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Inactivation of Horse Liver Alcohol Dehydrogenase by Modification of Cysteine Residue 174 with Diazonium-1*H*-tetrazole†

David C. Sogin‡ and Bryce V. Plapp*

ABSTRACT: Diazonium-1*H*-tetrazole was tested as a potential active-site-directed reagent for amino acid residues involved in catalysis by alcohol dehydrogenase. In a novel reaction with a protein, diazonium-1*H*-tetrazole inactivated the enzyme selectively, and almost stoichiometrically, by reacting with the sulfur of a cysteine residue, Cys-174. As a model compound, the tetrazole adduct of free cysteine was prepared. Elementary and spectral analyses of the adduct were consistent with the structure 5-tetrazoleazo-*S*-cysteine. The adduct absorbs light with a maximum at 316 nm, and is destroyed by irradiation at this wavelength. The inactivated enzyme still bound NADH as determined by difference spectroscopy, but did not enhance the fluorescence of the bound NADH as did native enzyme. X-ray crystallographic studies of free enzyme have shown that Cys-174 coordinates the zinc at the active site (Eklund, H., Nordström, B., Zeppezauer, E., Söderlund, G., Ohlsson, I., Boiwe, T., and Brändén, C.-I. (1974), *FEBS Lett. 44,* 200–204). The modified enzyme is probably inactive because the large, negatively charged tetrazole ring interferes sterically or electrostatically with the binding of substrates or with hydride transfer.

Several amino acid residues located at the active sites of horse liver alcohol dehydrogenase (EC 1.1.1.1) have been identified by chemical modification studies and by x-ray crystallography, but only the zinc ions at the active sites are thought to be involved in the transfer of hydride ion from ethanol to NAD^+ in the ternary complex (Eklund et al., 1974). Some proposals for the mechanism of alcohol dehydrogenases involve removal of the proton from the hydroxyl group of the alcohol by a basic group, possibly with formation of a zinc alkoxide (Eklund et al., 1974; Wang, 1968; McFarland and Chu, 1975; Mildvan, 1970; Klinman, 1975). Since various pH dependency studies have shown that one or more groups on the liver enzyme with pK values from 6.4 to 7.6 must be unprotonated for maximum activity, the base may be an imidazole (Brooks et al., 1972; Plapp et al., 1973; Shore et al., 1974). To modify such a base, we chose to use diazonium-1*H*-tetrazole, which reacts with histidine and tyrosine residues to form readily identifiable products (Horinishi et al., 1964; Sokolovsky and Vallee, 1966; Takenaka et al., 1969). Furthermore, DHT[1] is similar to tetrazole, which is a competitive inhibitor against ethanol with an inhibition constant of 3.2 mM (Theorell et al., 1969). Thus, DHT might be an active-site-directed inactivator, binding to the enzyme–NAD^+ complex as does pyrazole (Theorell and Yonetani, 1963). Nitrogenous bases, such as imidazole and pyrazole, also bind (weakly) at the zinc site on the enzyme in the absence of NAD^+ (Sigman, 1967); thus, DHT might also bind to and react with free enzyme.

Experimental Procedure

Materials. Chemicals were obtained from the following sources: NAD^+ and NADH for kinetics, Boehringer Mannheim; NAD^+ and NADH, Sigma; cysteine (free base), Cyclo Chemicals; 5-amino-1*H*-tetrazole, Eastman or Aldrich; acetaldehyde, 2-mercaptoethanol, and pyrazole, Eastman; and ethylenimine and all sequencing reagents, Pierce. Horse liver alcohol dehydrogenase was purified by a modification of the procedure of Theorell et al. (1966).

Preparation of Tetrazole Azoenzyme. DHT was freshly prepared and adjusted to pH 2 to 3 with 6 N NaOH (Horinishi et al., 1964). The general modification procedure was to add DHT (2.4 to 250 μM in water) to an equal volume of alcohol dehydrogenase (at a concentration of 1 to 10 mg per ml in 1.0 M potassium phosphate buffer, pH 6.8), while the

† From the Department of Biochemistry, The University of Iowa, Iowa City, Iowa 52242. *Received September 9, 1975.* A preliminary report has appeared (Sogin and Plapp, 1975a). This work was supported by Research Grant No. AA00279 from the U.S. Public Health Service, National Institute on Alcohol Abuse and Alcoholism. The JEOL Sequenator was purchased in part with funds from the National Science Foundation (GB 39084) and the National Institutes of Health (GM 550).
‡ Supported by Training Grant No. GM 550 from the National Institutes of Health and by a predoctoral fellowship from the National Institutes of Mental Health, MH 58593. Present address: The Rockefeller University, New York, N.Y. 10021.

[1] The abbreviation used is: DHT, diazonium-1*H*-tetrazole.

