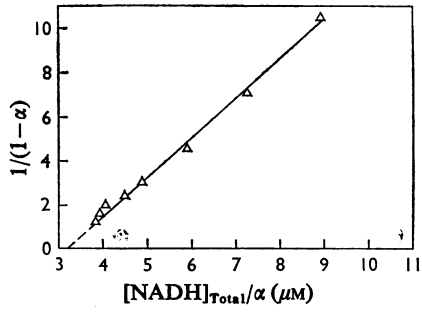

Fig. 6. *Scatchard* (1949) *plot for binding of NADH to alcohol dehydrogenase in the ternary complex*

Scatchard (1949) plot for binding of NADH to the ternary (△) complex has been evaluated from the calculated titration curve for the ternary complex shown in Fig. 5. Final difference reading on the ordinate scale was 60 (----). α is defined as the fraction of the completely liganded enzyme concentration, or the fractional saturation (Gutfreund, 1972). Enzyme concentration and NADH-binding capacity with dissociation constant are shown in Table 2.

Reduced coenzyme is tightly bound to the enzyme in the ternary complex ($K = 0.5\,\mu$M). Enhancement in fluorescence of NADH bound to alcohol dehydrogenase in the presence of acetamide is 920% compared with the enhancement of fluorescence in the absence of acetamide, at the same enzyme concentration (Fig. 5). The value for NADH-binding capacity of enzyme is in accordance with the similar value obtained by Dickinson (1970), under comparable experimental conditions (Table 2). Since the instrument settings and blank titration curves of NADH alone, were identical or very similar in all experiments shown in Table 2, fluorescence yield per μM enzyme for different experiments can be directly compared.

Fluorescence of NADH bound to 5,5′-dithiobis-(2-nitrobenzoate)-modified alcohol dehydrogenase was monitored in several stages of the modification reaction. The NADH-binding capacity and dissociation constant of NADH from the ternary complex is unaffected if 0.8 thiol group/monomer is modified. It appears that the 5,5′-dithiobis-(2-nitrobenzoate)-reactive thiol group per monomer, which is essential for catalytic activity of the enzyme, does not participate in the formation of the ternary non-productive complex with acetamide. If the second thiol group per monomer is modified with 5,5′-dithiobis-(2-nitrobenzoate), the capacity of the enzyme to bind NADH in the ternary complex is virtually abolished (see the Discussion section). Thus a single thiol group per monomer, which is most reactive towards 5,5′-dithiobis-(2-nitrobenzoate) in the native enzyme, can be distinguished from other 5,5′-dithiobis-(2-nitrobenzoate)-reactive thiol groups in several ways: (*a*) it is threefold more reactive towards 5,5′-dithiobis-(2-nitrobenzoate) than other thiol groups; (*b*) its modification with 5,5′-dithiobis-(2-nitrobenzoate) abolishes completely the activity of the enzyme; (*c*) the capacity of the enzyme to form a ternary complex with NADH and acetamide remains virtually unaffected.

To study the binding of NADH to diethyl pyrocarbonate-modified enzyme, 80%-inhibited derivative was prepared, which contained 0.85 ethoxycarbonylhistidine residue/monomer. Although a single histidine residue/monomer is responsible for catalytic activity, in such ligand-binding studies it is always advisable to prepare enzyme derivatives which have less than 0.80–0.85 histidine residue modified/monomer, since a deviation from linear relationship was observed above 85% inhibition (Fig. 2). The diethyl pyrocarbonate–alcohol dehydrogenase derivative, prepared in this way, produces an enhancement in NADH fluorescence in the presence of acetamide, which is comparable in magnitude with that produced by native enzyme in the absence of acetamide (Table 2). From this observation we conclude that the capacity of diethyl pyrocarbonate-modified enzyme to form a ternary complex is completely abolished. Since the formation of binary

Table 2. *Binding of reduced coenzyme to native and chemically modified alcohol dehydrogenase*

Experimental conditions for fluorescence titration of native alcohol dehydrogenase are given in Figs. 5 and 6. Experimental conditions for titrations of modified alcohol dehydrogenase are given in the Experimental section. Under these conditions the fluorescence yield of 1 μM-NADH was 1.44 arbitrary fluorescence units. The maximum fluorescence reading in each experiment shown in the Table is evaluated from the calculated curve for bound enzyme (an example of such a calculation is given in Fig. 5). To obtain maximum fluorescence yield/μM enzyme, maximum fluorescence readings on the ordinate scale were divided by the micromolar concentrations of enzyme.

