Studies on the Active-Site Sulfhydryl Groups of Yeast Alcohol Dehydrogenase†

Jer-shung Twu, Christopher C. Q. Chin, and Finn Wold*

ABSTRACT: Yeast alcohol dehydrogenase has previously been shown to contain two distinct active-site sulfhydryl groups, one which reacts specifically with iodoacetate (and by inference also with iodoacetamide) and one which reacts with butyl isocyanate. The characteristics of the reactions of these sulfhydryl groups with butyl isocyanate and with iodoacetamide and some properties of the inactive derivatives have been studied further. The inactivation of dehydrogenase by the two reagents was compared in parallel experiments and found to be affected very similarly by protective agents such as NAD^+, NADH, NAD^+ + pyrazole, NADH + acetamide. The two sulfhydryl groups per active site cannot both be modified; when one has reacted, the other becomes unreactive towards either reagent, and it is concluded that the two sulfhydryl groups are closely associated in the active site. The reaction with iodoacetamide at pH 6.5 was found to involve both sulfhydryl groups, thus providing further evidence for the existence of two distinct "essential" sulfhydryl groups, and also establishing that the specificity of a given reagent for a given sulfhydryl group is not absolute. Some spectral properties of binary and ternary coenzyme and analog complexes of inactive butylcarbamoyl-dehydrogenase and carboxamidomethyl-dehydrogenase were determined and compared to those of the active enzyme. These properties reveal a striking similarity between the two inactive derivatives; they were both found to bind NADH, but had both lost the site responsible for binding of the second ligand in the formation of ternary complexes such as enzyme–NADH–acetamide, enzyme–NAD^+–pyrazole and enzyme–(pyridinecarbaldehyde–adenine dinucleotide)–hydroxylamine. Based on the fluorescence yield observed with the derivatives, it was concluded that the covalently bound butylcarbamoyl groups or carboxamidomethyl groups partly substituted for acetamide as partners in the enzyme–NADH–acetamide ternary complex.

Yeast alcohol dehydrogenase is a tetramer made up of four identical or very similar subunits (Pfleiderer and Auricchio, 1964; Harris, 1964a). Based on coenzyme binding (Hayes and Velick, 1954), on Zn binding (Kagi and Vallee, 1960), and on the specific inactivation of the enzyme by the reaction of 4 mol of sulfhydryl groups/mol of enzyme (Whitehead and Rabin, 1964), it has been concluded that the tetramer contains four active sites. Dickinson (1970), however, showed that the ternary NADH–acetamide–enzyme complex only formed with three of the active sites, and consequently suggested that negative interaction between sites may make the fourth site essentially inoperative in the normal action of the enzyme. We recently reported (Twu and Wold, 1973) that butyl isocyanate acts as an active-site-specific reagent for yeast alcohol dehydrogenase and in support of Dickinson's hypothesis found that the enzyme was completely inactivated after the incorporation of only 3 mol of reagent/mol of enzyme. The butylcarbamoylation was specific for sulfhydryl groups, but rather surprisingly the isolated butylcarbamoylated peptide was found to be different from that isolated from dehydrogenase inactivated with iodoacetate (Harris, 1964b). Thus, it was proposed that each of the active sites of yeast alcohol dehydrogenase contains two reactive sulfhydryl groups, one which reacts preferentially with butyl isocyanate and appears to reflect the proposed anticooperative interaction (for clarity of discussion this group will be given the designation X) and one which reacts preferentially with iodoacetate and which appears not to be affected by subunit interaction (designated Y).

The present work was undertaken in an attempt to provide direct evidence for this model involving two distinct sulfhydryl groups in the active site of dehydrogenase. The specific questions we would like to answer are the following. How specific is the butyl isocyanate reaction for the X-sulfhydryl groups, and the iodoacetamide reaction for the Y-sulfhydryl group? Do the two reactive sulfhydryl groups react in a mutually exclusive fashion? How similar are the two inactive enzyme derivatives (*S*-carboxamidomethylated enzyme and *S*-butylcarbamoylated enzyme) with respect to binding of coenzymes and other ligands? The results confirm the presence of two distinct sulfhydryl groups and suggest that very similar inactive derivatives are obtained with either sulfhydryl reagent.

Experimental Section

Materials. Yeast alcohol dehydrogenase (crystallized and lyophilized; 400 units/mg), NAD, NADH, and 3-pyridinecarbaldehyde adenine dinucleotide (PAAD) were purchased from Sigma Chemical Co. Acrylamide gel electrophoresis did not reveal any significant impurities in the enzyme preparation used. [^{14}C]Butyl isocyanate (labeled in the carbonyl carbon, 3.25 Ci/mol) and [1-^{14}C]iodoacetamide (12.2 Ci/mol) were obtained from New England Nuclear.

Assays and Methods. The procedures for enzyme assays, protein assays, and amino acid analyses, and determination of radioactivity have been described in the previous paper (Twu and Wold, 1973). The protein degradation and peptide isolation also followed published procedures (Chin and Wold, 1972; Twu and Wold, 1973). All spectrophotometric studies were carried out with a Cary 15 spectrophotometer maintained at constant temperature (25°). Fluorescence measurements were made with a Hitachi Perkin-Elmer fluorescence spectrophotometer, Model MPF-2A also maintained at 25°.