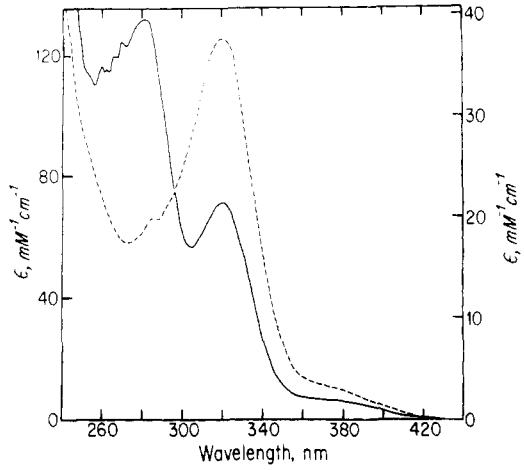


FIGURE 1: Spectra of alcohol dehydrogenase modified with diazonium-1*H*-tetrazole. The spectrum of the product (10% enzyme activity) obtained from reaction with 2.2 mol of DHT per mol (80 000 g) of dimeric enzyme is given by the solid line (read scale on the right). The spectrum of the product (no detectable activity) obtained from the reaction with 19 mol of DHT per mol of enzyme is given by the dashed line (read scale on the left). The extinction coefficients were calculated on the basis of the number of moles of enzyme. The buffer was 33 mM sodium phosphate (pH 8.0).

solution was stirred vigorously with a magnetic stirrer. All solutions were cooled to 0 °C prior to and during the reaction, which was complete in less than 1 min. The concentration of DHT was chosen so that, depending on the purpose of the experiment, 0.1 to 10 equiv of DHT was added per subunit of enzyme. Use of concentrations of protein higher than 10 mg/ml led to less loss of activity for the same amount of incorporation of azotetrazole. For spectral studies and kinetics, the reaction mixture was desalted by gel filtration on a column of Sephadex G-50 equilibrated with 33 mM sodium phosphate buffer (pH 8). In subsequent steps, the tetrazole azoenzyme was protected from light. Variations of this procedure for particular experiments are described in the appropriate figure legends.

Analysis of Proteins. Enzyme activity, protein concentration, and amino acid compositions were determined as described previously (Plapp, 1970; Sogin and Plapp, 1975b). A Cary 118C spectrophotometer was used for spectral measurements and for kinetics. A Perkin-Elmer Hitachi MPF-2A spectrofluorimeter was used for fluorescence measurements.

Synthesis of 5-Tetrazoleazo-2-thioethanol. A solution of DHT was prepared by dissolving 5-amino-1*H*-tetrazole (1.0 g, 10 mmol) in 23 ml of 1.6 N HCl at 0 °C. $NaNO_2$ (0.7 g, 10 mmol) was added with gentle stirring and allowed to dissolve and react for 5 min at 0 °C. Under these conditions, the DHT seemed to be stable, but at a higher temperature or concentration, DHT can explode. 2-Mercaptoethanol (0.7 ml, 10 mmol) was added and allowed to react 5 min. The reaction mixture was extracted with four 70-ml portions of diethyl ether, and the yellow extract was concentrated by evaporation to 20 ml. (The free acid obtained exploded when vacuum distillation was attempted.) Aqueous 6 N NaOH was added until the ether layer began to turn colorless. The pH of the aqueous layer was kept below 6. The ether layer was decanted, and the aqueous layer was washed several times with ether to remove any free acid of the product. Ethanol was added to dissolve any of the precipitated product and the solution was put at −15 °C. After 1 to 2 days the crystals were collected, dried 4 h over P_2O_5 at 65 °C at reduced pressure, and dissolved in a minimum amount of ethanol. The solution was filtered to remove any contaminating salt. The ethanol was removed by evaporation and the residue was recrystallized from water–2-propanol (1:1) heated no higher than 40 °C. The product was dried over P_2O_5 at 25 °C at reduced pressure to give 0.8 g (35%) of the sodium salt of 5-tetrazoleazo-2-thioethanol dihydrate: mp 107–109 °C. Anal. Calcd for $C_3H_9N_6O_3S_1Na$: C, 15.52; H, 3.91; N, 36.19; S, 13.81. Found: C, 15.29; H, 3.82; N, 36.40; S, 14.01.

Synthesis of 5-Tetrazoleazo-S-cysteine. Cysteine (1.21 g, 10 mmol of the free base) was added at 0 °C to the solution of DHT prepared as described above. After 15 min at 0 °C, the product that precipitated was collected and washed with 1 N HCl, H_2O, ethanol, and diethyl ether. The product was dried over P_2O_5 at reduced pressure to give 1.4 g (65%) of 5-tetrazoleazo-*S*-cysteine. Anal. Calcd for $C_4H_7N_7O_2S_1$: C, 22.18; H, 3.25; N, 45.14; S, 14.76. Found: C, 21.96; H, 3.26; N, 44.69; S, 14.90.