Enzyme preparation		Dissociation constant of NADH (μM)	NADH-binding capacity per tetramer	Enzyme concentration (μM)	Fluorescence yield per μM enzyme (units/μM)
Native alcohol dehydrogenase	Binary complex	—	—	1.83	3.7
	Ternary complex	0.5	1.75	1.83	33.0
5,5'-Dithiobis-(2-nitrobenzoate)-modified alcohol dehydrogenase; ternary complex with acetamide	0.8 thiol group modified per monomer	0.5	2.10	1.34	34.5
	1.8 thiol groups modified per monomer	—	—	0.73	6.1
	3.0 thiol groups modified per monomer	—	—	1.04	2.9
Diethyl pyrocarbonate-modified alcohol dehydrogenase; 0.85 histidine modified per monomer; 80% inhibition	Binary complex	—	—	4.80	2.0
	Ternary complex	—	—	2.12	6.6

complex still slightly intensifies the fluorescence of NADH, we conclude that diethyl pyrocarbonate-modified enzyme is still capable of binding reduced coenzyme. NADH-binding capacities and NADH-dissociation constants have not been calculated for most modified alcohol dehydrogenase preparations, since a low enhancement of NADH fluorescence which is observed with chemically modified enzyme makes such calculations inaccurate, especially at low enzyme concentrations and in the absence of acetamide.

In order to further investigate the accuracy of our fluorescence titrations we examined blank reactions of enzyme with NADH in the absence and presence of acetamide (Fig. 7).

Concentrations of enzyme, coenzyme and acetamide in the experiment shown in Fig. 7 were comparable with their concentrations in experiments shown in Table 2 and Fig. 5. Decrease in fluorescence in the presence of acetamide at both high (Fig. 7, ●) and low (Fig. 7, ○) concentrations of NADH is less than 5% during the first 15–20 min of reaction. Thus blank reaction between enzyme and NADH in the presence of acetamide is too small to influence the end point in fluorescence titrations. In the absence of acetamide and at low concentration of NADH, decrease in fluorescence owing to the blank reaction is 12% in 15–20 min (Fig. 7, △). This value is lower at higher NADH concentrations. Acetamide at both low and high concentrations of NADH retards the blank reaction of NADH with the enzyme, probably acting as a competitive inhibitor in this reaction. It appears that substantial corrections might be necessary for titrations at high enzyme concentrations in

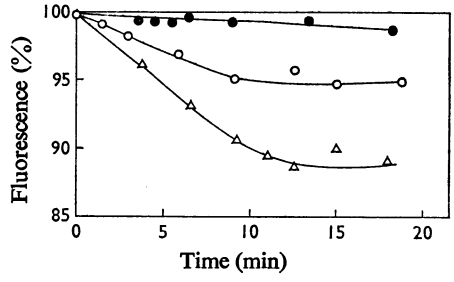

Fig. 7. *Blank reactions between alcohol dehydrogenase and NADH*

Blank reactions of the enzyme with NADH were followed spectrophotofluorimetrically in the presence and in the absence of acetamide in three separate measurements: enzyme (1 μM)+NADH (45 μM)+acetamide (0.3 M) (●); enzyme (1.1 μM)+NADH (7.3 μM)+acetamide (0.3 M) (○); enzyme (1.1 μM)+NADH (7.3 μM) (△). Final volume of test mixtures was 2.03–2.20 ml. Excitation was at 340 nm and emission was measured at 433 nm at the times indicated. Fluorescence signals in three measurements cannot be directly compared, as each measurement was performed with different instrument settings; therefore fluorescence is expressed as a percentage.