† From the Department of Biochemistry, University of Minnesota, Minneapolis, Minnesota 55455. *Received March 6, 1973.* This work was supported by a U. S. Public Health Service research grant from the National Institutes of Health (GM 15053).

Reaction of Enzyme with Butyl Isocyanate and with Iodoacetamide. The reaction of dehydrogenase with butyl isocyanate at pH 5.7 and 0° has been described (Twu and Wold, 1973). The inactivation with iodoacetamide was carried out under somewhat different conditions, allowing for the much slower reaction with this reagent. The enzyme (20–45 μM) was typically incubated at room temperature in 0.1 M potassium phosphate buffer (pH 6.5) with an excess of iodoacetamide (initial concentration 20–100 mol of reagent/mol of enzyme). At different times an aliquot was removed for the determination of activity in comparison with reagent-free controls and a second aliquot (0.5 ml) was also collected for the determination of reagent incorporation. This second aliquot was subjected to gel filtration on a 1 × 45 cm column of Sephadex G-25 (coarse) using 0.1 M potassium phosphate buffer (pH 6.5) as eluent. The excluded peak was collected and protein and radioactivity were determined. When complete inactivation was achieved (usually after 6–7 hr of incubation), the remainder of the sample was freed of excess reagent by gel filtration. At this stage some insoluble protein was generally encountered (up to 20% of the total sample); this was removed by centrifugation and the clear supernatant was saved for further studies.

Results

A Comparison of the Inactivation of Yeast Alcohol Dehydrogenase by Butyl Isocyanate and Iodoacetamide. Typical inactivation patterns for the two reagents are given in Figure 1. It must be emphasized that the two reactions are run under very different conditions, and therefore are not directly comparable. The reaction with the very unstable butyl isocyanate was carried out at pH 5.7 and at 0°, by successive additions of reagent, while the reaction with iodoacetamide was carried out at pH 6.5 and at 25°, following loss in activity with time after a single addition of reagent at zero time. These were the methods used for the production of inactive enzyme derivatives and it is important to note that although the extrapolated end point of the inactivation curve for butyl isocyanate is at 3 mol/mol of enzyme, completely inactivated samples of enzyme typically contained between 3.5 and 4 mol of reagent incorporated with either reagent. A sample of the carboxamidomethylated enzyme was subjected to exhaustive enzymatic digestion by agarose-bound proteases (Pronase, aminopeptidase M, and prolidase) (Brown and Wold, 1973). The digestion mixture was subjected to amino acid analysis on the long column of the Spinco (Model 120C) amino acid analyzer, and 57% of the total radioactivity was recovered in the position of *S*-carboxamidomethylcysteine (4 min behind Asp) and 28% of the radioactivity in the position of cysteic acid, which also corresponds to the elution position of oxidized *S*-carboxamidomethylcysteine. Although we have no explanation for the extensive oxidation in this case, we feel that this experiment demonstrated that sulfhydryl groups were the primary reaction sites for iodoacetamide under the conditions used.

Since both reagents are considered to be active-site specific, it was deemed important to compare their reactivity toward enzyme protected by coenzyme and substrate analogs. Either NAD^+ or NADH will form moderately stable complexes with the active site of the enzyme, but in the presence of a third component more stable ternary complexes have been observed. Two such systems were explored in this work, the NAD^+ + pyrazole ternary complex described by Theorell and Yonetani (1963) and the NADH + acetamide ternary complex reported by Dickinson (1970). For this comparison

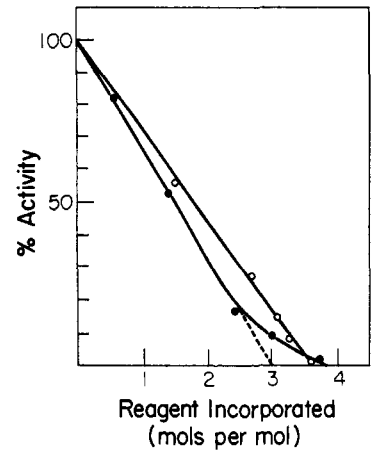

FIGURE 1: The inactivation of yeast alcohol dehydrogenase with butyl isocyanate at pH 5.7 and 0° (●) and with iodoacetamide at pH 6.5 and 25° (○). For the isocyanate reaction the enzyme (3×10^{-5} M) in 0.1 M potassium phosphate buffer (pH 5.7) was treated with successive additions of [^{14}C]butyl isocyanate to a total of 1, 2, 4, 10, and 20 mol of reagent per mol of enzyme. After each addition, the samples were incubated for 15 min and samples were then removed for activity determination and for radioactivity determination and protein assay after gel filtration on Sephadex G-25 to remove excess reagent. For the iodoacetamide reaction the enzyme (4.5×10^{-5} M) in 0.1 M potassium phosphate buffer (pH 6.5) was treated with 30 mol of [^{14}C]iodoacetamide/mol of enzyme. Samples were removed at 35, 95, 155, 215, and 450 min for determination of activity and of radioactivity after gel filtration.

it was felt that the reaction conditions should be as similar as possible and both inactivation reactions were consequently carried out at pH 6.5. The results in Table I reveal a very similar protection pattern for the inactivation by the two reagents. Pyrazole and acetamide by themselves give no protection, NAD^+ and NADH alone give some protection, con-