When tetrazoleazo-*S*-cysteine was applied directly to a column of Bio-Rad Aminex A-4 resin (0.9 × 50 cm) and developed with the pH 3.25 buffer at 70 ml/h and 50 °C on a Beckman 120C analyzer, it eluted 3 min after cysteic acid, which elutes with the void volume of the column (Spackman et al., 1958). After hydrolysis for 22 h in 6 M HCl at 110 °C, 60% of the 5-tetrazoleazo-*S*-cysteine was converted to half-cystine, and 5 to 20% to cysteic acid. A small peak in the same position as isoleucine had an area 10% the area of the half-cystine peak. No peaks were observed between cysteic acid and half-cystine.

Results

Reaction of DHT with Protein. Reaction of DHT with alcohol dehydrogenase produced a protein derivative with almost no activity and a new absorption peak at 320 nm (Figure 1). When additional molecules of DHT reacted, the absorbance at 320 nm increased, but products absorbing above 400 nm were not formed. Since the ratio of absorbance at 320 nm to 370 nm was constant, it appears that only one type of product formed, even with a 19-fold molar excess of reagent. The spectrum of the more extensively modified derivative in 8 M urea was essentially the same as that in the absence of urea. Furthermore, unlike the spectra for the products of the reaction of DHT with histidine or tyrosine (see below and Sokolovsky and Vallee, 1966; Takenaka et al., 1969), the spectrum of the extensively modified enzyme was essentially unchanged from pH 3 to 10. The modified protein was light sensitive in that intense irradiation at 320 nm decreased its absorbance at 320 nm, but irradiation (at 320 or 385 nm) had no effect on activity. As a precaution, therefore, solutions of modified protein were protected from light.

Model Reactions. Most aromatic diazonium compounds react with amino acids to give azo products that absorb visible light (Means and Feeney, 1971). To identify the amino acid residue on the protein reacting with DHT to give a product absorbing at 320 nm, we determined the spectra of products of DHT reacting with histidine, tyrosine, tryptophan, glycine, lysine, 2-mercaptoethanol, and cysteine. The monoazo and bisazo products of reaction with histidine and tyrosine absorb light at wavelengths greater than 400 nm, but monoazohistidine has no definite peak above 400 nm at pH 8.8 to 10 (Horinishi et al., 1964; Sokolovsky and Vallee, 1966; Takenaka et al., 1969). We found that the spectrum for monoazo-*N*-acetylhistidine at pH 7 had a peak at 350

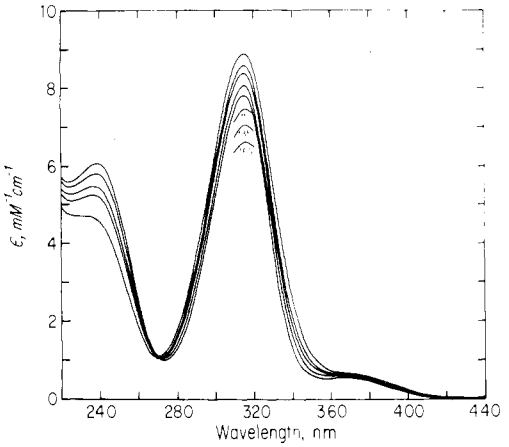


FIGURE 2: Spectra of 5-tetrazoleazo-*S*-cysteine in 50 mM potassium phosphate buffer (pH 6.8) and 0.1 mM EDTA. For the several spectra, the increasing absorbance at 316 nm is due to irradiation at 385 nm in a Hitachi MPF-2A spectrofluorimeter with an excitation slit of 20 nm. The cuvette was placed 9 cm from the exit window of the excitation monochrometer. The cysteine adduct was irradiated for 0 (lowest unnumbered line), 2, 5, 8, and 16 min. After 16 min the absorbance at 316 nm decreased as indicated by the lower, short traces at 110, 135, and 160 min.

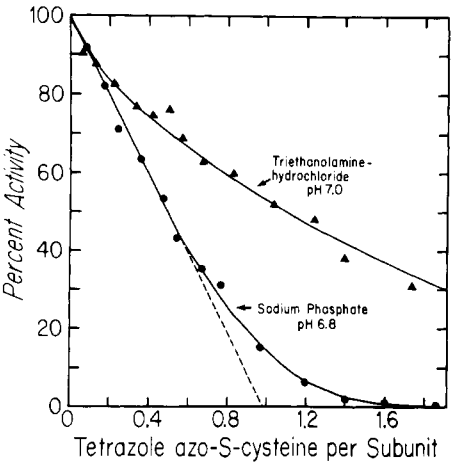
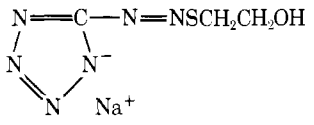


FIGURE 3: Correlation between inactivation of alcohol dehydrogenase and formation of tetrazoleazo-*S*-cysteine. About 1 mg of enzyme in 0.5 ml of 1.0 M triethanolamine hydrochloride buffer (pH 7) (▲) or in 1.0 M potassium phosphate buffer (pH 6.8) (●) was treated with 0.1- to 1.8-fold equiv of DHT (in 0.5 ml of H_2O) at 0 °C. Using an extinction coefficient at 320 nm of 9.1 mM^{-1} cm^{-1}, the formation of tetrazoleazo-*S*-cysteine was calculated from the spectra of the reaction mixtures compared directly to a solution of protein at the same concentration. DHT appears to react almost quantitatively with the protein and does not interfere with the spectral measurements.