the absence of acetamide. However, since the fluorescence of NADH bound to the enzyme is intensified by the presence of acetamide nearly tenfold, such corrections cannot influence the qualitative conclusions about the capacity of native and modified enzyme preparations to form the ternary complex, which we have drawn from the titration data presented in Fig. 5 and Table 2. On the other hand, such corrections are likely to influence the calculation of NADH-binding capacities and K values only at high enzyme concentrations in the absence of acetamide. Therefore blank reactions of alcohol dehydrogenase with NADH were not accounted for in our fluorescence measurements.

Discussion

Yeast alcohol dehydrogenase is a tetramer made up of four identical or nearly identical subunits (Harris, 1964; Pfleiderer & Auricchio, 1964). On the basis of coenzyme binding (Hayes & Velick, 1954), Zn^{2+} binding (Kagi & Vallee, 1960) and specific inactivation of the enzyme by reaction of 4mol of thiol groups/mol of enzyme (Whitehead & Rabin, 1964), it has been concluded that the tetramer contains four active sites. Dickinson (1974), however, has found by three independent methods, that yeast alcohol dehydrogenase has only two binding sites for reduced coenzyme; this author has also found that under certain conditions reaction of 2equiv. of iodoacetate with the alcohol dehydrogenase molecule produces 90–95% loss of activity. The latter observation suggests two essential thiol groups/molecule.

Our estimation of two NADH-binding sites, based on fluorescence titration, is in full agreement with the results obtained by Dickinson (1974). Fluorescence titration curve in Fig. 5 has been analysed by the Scatchard (1949) graphical method over almost the entire saturation range. The lowest point on the Scatchard (1949) plot (Fig. 6) is calculated for approx. 10–15% saturation of enzyme with NADH, and the highest point is calculated for approx. 90–95% saturation. Thus even in the absence of corrections for inner filter effects we feel that our value of NADH-binding capacity and K value are essentially correct. Linearity of the Scatchard plot indicates equivalence and independence of two binding sites of this tetrameric enzyme.

Although the work of Dickinson (1974) indicates the presence of only two essential iodoacetate-reactive thiol groups per tetramer, the sequence work of Harris (1964) on a 21-residue peptide around the iodoacetate-reactive thiol group incidates that this thiol peptide is to be found in each of four subunits. Some time ago we were able to show that the sequence around the essential thiol peptide of pig heart lactate dehydrogenase is similar to the essential thiol peptides of yeast alcohol dehydrogenase, horse liver alcohol dehydrogenase and glyceraldehyde 3-phosphate dehydrogenase (Holbrook *et al.*, 1967). It appears that a sequence around this essential thiol group is conservatively preserved in dehydrogenases. Twu & Wold (1973) have found that butyl isocyanate acts as an active-site-specific reagent for thiol groups in yeast alcohol dehydrogenase, but rather surprisingly the isolated butylcarbamoyl-peptide was found to be different from that isolated from iodoacetate-labelled alcohol dehydrogenase. These authors propose that each active site of yeast alcohol dehydrogenase has two distinct essential thiol groups.