TABLE I: Effect of NAD^+ + Pyrazole and of NADH + Acetamide on the Inactivation of Yeast Alcohol Dehydrogenase with Butyl Isocyanate and Iodoacetamide.[a]

Protective Agents Added	Act. Remaining (% of Control) after Treatment with	
	Butyl Isocyanate	Iodoacetamide
None	0	0
NAD^+ (5.8×10^{-3} M)	30	19
Pyrazole (1.2×10^{-2} M)	2	0
NAD^+ + pyrazole	98	95
NADH (2.5×10^{-4} M)	32	60
Acetamide (0.5 M)	0	0
NADH + acetamide	68	80

[a] Aliquots of a 5×10^{-6} M enzyme solution in 0.1 M potassium phosphate buffer (pH 6.5), containing the various protective agents as indicated were treated with 20 mol of either butyl isocyanate or iodoacetamide per mol of enzyme. With butyl isocyanate the reactions were run at 0° and the activity was determined after 15 min; with iodoacetamide the reactions were run at 25° and the activity was determined after 7-hr incubation. In both cases the activity was compared to reagent-free controls incubated with the experimental samples.

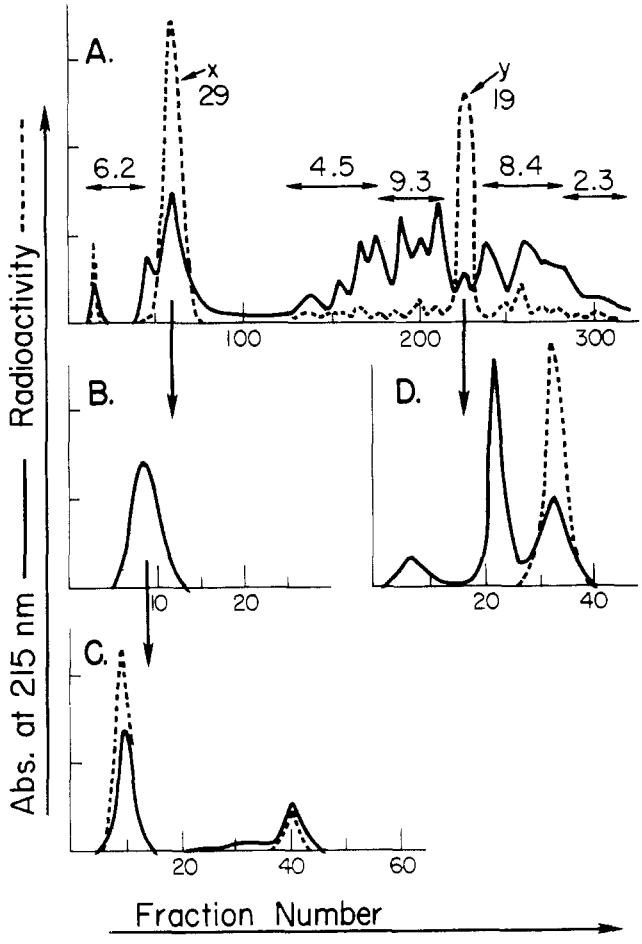

FIGURE 2: The purification of the two major radioactive peptides (X and Y) from a peptic digest of carboxamidomethyl-dehydrogenase. (A) Fractionation of the initial peptic digest on a 1.5 × 40 cm column of phosphocellulose eluted with 0.025 N H_3PO_4 (through fraction 50), then with a three-chamber gradient of 350 ml each of 0.025 N H_3PO_4, 0.01 M KCl in 0.025 N H_3PO_4 and 0.2 M KCl in 0.025 N H_3PO_4 (through fraction 270) and finally with 0.5 M KCl in 0.025 N H_3PO_4 titrated to pH 7.0 with KOH. The two major peaks, designated X and Y contained 29 and 19% of the starting radioactivity, respectively. In addition, minor radioactive components were found throughout the chromatogram as indicated by the percentages given in the figure accounting for a total of 80% of the starting radioactivity. (B) Peak X was lyophilized, desalted on Sephadex G-10, and subjected to chromatography on a TEAE-cellulose column eluted with 0.005 M NH_4OH. A single peak was obtained. (C) The peak from the TEAE column was lyophilized and dissolved in 0.05 M pyridine acetate buffer (pH 3.3) (molarity based on pyridine) and subjected to chromatography on a 1.5 × 45 cm column of Dowex 50W-X2 eluted with the same buffer through fraction 24 and then with a 0.2 M pyridine acetate buffer (pH 4) (molarity based on pyridine). The major peak containing 70% of the radioactivity in the original X-peptide fraction was collected, lyophilized and analyzed as purified X-peptide. (D) The Y-peptide fraction from A was concentrated, desalted on Sephadex G-10 and lyophilized. The dry residue was dissolved in 0.025 N H_3PO_4 and subjected to chromatography on a 0.9 × 77 cm column of phosphocellulose, eluted with 0.025 N H_3PO_4 through fraction 15 and then with 0.085 M KCl in 0.025 N H_3PO_4 through fraction 40. All the radioactivity coincided with the third peak which was collected and analyzed as purified Y-peptide.

TABLE II: Amino Acid Composition[a] of Peptic Peptides X and Y from Carboxamidomethylated Dehydrogenase.