nm and, at pH 10, two peaks and increased absorbance at higher wavelengths. Glycine, lysine, and tryptophan did not appear to react. Both thiol compounds reacted (in less than 1 min at pH 7–9 and room temperature) to give products with spectra above 310 nm similar to the spectra for the modified protein (compare Figure 1 to Figure 2). Since 2-mercaptoethanol has no amino or carboxyl groups and gave almost the same spectrum as cysteine, it appears that DHT reacts with the sulfhydryl group. The adduct obtained from 2-mercaptoethanol had an elementary analysis consistent with the following structure:[2]



5-tetrazoleazo-2-thioethanol (sodium salt)

Since it migrates more rapidly than picric acid toward the anode during electrophoresis at neutral pH, the adduct is negatively charged. Other tetrazole compounds have p*K* values in the range of 3–4 for the loss of a proton (Charton, 1969). The elementary analysis of the cysteine adduct was consistent with the analogous structure.

The spectra of both DHT-thiol adducts were affected similarly by irradiation. When 5-tetrazoleazo-*S*-cysteine was dissolved in 20 mM potassium phosphate buffer at pH 6.8 and irradiated at 385 nm the spectra in Figure 2 were obtained: the absorbance at 316 nm increased while the absorbance at 240 nm decreased with an isosbestic point at 265 nm. After the absorbance at 316 nm increased to a maximum, further irradiation at 385 nm caused an exponential loss of absorbance at 316 nm with a half-life of 300 min. The maximum extinction coefficient at 316 nm can be estimated to be 9.7 mM^{-1} cm^{-1}, if it is assumed that the cysteine adduct is converted to a higher absorbing isomer and then destroyed with first-order kinetics. Similar spectral changes were observed for the mercaptoethanol adduct, and an extinction coefficient at 318 nm of 9.7 mM^{-1} cm^{-1} was estimated. For either thiol adduct, when irradiation was stopped, no further changes in absorbance at 316 or 318 nm and no reversion to the original spectrum were observed. The absorbance changes caused by irradiation may be due to photocatalyzed cis–trans isomerism and decomposition (Calvert and Pitts, 1966; Jaffe and Orchin, 1962), but further work is required to substantiate this possibility.

The extinction coefficient used for determining the incorporation of azotetrazole groups into the protein was estimated from the apparent extinction coefficient of a solution of 1 mM cysteine and 0.1 mM DHT that had reacted at the same pH used for the protein modification. The value obtained, 9.1 mM^{-1} cm^{-1} at 316 nm, is probably within 10% of the true value, but it is an estimate because the apparent extinction coefficients of both the cysteine and protein adducts increase upon irradiation at 385 nm. Furthermore, with 2-mercaptoethanol, the apparent extinction coefficient was 8.2 mM^{-1} cm^{-1} at 318 nm.

Various other treatments also caused destruction of the thiol adducts as indicated by loss of absorbance at 316 or 318 nm. The thiol adducts were rapidly destroyed by irradiation at 320 nm (half-life 4 to 5 min) or by exposure to extremes of pH, particularly less than pH 2. Occasionally, solutions of the adducts lost absorbance even in the dark, but this was prevented by the addition of EDTA. In practice, if solutions of the adducts were kept dark and near neutrality, the instability of the adducts presented no difficulties for the work described below.

Incorporation of Azotetrazole into Protein. Extrapolation of the linear portion of a plot of activity remaining against the formation of tetrazoleazocysteine per subunit (lower curve in Figure 3) indicates that the incorporation of one azotetrazole group per active site is sufficient to completely inactivate the enzyme. As seen in the upper curve, 0.5 M triethanolamine-HCl (pH 7) was a poor buffer for obtaining minimum incorporation and maximum inactivation. Chloride anions bind at two sites within the active site (Coleman and Weiner, 1973) and will protect the enzyme against carboxymethylation of cysteine-46 (Eklund et al.,

[2] The structure of the 5-tetrazoleazo-2-thioethanol has been confirmed by x-ray crystallography of a zinc complex; A. Arnone, unpublished results.