This conclusion is supported by our experiments with 5,5′-dithiobis-(2-nitrobenzoate). After modification with 5,5′-dithiobis-(2-nitrobenzoate) of a single thiol group per monomer, the binding properties of alcohol dehydrogenase for coenzyme and substrate analogues remain unaffected, with a concomitant loss of activity. After further modification of thiol groups with 5,5′-dithiobis-(2-nitrobenzoate), the capacity of the enzyme to enhance the fluorescence of bound NADH in the presence of acetamide is greatly decreased. It is interesting to compare this finding with the observation of Twu *et al.* (1973) on the fluorescence properties of iodoacetamide- and butyl isocyanate-modified alcohol dehydrogenase. Fluorescence yield (in the ternary complex with NADH and acetamide and in the binary complex with NADH) of butyl isocyanate–dehydrogenase prepared by these authors is 300% higher than that of the native alcohol dehydrogenase–NADH complex, whereas Dickinson (1972) observed only a 50% higher fluorescence for the carboxamidomethyl-dehydrogenase–NADH complex compared with the unmodified dehydrogenase–NADH complex. Butyl isocyanate is known to label predominantly thiol groups in the sequence: Cys-Ala-Gly-Ile-Thr-Val (Twu & Wold, 1973). It appears that under the conditions of Dickinsons's (1972) experiment iodoacetate reacts predominantly with the essential thiol groups in the sequence: Cys-His-Thr-Asp-Leu-His-Ala-Trp (Harris, 1964). Twu *et al.* (1973) feel that in their experiments the substituent on the thiol group in the Cys-Ala-Gly sequence has the same effect as free acetamide in forming the proper 'ternary' complex, whereas the substituent on the thiol group in the Cys-His-Thr sequence has no effect on the NADH complex. These observations are in accordance with our finding that enhancement of NADH fluorescence is not abolished after the modification of the first 5,5′-dithiobis-(2-nitrobenzoate)-reactive thiol group per monomer, but only after the modification of the second group. Differences in fluorescence properties of treated enzymes are much more clear-cut in modifications with 5,5′-dithiobis-(2-nitrobenzoate) than they are in modifications with iodoacetamide and butyl isocyanate, because of the absolute specificity of 5,5′-dithiobis-(2-nitrobenzoate) for thiol groups.

Thus it is probable that 5,5'-dithiobis-(2-nitrobenzoate) reacts first with a single -SH group per monomer in the Cys-Ala-Gly sequence. It is difficult to rationalize this inhibitory effect of 5,5'-dithiobis-(2-nitrobenzoate), since the modified enzyme can apparently form a ternary complex. It is possible that this inhibition is due to a failure of the enzyme to catalyse hydride transfer within a ternary complex. The possibility that a covalently bound 3-carboxy-4-nitrophenylmercaptide group can substitute for acetamide as a partner in a highly fluorescent ternary complex can be excluded, since there is no enhancement of fluorescence in the absence of acetamide. In the absence of acetamide, the binary complex NADH–5,5'-dithiobis-(2-nitrobenzoate)-modified dehydrogenase (0.8 thiol group/monomer) has the same low fluorescence yield/μM enzyme as the binary complex NADH–native dehydrogenase (V. Leskovac, unpublished work).

The second 5,5'-dithiobis-(2-nitrobenzoate)-reactive thiol group appears to be in the Cys-His-Thr sequence, the same sequence that is labelled with iodoacetate or iodoacetamide. This modification occurs in or near the substrate-binding site, as the capacity of treated enzyme to bind acetamide is completely abolished. It should be pointed out that Twu *et al.* (1973), who were able to label both active-site thiol groups, came to the conclusion that they are closely associated in the active site. Obviously, the proposition that two 5,5'-dithiobis-(2-nitrobenzoate)-reactive essential thiol groups per monomer are the same essential thiol groups that are reacting with iodoacetate and butyl isocyanate needs further experimental support.