	Molar Ratio			
	X-peptide		Y-peptide	
	Calcd[c]	Found	Calcd[d]	Found
Aspartate	0	0.2	2	2.1
Threonine	1	0.95	1	0.95
Serine	0	0.2	1	0.95
Glutamate	0	0.7	0	0.45
Proline	0	0	0	trace
Glycine	1	1.0	1	1.35
Alanine	1	1.5	0	0.45
$^1/_2$-Cystine[b]	1	1.0	1	0.8
Valine	1	*1.0*	2	*2.0*
Methionine	0	0	0	0
Isoleucine	1	0.85	1	1.0
Leucine	0	0	1	1.05
Tyrosine	0	0	1	0.7
Phenylalanine	0	0	0	0
Lysine	0	0	1	1.0
Histidine	0	0	1	0.5
Arginine	0	0	0	0

[a] Calculated relative to valine. [b] Analyzed as cysteic acid after performic acid oxidation in X-peptide and as *S*-carboxymethylcysteine in Y-peptide. [c] Calculated for the sequence Cys-Ala-Gly-Ile-Thr-Val (Twu and Wold, 1973). [d] Calculated for the sequence (Asp,Ile,Val) Lys-Tyr-Ser-Gly-Val-Cys-His-Thr-Asp-Leu derived from the tryptic peptide-Tyr-Ser-Gly-Val-Cys-His-Thr-Asp-Leu-His-Ala-Trp-His--- (Harris, 1964b) assuming peptic cleavage at the C terminus of Leu and the absence of the tryptic cleavage after Lys. (The four amino acids Asp, Ile, Val, and Lys have been added to the N terminus of the tryptic peptide to account for the observed amino acid composition.)

sistent with their relative affinity for the enzyme, and the combination of NAD^+ and pyrazole or NADH and acetamide greatly enhance this protection. Because of the differences in reaction rates (the butyl isocyanate reaction is probably complete within a minute while the iodoacetamide reaction requires hours) it does not seem reasonable to try to interpret the quantitative differences between the two reactions. Even if there is a real difference in the availability of two different reaction sites (sulfhydryl groups) to the two different reagents, this is certainly a minor one, and the main conclusion from this experiment must be that the sulfhydryl groups are quite similarly exposed to the two reagents, both in the free enzyme and in the enzyme–coenzyme or enzyme–coenzyme–analog complexes.

Isolation of Labeled Peptides from [^{14}C]Carboxamidomethylated Dehydrogenase. Because of the similarity in the inactivation of dehydrogenase by the two reagents and because of the fact that the peptide characterized by Harris (1946b) was obtained from carboxymethylated enzyme, it was felt important to establish whether carboxamidomethylation at pH 6.5 and carboxymethylation at pH 7.5 indeed do involve a common reactive sulfhydryl group (Y), which is distinct from the one which reacts with butyl isocyanate (X). To this end 30 mg of dehydrogenase was inactivated with [^{14}C]iodoacetamide according to the procedure given in Figure 1. After removal of excess reagent, the protein, containing slightly less than 4 mol of reagent/mol of enzyme was digested with pepsin and subjected to purification under the same conditions as those used in the isolation of the butylcarbamoylated peptide (Twu and Wold, 1973). The purification steps are summarized in

Figure 2, and show that two major radioactive peptides were obtained. The amino acid analyses of these two peptides (Table II) strongly suggest that the major one (29%) is identical with the butylcarbamoyl peptide (X), while the lesser one (19%) appears to correspond to the sequence obtained by Harris (1964b) if one allows for the different cleavages with pepsin and trypsin. Thus, the composition of the Y peptide obtained here is consistent with a peptide containing the first nine amino acids of Harris' (1964b) peptide with an additional (Asp,Ile,Val)-Lys tetrapeptide added at the N-terminal end. This tetrapeptide would be expected to be removed by tryptic digestion. This peptide assignment based simply on amino acid composition can only be considered as tentative especially since apparently neither peptide was obtained in pure form. (Both peptides contain significant quantities of Glu, Ala, and Asp which are not accounted for by the suggested sequences.) However, the low Ala content of the Y-peptide and the low Ser and absence of His in the X-peptide eliminate the possibility that the Y-peptide merely is an extension of the X-peptide; and the important conclusion, that the two peptides represent distinct sulfhydryl groups rather than overlapping sequences containing the same sulfhydryl group, should be valid even if the Y-peptide assignment is incorrect.

Can Both Sulfhydryl Groups Be Modified Simultaneously? Even if neither reagent is specific for the X- or the Y-sulfhydryl group it is important to establish whether the inactive butylcarbamoylated enzyme derivative contains another set of sulfhydryl groups available for reaction with iodoacetamide, or conversely whether the carboxamidomethylated enzyme contains another set of sulfhydryl groups which can be modified by butyl isocyanate. To obtain the answer to these questions, dehydrogenase samples were reacted with each of the two reagents (nonradioactive) under the conditions given in Figure 1 except that the butylcarbamoylation was carried out at pH 6.5. The reactions were allowed to procede until complete inactivation was achieved. At this stage the carboxamidomethylated sample was centrifuged to remove insoluble protein, cooled to 0° and treated with 10 mol of [^{14}C]butyl isocyanate per mol of protein. After incubation for 15 min, the reaction mixture was subjected to gel filtration and the radioactivity incorporated was determined. Similarly, the butylcarbamoylated enzyme was treated with 12 mol of [^{14}C]-iodoacetamide/mol of protein for 7 hr at 25°, gel filtered, and analyzed for radioactivity incorporated. The results are given in Table III and clearly show that it is not possible to modify both sets of sulfhydryl groups under the conditions that are used to inactivate the enzyme with each individual reagent.