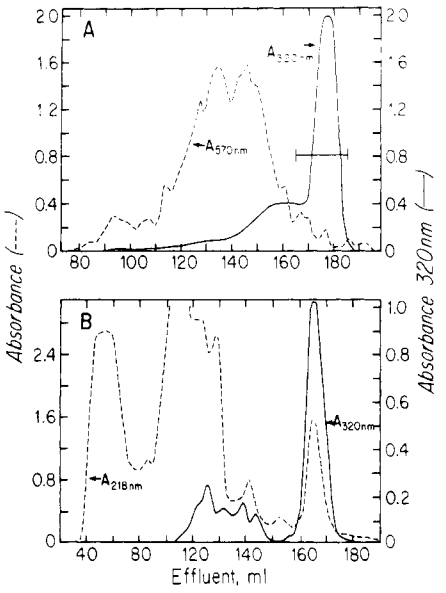


FIGURE 4: Isolation of 5-tetrazole azopeptide. Tetrazole azoenzyme was prepared by the addition of 20 ml of 350 μM DHT to 20 ml of 250 μN enzyme in 0.4 M potassium phosphate buffer (pH 6.8) with vigorous stirring at 0 °C. The azoenzyme was dialyzed against 6 M urea in 0.67 M Tris-HCl buffer (pH 8.6) and aminoethylated by adding 2-mercaptoethanol to a final concentration of 0.1 M, waiting 30 min, and adding ethylenimine to a final concentration of 0.47 M. After 2 h the reaction appeared complete as indicated by a negative nitroprusside reaction (Cole, 1967), and 2-mercaptoethanol was added to a final concentration of 0.7 M to react with the remaining ethylenimine. The reaction mixture was dialyzed against several changes of water to remove salt. S-Aminoethylated tetrazole azoprotein was digested simultaneously with trypsin and chymotrypsin (each totaling 2% of the concentration of protein) at pH 8 and 37 °C in a Radiometer pH stat for 6 h. The mixture of peptides was freeze-dried and dissolved in water adjusted to pH 10 with NH_4OH. (A) The peptides were filtered through a column (1.2 × 144 cm) of Sephadex G-25 developed at room temperature with H_2O adjusted to pH 10 with NH_4OH at a flow rate of 43 ml/h. Absorbance at 320 nm is indicated by a solid line and absorbance at 470 nm due to ninhydrin analysis of alkaline hydrolysates (Fruchter and Crestfield, 1965; Moore, 1968) is indicated by a dashed line. (B) The material from A was applied to a column (1.2 × 20 cm) of Whatman DE-52 DEAE-cellulose and developed with a 500-ml linear gradient from 0 to 1 M KCl with 1 mM Tris-HCl buffer (pH 8) at a flow rate of 58 ml/h. Absorbance at 218 nm is indicated by a dashed line.

1974; Reynolds and McKinley-McKee, 1969). Chloride ion may also protect against reaction with DHT. In experiments such as those in Figure 3, selective inactivation was favored by the low temperature and low pH, which slowed the reaction and allowed more efficient mixing. Even so, the adduct formed within 1 min. (Preliminary studies on the pH dependence of the reaction of DHT with alcohol dehydrogenase, *N*-acetylhistidine, and *N*-acetyltyrosine methyl ester showed sharply increasing rates of reaction above pH 6 and below pH 9.5.)

In an attempt to show that DHT inactivated the enzyme by reacting at the active site, the protective effects of coenzymes and substrate analogues were studied. NAD^+ (0.1 mM) and pyrazole (1 mM) should almost saturate the binding sites and protect the enzyme. Together, however, they only prevented 50% of the inactivation in the reaction of 1 equiv of enzyme with 1.4 equiv of DHT, while the amount of azocysteine formed was the same as in the control. NAD^+ (0.1 mM) alone did not protect. Higher concentrations of NAD^+ (1 mM) or NADH (0.1 mM) protected somewhat, but this was probably due to the reaction of DHT with the free coenzymes.

Table I: Sequence Determination of 5-Tetrazole Azopeptide.[a]

Step	A	nmol	B	nmol	nmol of A plus nmol of B in Previous Step	
1	Leu	228	Ile	100	Leu	228
2	Ile	185	Gly	78	Ile	285
3	Gly	141	Ala[b]	62	Gly	219
4	Ala[b]	17	Gly	81	Ala[b]	79
5	Gly	49	Phe	31	Gly	130
Cup[c]	Phe	61	Gly	91	Phe	92

[a] About 600 nmol of peptide was coupled with 2-amino-1,5-naphthalenedisulfonic acid (to prevent extraction of the peptide (Foster et al., 1973)) and degraded by the Edman procedure in a JEOL JAS 47K automatic sequenator using the standard JEOL short peptide program. Amino acids were determined after each step after hydrolysis in 57% HI for 12 h at 125 °C (Smithies et al., 1971). The amino acid present in the largest amount is indicated in column A and in the second largest amount in column B. Column B appears to foreshadow the amino acid for the next step; in the last column the amino acid present in the largest amount in A was added to the amount in B from the previous step. [b] The alanine probably arises from the 5-tetrazoleazo-*S*-cysteine. Normally, free cysteine is converted to alanine during hydrolysis in HI (Smithies et al., 1971). [c] Total contents of the aqueous phase of step 5 were hydrolyzed 22 h in 6 N HCl at 110 °C. The source of the glycine is unknown; the amino acid composition indicates the presence of only two glycine residues.