Diethyl pyrocarbonate inhibits specifically and stoicheiometrically the enzyme at pH 6 by modification of a single histidine residue per monomer. We have attempted to estimate the pH-dependence of the bimolecular rate constant (k_2) for this reaction. The extinction at 240nm was displayed on a chart recorder of a self-recording double-beam spectrophotometer, after manual mixing of the enzyme with inhibitor in the cuvette of the spectrophotometer in 0.1M-sodium phosphate buffer, pH 6–7.8. From the chart records the following approximate k_2 values have been calculated: at pH 6, 90$M^{-1}\cdot s^{-1}$; at pH 7, 120$M^{-1}\cdot s^{-1}$; at pH 7.8, over 300$M^{-1}\cdot s^{-1}$. At alkaline pH values the rate of reaction was too fast for manual measurement, and for exact measurement of reaction constants stopped-flow or related techniques are needed. Holbrook & Ingram (1973) have reported that the inhibition of pig heart lactate dehydrogenase with diethyl pyrocarbonate is due to the acylation of a unique histidine residue in the active site, which is tenfold more reactive than free histidine. These authors concluded that there was no reaction with protonated histidine, and calculated the bimolecular rate constant for unprotonated enzyme (214$M^{-1}\cdot s^{-1}$) and free histidine (24$M^{-1}\cdot s^{-1}$). Since the apparent pK_a of free histidine and essential histidine in lactate dehydrogenase was 6.8, the essential histidine residue of the enzyme is abnormally reactive. Obviously, it would also be important, from the mechanistic point of view, to determine the apparent pK_a of an essential histidine residue in yeast alcohol dehydrogenase. Since the modification of an essential histidine prevents binding of acetamide, we assume that this residue is in close vicinity to the substrate-binding site of alcohol dehydrogenase.

In connexion with Fig. 2 it should be pointed out that Dickenson & Dickinson (1973) have reported a very similar pattern of inactivation of yeast alcohol dehydrogenase with diethyl pyrocarbonate. These authors have investigated the inhibition of the enzyme with diethyl pyrocarbonate at pH 7.0, and found that if the number of diethyl pyrocarbonate-modified histidine residues as a function of enzyme activity is extrapolated to 100% inhibition, 1,2 histidine residues/subunit are obtained. Comparing our results (Fig. 2) with those of Dickenson & Dickinson (1973) we conclude that the reaction of diethyl pyrocarbonate with histidine residues of the enzyme is more specific at pH 6 than at pH 7. At pH 6 and nearly 80% inhibition, histidine modification and inhibition are still linearly proportional (Fig. 2), whereas at pH 7 deviation from linearity is starting already below 50% inhibition (Dickenson & Dickinson, 1973). With the aid of fluorescence titration Dickenson & Dickinson (1973) reported a qualitative finding that the enzyme partially modified with diethyl pyrocarbonate cannot form an enzyme–NADH–acetamide complex although it can still bind NADH. This finding is confirmed by our fluorescence titration data in Table 2.

At the present time, the available information on the number of active sites per alcohol dehydrogenase molecule is apparently contradictory. Zn^{2+} binding (Kagi & Vallee, 1960), reaction of thiol groups with 5,5'-dithiobis-(2-nitrobenzoate), reaction of histidines with diethyl pyrocarbonate and structural studies (Harris, 1964) indicate the presence of four very similar subunits with equal catalytic properties. Inhibition with iodoacetate and coenzyme-binding studies (Dickinson, 1974) indicate the presence of two binding sites per molecule. Complete inhibition of enzyme with butyl isocyanate is correlated with the incorporation of 3mol of inhibitor/mol of enzyme (Twu *et al.*, 1973). If yeast alcohol dehydrogenase is made up symmetrically of four similar subunits with only two catalytically active sites, it will raise interesting questions about the mechanism of action of this enzyme. NAD^+-linked dehydrogenases are usually made up of even numbers of subunits, with a number of active sites corresponding to the number of subunits (Sund, 1968). The exception to this rule is yeast aldehyde dehydrogenase with only 2 binding sites/

molecule despite its tetrametic structure (Bradbury & Jakoby, 1971).

From the results presented in this paper, we conclude that at least one histidine and one cysteine residue are closely associated in the substrate-binding site of alcohol dehydrogenase. We are currently planning to investigate the relationship of Zn^{2+} to the active-site histidine and cysteine residues, with the hope of establishing their exact mutual relationship in the catalytic mechanism of yeast alcohol dehydrogenase.

This investigation has been financially supported by the Provincial Research Council of Vojvodina. We sincerely thank Mrs. V. Prodanov for excellent technical assistance and Professor M. Miskovic for permission to use his spectrophotofluorimeter.

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