Comparison of the Properties of S-Butylcarbamoyl-Dehydrogenase and S-Carboxamidomethyl-Dehydrogenase. The model that seems to best fit this data is one depicting an active site with two reactive sulfhydryl groups (designated arbitrarily X and Y) positioned in such a manner that if one reacts, the other is rendered unreactive. One of these sulfhydryl groups (X) appears to react preferentially with butyl isocyanate at pH 5.7 and the other (Y) with iodoacetate at pH 7.5 (Harris, 1964b). At pH 6.5, either the X- or the Y-sulfhydryl group can react with iodoacetamide, with a slight preference for X over Y (3:2). With this model in mind, it is relevant to ask whether an active site with a butylcarbamoyl group covalently linked at the X-sulfhydryl group has the same properties as an active site with a carboxamidomethyl group covalently attached at either the X-sulfhydryl group or the Y-sulfhydryl group. Since both derivatives are inactive, this question had to be explored by determining the facility with which binary and ternary complexes between enzyme derivatives and coenzymes and analogs can be formed. Three such systems were investigated. The first of these is the strongly fluorescent ternary complex formed between dehydrogenase, NADH and acetamide first described by Dickinson (1970), and later used by him to study the properties of carboxamidomethyl-dehydrogenase (Dickinson, 1972). One interesting feature of this system is that it permits one to ask whether the covalently bound "acetamide" or butylcarbamoyl groups in the two derivatives will play the same role as the noncovalently bound acetamide in forming the fluorescent ternary complex. The results of one such an experiment are given in Figures 3 and 4 and show that whereas the two inactive enzyme derivatives give considerably higher fluorescence enhancement than does free dehydrogenase when added to NADH, the further fluorescence increase upon addition of acetamide, signifying the formation of ternary complex, is abolished in the two derivatives. The conclusion of this experiment is that the binding site for acetamide is no longer available in the derivatives, and that the two covalently bound groups (butylcarbamoyl and carboxamidomethyl) only partly behave as does free acetamide in terms of the interaction with the enzyme–NADH complex to give the strongly fluorescent complex. Based on the similarity of the fluorescence spectra of the NADH complexes, it is also concluded that the NADH environment in the two derivatives is very similar.

Two other systems were explored in the comparison of the two dehydrogenase derivatives, the formation of enzyme–PAAD$^+$–hydroxylamine complex (van Eys *et al.*, 1957; Dickinson, 1970, 1972) and the formation of the enzyme–NAD$^+$–pyrazole complex (Theorell and Yonetani, 1963). The results in Figures 5 and 6 show that the two dehydrogenase derivatives have lost the ability to form both ternary complexes. Since coenzyme binding *per se* is not abolished (Dickinson, 1971; Auricchio and Bruni, 1969) by chemical modification of the active site sulfhydryl groups (see also Figures 3 and 4), the explanation for all these findings must be that it is the

TABLE III: Attempts to Incorporate Both Butyl Isocyanate and Iodoacetamide into the Active Site of Yeast Alcohol Dehydrogenase.[a]

Enzyme–Sample Reagent	Act. (%)	Mol of Reagent Incorpd/Mol of Enzyme: [^{14}C]Butyl Isocyanate (A)	Mol of Reagent Incorpd/Mol of Enzyme: [^{14}C]Iodoacetamide (B)
E + none (control)	100		
E + ^{14}C-A	0	3.7	
E + ^{12}C-A	0	0[b]	
E-^{12}C-A + ^{14}C-B	0		1.10
E + ^{14}C-B	0		3.6
E + ^{12}C-B	0		0[b]
E-^{12}C-B + ^{14}C-A	0	0.75	

[a] The experimental details are given in the text. [b] No determination of reagent incorporation was made on these samples. Based on the known incorporation of ^{14}C-labeled reagent under identical conditions (values immediately above), it was assumed that the E–[^{12}C]A derivative contained 3.7 mol of butylcarbamoyl groups/mol of enzyme and that the E–[^{12}C]B derivative contained 3.6 mol of carboxamidomethyl groups/mol of enzyme (Figure 1).

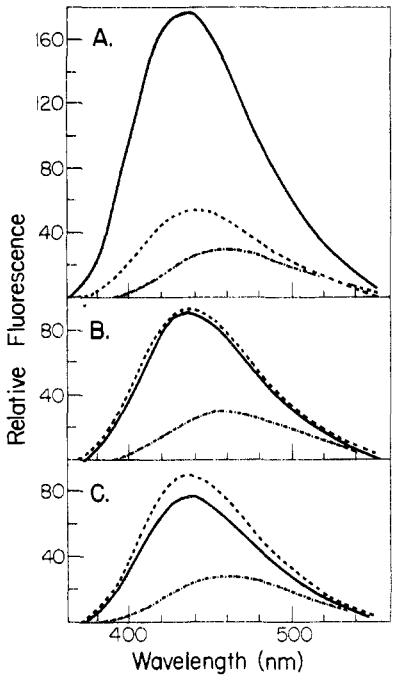

FIGURE 3: Uncorrected fluorescence spectra (activation at 340 nm) of NADH (13. 6 μM) alone (·····), in the presence of 5.5 μM enzyme (-----) and in the presence of 5.5 μM enzyme + 0.5 M acetamide (—) in potassium phosphate buffer (pH 6.5) and at 25°: (A) native enzyme; (B) butylcarbamoyl-enzyme; (C) carboxamidomethyl-enzyme.