Identification of the Cysteine Residue Modified by DHT. The amino acid modified during inactivation of alcohol dehydrogenase by DHT was identified by sequence work on a preparation of enzyme that had 1.2 molecules of azocysteine per subunit and a residual activity of 10%. As described in Figure 4, we isolated a tryptic–chymotryptic peptide in an overall yield of 23% based on the amount of protein digested. The peptide bound tightly to the DEAE-cellulose and was the last peptide to elute. (A similar concentration of salt was required to elute 5-tetrazoleazo-2-thioethanol.) No other peptide absorbing at 320 nm was obtained in excess of 5% of the total amount of material digested. The molar ratios of the amino acids in the major peptide were Gly 2.0, half-Cys 0.5, Ile 1.0, Leu 0.86, and Phe 1.1, which fits the sequence of residues 171–176, Leu-Ile-Gly-Cys-Gly-Phe (Jörnvall, 1970), if a 50% yield of half-cystine from tetrazoleazocysteine is expected. Further analysis by automatic Edman degradation (Table I) indicated that the peptide probably was a mixture of the hexapeptide containing residues 171–176 and the pentapeptide containing residues 172–176. The spectrum of the isolated peptide was very similar to the spectrum of 5-tetrazoleazo-*S*-cysteine. Thus, Cys-174 appears to be the residue modified in the protein.

Mechanism of Inactivation. Tetrazole azoenzyme appears to be completely inactive. The Michaelis and inhibition constants obtained by using product inhibition studies as described previously (Plapp, 1970) at pH 8 and 25 °C for a preparation of enzyme with 1.5 azotetrazole groups incorporated per subunit and 2% residual activity were indistinguishable from native enzyme. If tetrazole azoenzyme had any intrinsic activity, these kinetic constants probably would have differed from native enzyme, as has been found for partially inactivated phosphopyridoxyl alcohol dehydrogenase (Sogin and Plapp, 1975b).

The coenzyme-binding characteristics of the azoenzyme were also studied. In a preparation of azoenzyme with 1.1 cysteines modified per subunit and 20% residual activity, all

of the subunits could bind NADH as measured by difference spectral titrations (Theorell and Yonetani, 1964) where aliquots of NADH were added to the enzyme until the maximum absorbance difference was obtained. The end point occurred when about 90% of the protein was titrated (estimated accuracy ±10%). The final difference spectrum for a titration is shown in Figure 5. This spectrum is similar to that obtained with native (Theorell and Yonetani, 1964) or picolinimidylated enzyme (Plapp et al., 1973); small differences from 300 to 340 nm may be due to perturbation of the chromophoric azotetrazole group. The difference extinction coefficients at 353 nm in the presence or absence of isobutyramide were 2.7 and 2.2 mM^{-1} cm^{-1}, respectively, quite similar to the value of 2.5 mM^{-1} cm^{-1} found for native enzyme. When this azoenzyme was titrated fluorometrically with NADH (Winer and Theorell, 1960), with excitation at 330 nm and emission at 420 nm, the total change in fluorescence occurred when an amount of NADH was added that was equivalent to the concentration of competent sites calculated from the residual activity in a standard assay. Apparently the modified enzyme has somewhat reduced affinity for NADH so that NADH binds first to the residual native enzyme, giving the total change in fluorescence before all of the binding sites are occupied. Similarly, when the azoenzyme was titrated spectrophotometrically with NAD^+ in the presence of pyrazole (Theorell and Yonetani, 1963), the increased absorbance at 300 nm could be accounted for by the number of enzymically competent sites. Since the specific environment of the reduced nicotinamide ring is critical to the enhancement of fluorescence of bound NADH (Sarma and Woronick, 1972), and since pyrazole and NAD^+ must bind together to give the increased absorbance, our results suggest that the binding of the nicotinamide ring or the substrate analogues, or both, is altered in the modified enzyme.

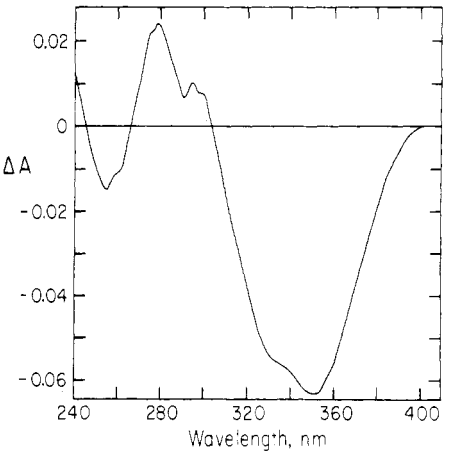


FIGURE 5: Double-difference spectrum for the complex of tetrazole azoenzyme and NADH. In the reference beam, one compartment of the cuvette contained 50 μN tetrazole azoenzyme and the other compartment contained 100 μM NADH and 200 mM isobutyramide. In the sample beam, one compartment contained 50 μN enzyme, 100 μM NADH, and 200 mM isobutyramide, and the other compartment contained buffer alone. All compartments contained 33 mM sodium phosphate buffer (pH 8) with 0.25 mM EDTA. Each compartment had a pathlength of 4.5 mm. The spectrum shown was adjusted for small deviations from the baseline observed before the NADH was added to the compartments. A control experiment showed that addition of NADH to compartments with buffer alone did not alter the baseline.