binding of the third component (acetamide, hydroxylamine, or pyrazole) that is blocked in the two derivatives. The experiment in Figure 6 also confirms the observation (Twu and Wold, 1973) that the butylcarbamoylation is reversible. After incubating the derivative under conditions which are known to cleave *S*-(butylcarbamoyl)cysteine derivatives, the normal ternary complex could form. As is shown in Figure 7, the reappearance of the ability to complex with NAD^+ and pyrazole paralleled the recovery of catalytic activity. Similar treatment of the carboxamidomethyl derivative gave neither complex formation nor activity recovery. These results are consistent with the known stability of the two derivatives.

Discussion

One of the major problems in planning this work was to design experiments as basis for meaningful comparison of the two derivatives and yet be able to draw as much as possible on earlier studies. In the original work with butyl isocyanate, pH 5.7 was arrived at as the best compromise for efficient inactivation of yeast alcohol dehydrogenase in the active pH range (Twu and Wold, 1973). Although a higher pH can be used, larger quantities of reagent are required as the pH is increased, and above pH 7.0, the inactivation becomes very inefficient because of rapid hydrolysis of the derivative. The labeled peptide (designated X-peptide in this paper), which was isolated and tentatively determined to be Cys-Ala-Gly-Ile-Thr-Val, was obtained from dehydrogenase inactivated with butyl isocyanate at pH 5.7 (Twu and Wold, 1973).

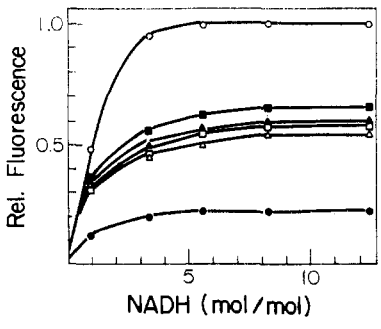

FIGURE 4: Titration of 5 μM native dehydrogenase (○,●) 5 μM butylcarbamoyl-dehydrogenase (△,▲) and 5 μM carboxamidomethyl-dehydrogenase (□,■) with NADH in the absence (filled symbols) and presence (open symbols) of 0.5 M acetamide. The experiments were all carried out in potassium phosphate buffer (pH 6.5) and at 25°, with an activating wavelength of 340 nm. Emission was read at 435 nm.

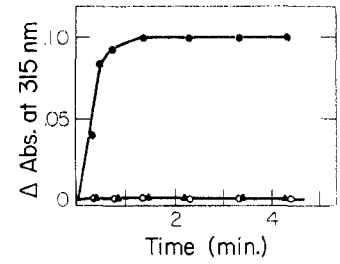

FIGURE 5: Time course for the formation of the ternary complex of enzyme (11 μM) pyridinecarbaldehyde adenine dinucleotide (330 μM) and hydroxylamine (20 mM) in 0.1 M potassium phosphate buffer (pH 6.5) and at 25°: native enzyme (●), butylcarbamoyl-enzyme (○); carboxamidomethyl-enzyme (▲).

The data on the inactivation by alkylation and the characterization of the alkylated derivatives are considerably more complicated. The original determination of the stoichiometry of the reaction of yeast alcohol dehydrogenase with iodoacetate and iodoacetamide was carried out at pH 9, but it was also established that the rate of inactivation with both reagents was independent of pH between 4.5 and 9 (Whitehead and Rabin, 1964; Rabin *et al.*, 1964). The work leading to the isolation of the labeled active-site (tryptic) peptide was carried out with a derivative prepared with [^{14}C]iodoacetate at pH 7.5, and the carboxymethylated sequence which accounted for essentially all the radioactivity of the inactive enzyme was determined to be Tyr-Ser-Gly-Val-Cys*-His-Thr-Asp-Leu-His-Ala-Trp-His-Gly-Asp(Trp,Pro,Leu,Pro,-Thr)Lys (Harris, 1964b). Subsequent studies on the coenzyme binding to inactivated dehydrogenase used a derivative prepared by reaction with iodoacetamide at pH 7.0 (Dickinson, 1972).

In order to carry out the comparison of the effect of coenzymes and substrate analogs as protective agents in the most meaningful manner, we felt that at least one set of inactivation experiments should be carried out at the same pH for both reagents. The upper limit for the isocyanate reaction is at pH 6.5, and that was the pH selected for the protection experiment. For the sake of internal consistency that was also the pH used for all the other reactions of dehydrogenase with iodoacetamide. The butylcarbamoyl derivatives used in the spectral studies were prepared at pH 5.7 according to the original procedure, however (Twu and Wold, 1973).

The results presented in this paper confirm the finding that there are two reactive sulfhydryl groups in or near the active sites of yeast alcohol dehydrogenase. The results in Table I show that the pattern of protection by compounds which bind in the active site of dehydrogenase is very similar for the two reagents and thus that the reactive sulfhydryl groups are positioned in very similar environments in both the native enzyme and in the various binary and ternary complexes.

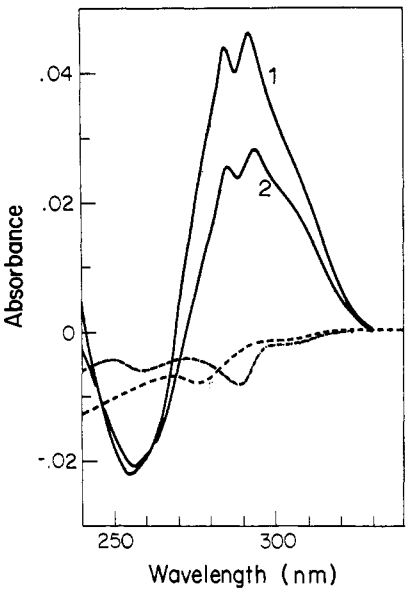

FIGURE 6: Difference spectra in 0.1 M potassium phosphate buffer (pH 6.5) and at 25° of the ternary complex of enzyme (2.8 μM), NAD^+ (30 μM), and pyrazole (2.5 mM) read against the same concentration of enzyme plus NAD^+: with native enzyme (—); butylcarbamoyl-enzyme (----); carboxamidomethyl enzyme (-···-) and the same butylcarbamoyl-enzyme sample after 24-hr incubation at room temperature (—2).

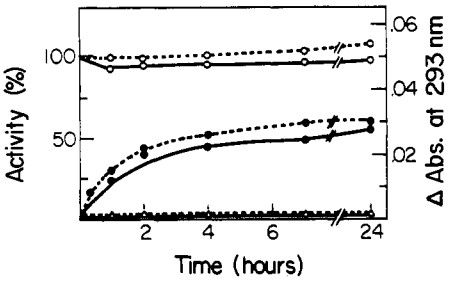

FIGURE 7: The time course of reactivation (solid lines) and recovery of 293-nm absorbance (ability to form ternary complex with NAD^+ and pyrazole) (dotted lines) of butylcarbamoyl-enzyme (●) and of carboxamidomethyl-enzyme (△) in comparison to native enzyme control (○) during incubation at room temperature and in 0.1 M potassium phosphate buffer (pH 6.5).

This latter conclusion is based on the assumption that the relative reactivity of the X- and Y-sulfhydryl groups is not affected by coenzyme and the second ligand, and that the product obtained with butyl isocyanate at pH 6.5 is primarily X-*S*-(butylcarbamoyl)-enzyme and the product with iodoacetamide has the same distribution of X-*S*- and Y-*S*-carboxamidomethyl-enzyme whether or not the protective agents are present. This point warrants further investigation. If the presence of coenzyme for example should be found to protect one sulfhydryl group specifically, a good deal of information about the role of the two sulfhydryl groups in the dehydrogenase action would become experimentally accessible.

The experiment designed to establish whether both sulfhydryl groups may be modified simultaneously (Table III) is meaningful in a broad sense even without precise knowledge of the reagent distribution at the X- and Y-sulfhydryl groups after the first step of inactivation. The inactivation patterns in Figure 1 clearly show that only one set of sulfhydryl groups reacts readily with either reagent, and the results in Table III unequivocally eliminate the possibility that the second set still is uniquely reactive with the other reagent. This is the expected result if both reagents are indeed active-site specific, which by definition means that their unique specificity requires recognition and binding at an intact, functional active site. Since butyl isocyanate and iodoacetate appear to have a different selectivity for the two available sulfhydryl groups in each active site, it was felt that the possibility of having two noninteracting reactive sulfhydryl groups was real and should be tested. The additional incorporation of about 1 mol of reagent/mol of enzyme in the second step in Table III is undoubtedly due to the nonspecific reaction which has been observed at high reagent concentration (Rabin *et al.*, 1964; Twu and Wold, 1973).

The interpretation of the results obtained from the comparison of the spectral properties would be most meaningful if the exact distribution of each reagent between the two sulfhydryl groups were known. Based on the available data, we can only estimate that the inactive butylcarbamoyl derivative (3.7 mol of reagent/mol of enzyme) actually had the reagent composition X-*S*-(butylcarbamoyl)$_{3.1}$–Y-*S*-(butylcarbamoyl)$_{0.6}$–E (based on the finding that 77% of the recovered radioactivity was associated with the X-peptide and 9–14% of the radioactivity with a peptide eluting in the Y-peptide position (Twu and Wold, 1973)) and that the inactive carboxamidomethyl derivative (3.6 mol of reagent/mol of enzyme) had the composition X-*S*-(carboxamidomethyl)$_{2.2}$–Y-*S*-(carboxamidomethyl)$_{1.4}$–E (based on the observed ratio of 3:2 of the two peptides in Figure 2A). Thus, the difference in distribution of the reagent in the two derivatives is probably not large enough to warrant a comparison of the X and Y positions, and one can only conclude from the experiments in Figures 3–6 that dehydrogenase derivatives with either butylcarbamoyl groups or carboxamidomethyl groups in the active site have very similar properties.