Discussion

DHT reacts rapidly with thiols to form a relatively stable product. Studies with model compounds indicate that the tetrazoleazothiol structure is more likely than the S-alkylated derivative in which the azothiol group has rearranged with elimination of N_2 as observed in reactions of other diazonium compounds with thiols (Stadler, 1884; Fiedlander and Chwala, 1907; Howard and Wild, 1957). The maximum absorbance band for 5-(methylmercapto)tetrazole is not at 320 nm, but there is a shoulder at 230 nm (Lieber et al., 1959). The elementary analyses indicate that the azocysteine and azomercaptoethanol products retain the diazonium nitrogens.

The reaction of DHT with a sulfhydryl group of a protein to form a relatively stable thiol azo bond is unusual, and our results provide the first evidence on the structure of the product. Shimada previously observed that DHT reacts with myosin and cysteine to produce products that absorbed at 320 nm, but further characterization was not reported (Shimada, 1970). In another protein containing sulfhydryl groups, glutamate dehydrogenase, DHT apparently reacts with amino groups, but the spectrum of the products was not reported (Deppert et al., 1973). Diazotized 3-aminopyridine adenine dinucleotide reacts with sulfhydryl groups in the active sites of yeast alcohol dehydrogenase. Acid hydrolysis of the product yields *S*-3-pyridylcysteine, which apparently forms by rearrangement with elimination of N_2 (Chan and Anderson, 1975).

DHT can react rapidly and almost stoichiometrically with many sulfhydryl groups in liver alcohol dehydrogenase. As shown in Figure 1, reaction of 19 molecules of DHT per molecule of enzyme modified about 14 cysteine residues, without detectable modification of histidine and tyrosine residues. However, reaction of about one DHT per subunit of enzyme selectively modified Cys-174 and inactivated the enzyme. Thus, Cys-174 may be an especially reactive residue or DHT may be an active-site-directed reagent. Unfortunately, the high reactivity of DHT precluded studies in which the rate of inactivation (in the presence or absence of NAD^+) was determined as a function of the DHT concentration, which could demonstrate that DHT has affinity for the enzyme or the enzyme-NAD^+ complex. Nevertheless, the fact that DHT inactivates free enzyme better than it inactivates the enzyme-NAD^+-pyrazole complex, and the observations that the binding sites for the reduced nicotinamide ring and pyrazole are altered in the inactivated azoenzyme suggest to us that DHT probably occupies a position close to the pyrazole (or ethanol) binding sites during the chemical reaction. (That there was some inactivation in the presence of NAD^+ and pyrazole may be due to modification of essential cysteine residues other than Cys-174.)

Recent x-ray crystallographic studies of the apoenzyme have shown that the sulfurs of Cys-46 and -174, the imidazole group of His-67, and a water molecule are coordinated to the zinc in the active site (Eklund et al., 1974). The structure of the enzyme suggests a possible explanation for the selectivity of DHT for Cys-174. Fitting a model of DHT into the three-dimensional structure shows that N-1 of the tetrazole ring could bind to the zinc ion and hold the diazonium group in a position favorable for reaction with the sulfur of Cys-174.[3] (Experimental evidence for the interaction of zinc ions with azo compounds comes from resonance Raman studies of a complex of the enzyme with zincon, which suggest that an azo linkage can bind to the zinc in the

[3] C.-I. Brändén, personal communication.

active site (McFarland et al., 1975). Furthermore, model complexes of zinc and tetrazoleazo-*N*-carbobenzoxytyrosine have been formed and studied (Johansen and Vallee, 1975).) It may be noted that iodoacetate specifically reacts with Cys-46, apparently because the guanidinium group of Arg-47 attracts the carboxylate of the reagent and orients it for reaction (Eklund et al., 1974; Lange et al., 1975). The unusual reactivities of the sulfurs of Cys-46 and Cys-174 may be relevant to the enzymatic mechanism.

Several mechanisms for the action of liver alcohol dehydrogenase have included roles for sulfhydryl groups in catalysis (Sund and Theorell, 1963; Evans and Rabin, 1968). However, the x-ray studies of Eklund et al. (1974) show that "There are no histidines, tryptophans, cysteines, aspartic acids, or glutamic acids that can directly participate in the catalytic action as deduced from the apoenzyme structure." Eklund et al. suggested that a hydroxide ion bound to the zinc could act as a base, but this suggestion is subject to the reservation that the conformation of the enzyme–coenzyme complexes is probably different than that of the apoenzyme (Zeppezauer et al., 1967). Nevertheless, nuclear magnetic resonance (NMR) studies on enzyme in which the zinc is replaced with cobalt also strongly implicate the involvement of water in ternary complexes (Sloan et al., 1975). Our results with DHT provide no new evidence on the role of zinc-bound water, but do raise the possibility that the sulfur of Cys-174 has a role in catalysis. If the zinc-bound sulfur has more nucleophilicity ("hyperreactive" toward DHT) than "normal" sulfhydryls elsewhere on the enzyme, it is conceivable that it could also function as a base. On the other hand, if the selectivity of DHT is due to its affinity for the zinc in the active site, the inherent reactivity of Cys-174 may be normal (or even less than normal) and no role for the residue in catalysis need be postulated.