It should be pointed out in this connection that our results on the fluorescence yield of the enzyme–NADH–acetamide complex do not agree quantitatively with those obtained by Dickinson (1972). Dickinson (1972) showed that blocking the active-site sulfhydryls with iodoacetamide at pH 7.0 gave a derivative with an unaltered affinity for NADH, and that acetamide no longer formed a ternary complex with NADH and the enzyme derivative. Qualitatively our results agree with these observations. However, whereas Dickinson observed only 50% higher fluorescence for the carboxamidomethyl-dehydrogenase–NADH complex compared to the unmodified dehydrogenase–NADH complex, our corresponding values for both butylcarbamoyl-dehydrogenase and carboxamidomethyl-dehydrogenase approach a value of 300% (Figure 4). The relative fluorescence yield of dehydrogenase–NADH–acetamide and dehydrogenase–NADH in a ratio of 10:2 (Figure 4) is in excellent agreement with that observed by Dickinson (1972) and we therefore feel that the difference for the carboxamidomethyl-dehydrogenase–NADH complex is real and probably reflects differences in the sulfhydryl groups modified. Thus, if Dickinson's derivative which was prepared at pH 7.0 were primarily the Y-sulfhydryl derivative, the high fluorescence yield in our work for both derivatives, known to involve primarily the X-sulfhydryl group, could suggest that the substituent on the X-sulfhydryl group has the same effect as free acetamide in forming the proper "ternary" complex, whereas the same substituent on the Y-sulfhydryl group has no effect on the NADH complex.

We are currently planning a systematic study of the effect of pH on the reaction specificity of both butyl isocyanate,

iodoacetate, and iodoacetamide for the X and Y sites in yeast alcohol dehydrogenase, with the hope of establishing the respective role of the two sulfhydryl groups in dehydrogenase action.

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Purification, Molecular, and Catalytic Properties of Pyruvate Phosphate Dikinase from the Maize Leaf†

Tatsuo Sugiyama

ABSTRACT: Pyruvate phosphate dikinase, which presumably is involved in photosynthetic C_4 dicarboxylic acid pathway in plant, has been purified to homogeneity from maize leaves. The enzyme has a sedimentation coefficient ($s_{20,w}$) of 8.86 S and a molecular weight, determined by sedimentation equilibrium, of 387,000 daltons. Dissociation of the dikinase and electrophoresis on sodium dodecyl sulfate–polyacrylamide gels yields a single stained protein band which corresponds to a subunit weight of 94,000 daltons. Thus it appears that the native enzyme is composed of four identical or similar polypeptide chains. In the absence of magnesium ion, the tetrameric enzyme molecule appears to reversibly dissociate into dimer having a molecular weight, assessed by Sephadex gel filtration, of approximately 195,000 daltons. The enzyme exhibits the property of cold lability, reversibly losing activity at 0°. The enzyme is able to catalyze a reversible conversion of pyruvate to P-enolpyruvate; the observed equilibrium constant of 204 at pH 7.24. At pH 7.5 and 22°, the native enzyme catalyzes the conversion of 2620 mol of pyruvate to P-enolpyruvate/min per mol of enzyme; under comparable conditions, the rate in the reverse (pyruvate formation) direction is 2420 mol/min per mol of enzyme. The enzyme is stimulated by ammonium ion; at pH 7.5, the activation constants being 0.25 and 5.0 mM for the direction toward P-enolpyruvate synthesis and pyruvate synthesis, respectively.

Pyruvate phosphate dikinase, previously named P-enolpyruvate synthase (Hatch and Slack, 1967, 1968), is a new type of enzyme catalyzing the following reaction: ATP + pyruvate + $P_i \rightleftharpoons$ AMP + P-enolpyruvate + PP_i. The dikinase was extracted originally from monocotyledonous tropical grasses such as maize, sugar cane, and *Sorghum* (Hatch and Slack, 1968). The enzyme has now been found in protozoa (Reeves, 1968), two different species of bacteria (Evans and Wood, 1968, 1971; Reeves *et al.*, 1968), and dicotyledonous plants (Johnson and Hatch, 1968). Recent studies in Wood's laboratory on the enzyme isolated from propionibacteria have produced evidence indicating a tri-(uni,uni) Ping-Pong mechanism involved in the enzyme reaction. This idea was further supported by a successful isolation of phosphoryl- and pyrophosphoryl-enzymes (Evans and Wood, 1968; Milner and Wood, 1972).

In the higher plant, this enzyme is believed to operate in the C_4 dicarboxylic acid pathway of photosynthesis (Hatch and Slack, 1966, 1968, 1969; Slack and Hatch, 1967; Johnson and Hatch, 1968). Moreover, the dikinase activity within the leaves of plants is somehow regulated photochemically. The decay rate of enzyme activity upon transferring the plants from light to darkness is characterized by a first-order reaction with a half-time of 15 min, which is rapidly reactivated upon reexposure of the leaves to light (Slack, 1968). In a subsequent paper, Hatch and Slack (1969) demonstrated that heat-labile component(s) present in crude plant extracts may participate in the regulation of dikinase activity in leaves. These findings and analytical data which reveal the same order of the dikinase activity as that of photosynthetic rates observed for tropical grasses (Hatch and Slack, 1970) support a thesis that this enzyme plays a pivotal role in the carbon dioxide fixation of the plants *via* the C_4 dicarboxylic acid pathway.

These observations on the plant dikinase in relation to its unique regulatory behavior led me to isolate the enzyme in a

† From the Research Institute for Biochemical Regulation, School of Agriculture, Nagoya University, Furocho, Chikusa, Nagoya, Japan. *Received March 16, 1973.*