Modification of Cys-174 with DHT may inactivate the enzyme simply by introducing a large, negatively charged substituent into the substrate binding site where hydride transfer occurs. Reductive alkylation of Lys-228 with pyridoxal phosphate (Sogin and Plapp, 1975b) or carboxymethylation of Cys-46 (Harris, 1964; Li and Vallee, 1964; Reynolds and McKinley-McKee, 1975) also introduces negative charges into the active site (Eklund et al., 1974) and mostly inactivates the enzyme, although significant residual activity remains. Selective alkylation of Cys-174 with the coenzyme analogues, nicotinamide 5-bromoacetyl-4-methylimidazole dinucleotide (Jörnvall et al., 1975a) and [3-(4-bromoacetylpyridino)propyl]adenosine pyrophosphate (Jörnvall, *et al.*, 1975b), also inactivates the liver enzyme, but in this case probably by blocking coenzyme binding. (An examination of the peptide labeling results indicates that DHT is somewhat more selective for Cys-174 than are either of the coenzyme analogues.) Interestingly, both of these coenzyme analogues inactivate yeast alcohol dehydrogenase by alkylating Cys-43, which is homologous to Cys-46 in the liver enzyme. That analogues with the reactive bromoacetyl group on either the adenine or nicotinamide moieties should exhibit such differential selectivity suggests that the active sites differ in certain critical aspects. In this connection, preliminary work indicates that DHT modifies predominantly Cys-43 in the yeast enzyme, although incorporation of two to three molecules of DHT per subunit of enzyme (cf. Figure 3) is required for complete inactivation.[4]

[4] B. V. Plapp, unpublished results.

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Ultraviolet Photoinactivation of Galactosyltransferase. Protection by Substrates†

David J. Clymer, Collis R. Geren, and Kurt E. Ebner*

ABSTRACT: Galactosyltransferase was irreversibly inactivated upon exposure to ultraviolet light and the rate of inactivation followed apparent first-order kinetics. Significant protection against inactivation was observed in the presence of various combinations of substrates. UDPgalactose and Mn^{2+} together gave the most protection. Amino acid analyses revealed the loss of 1 mol of tryptophan per mol of galactosyltransferase upon ultraviolet photoinactivation. Further evidence for an essential tryptophan was provided by difference spectra and by inactivation with 2-hydroxy-5-nitrobenzyl bromide and protection against this reagent by Mn^{2+} and UDPgalactose. The protection by UDPgalactose and Mn^{2+} was greater than that provided by UDPgalactose alone. Since Mn^{2+} provided no protection by itself, this suggested that the formation of the galactosyltransferase–Mn^{2+}–UDPgalactose complex caused a conformational change which was responsible for the observed protection of the essential tryptophanyl residue.

Bovine skim milk galactosyltransferase (UDPgalactose: D-glucose 1-galactosyltransferase, EC 2.4.1.22) catalyzes the transfer of galactose from UDPgalactose, forming β-1,4 linkages with glucose, GlcNAc,[1] or terminal GlcNAc groups of protein-bound β-glycosides (Brew et al., 1968; Schanbacher and Ebner, 1970; Fitzgerald et al., 1970a; Morrison and Ebner, 1971a). α-Lactalbumin is required to obtain significant rates in the reaction when glucose is the galactosyl acceptor, but inhibits the reaction when GlcNAc is the galactosyl acceptor (Schanbacher and Ebner, 1970; Morrison and Ebner, 1971a,b). In lactating mammary tissue, where α-lactalbumin is present in a significant concentration, the principal physiological role of galactosyltransferase is the production of lactose (Watkins and Hassid, 1962). Elsewhere, it is involved in glycoprotein biosynthesis (Schachter et al., 1970).

Based on the results of initial velocity and dead-end inhibition studies with GlcNAc as the galactosyl acceptor, Morrison and Ebner (1971c) have proposed an ordered mechanism for bovine milk galactosyltransferase with reactants adding in the order: Mn^{2+}, UDPgalactose, and GlcNAc. A further conclusion was that Mn^{2+} reacted with the free enzyme under conditions of thermodynamic equilibrium and did not dissociate after each turn of the catalytic cycle.

Magee and Ebner (1974) have demonstrated the existence of a critical sulfhydryl residue in galactosyltransferase by the use of sulfhydryl reagents which caused a distinct

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[1] Abbreviations used: GlcNAc, *N*-acetylglucosamine.