Relation of the Auramine O Binding Site to the Active Site of Horse Liver Alcohol Dehydrogenase*

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ABSTRACT: Auramine O, a diphenylmethane dye, binds to horse liver alcohol dehydrogenase at a location adjacent to the active site. By monitoring changes in auramine O binding and fluorescence properties upon addition of certain active site directed compounds, the location of the dye binding site relative to the active site was defined. A topographical map has been constructed showing the relationship between the binding sites of each of these compounds. The dye binding site overlaps the cyclohexanol binding site but not the ethanol binding site.

Horse liver alcohol dehydrogenase is the first purified enzyme shown to form a fluorescent complex with auramine O (Conrad, 1968). The characteristics of this interaction are: two binding sites per enzyme molecule, association constant of 1.0×10^5 M^{-1}, and noncompetitive inhibition of the enzymatic reaction with respect to both NAD and ethanol (Conrad *et al.*, 1970). Some other fluorescent dyes, including rose bengal and several arylaminonaphthalenesulfonate derivatives, also inhibit the activity of the enzyme, but they bind competitively with NAD (Brand *et al.*, 1967).

In this paper fluorescence, equilibrium dialysis, and inhibition kinetic experiments are described which further define the auramine O binding site on the enzyme. We also show the relationship between the auramine O binding site and the substrate and coenzyme binding sites of the enzyme. This work leads to a topographical mapping of the active-site region of horse liver alcohol dehydrogenase.

Materials and Methods

Crystalline LADH (EC 1.1.1.1) was obtained from C. F. Boehringer, Mannheim, West Germany, and dialyzed against 0.1 M sodium phosphate (pH 7.4) as previously described (Brand *et al.*, 1967). Enzyme concentrations were determined by 280-nm extinction (Sund and Theorell, 1963), NAD–pyrazole absorption titration (Theorell and Yonetani, 1963), and NADH–isobutyramide fluorescence titration (Winer and Theorell, 1960).

NAD, NADH, ADPR,[1] ADP, and AMP were obtained from Sigma Chemical Co., ST. Louis, Mo. Pyrazole was purchased from the Aldrich Chemical Co., Cedar Knolls, N. J. Isobutyramide was a product of Eastman Organic Chemicals, Rochester, N. Y. Potassium chloride was obtained from J. T. Baker Chemical Co., Phillipsburg, N. J. Auramine O was purchased from Allied Chemicals, New York, N. Y., and purified as described previously (Conrad *et al.*, 1970). N^1-Benzylnicotinamide chloride was prepared according to Karrer and Stare (1937). Pharmco USP 200 proof ethanol was obtained from Publicker Industries, Inc., Philadelphia, Pa. The water used for the preparation of all solutions had been passed through deionizing columns supplied by Hydro Service and Supplies, Inc., Durham, N. C.

Fluorescence spectra were obtained with a ratio fluorometer constructed in this laboratory (Witholt and Brand, 1968). Absorption spectra were obtained with a Cary 14 spectrophotometer. Fluorescence kinetics were run on an Aminco-Bowman spectrofluorometer. Reactions were followed by measuring the appearance of NADH by fluorescence with excitation at 340 nm and emission at 460 nm. Absorption of the emitted light by auramine O was corrected.

Equilibrium dialysis experiments were carried out in cells constructed in our machine shop. The capacity of each side of the cells was 2.5 ml; however, the volume used in each side was routinely 2.0 ml. The cells were equilibrated in a shaking water bath calibrated at 25°. Equilibrium was attained within 18 hr. The auramine O concentration was measured both inside and outside the membrane by reading the extinction at 430 nm, which is the isosbestic point between free and bound dye.

Results

The binding of auramine O to horse liver alcohol dehydrogenase (hereafter called dehydrogenase) was measured by means of equilibrium dialysis. Results of these experiments are shown in Figure 1, which indicates the moles of dye bound to dehydrogenase as a function of dye concentration. The middle curve shows the interaction between auramine O and dehydrogenase alone. The upper curve demonstrates enhanced binding in the presence of NAD. We have previously shown (Conrad *et al.*, 1970) that the fluorescence *decreases* when NAD is added to a mixture of auramine O and dehydrogenase. Thus dehydrogenase, auramine O, and NAD appear to form a ternary complex in which the dye binds more tightly than in the absence of coenzyme but with a lower fluorescence yield. The lower curve shows that auramine O does not bind to the ternary complex of dehydrogenase, NAD, and pyrazole.

Figure 2 shows the corrected fluorescence emission spectra of the auramine O–dehydrogenase complex alone (curve 1) and also in the presence of saturating NAD (curve 2) and NADH (curve 3). NAD causes a significant decrease in the

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[1] Abbreviation used is: ADPR, adenosine diphosphate ribose.

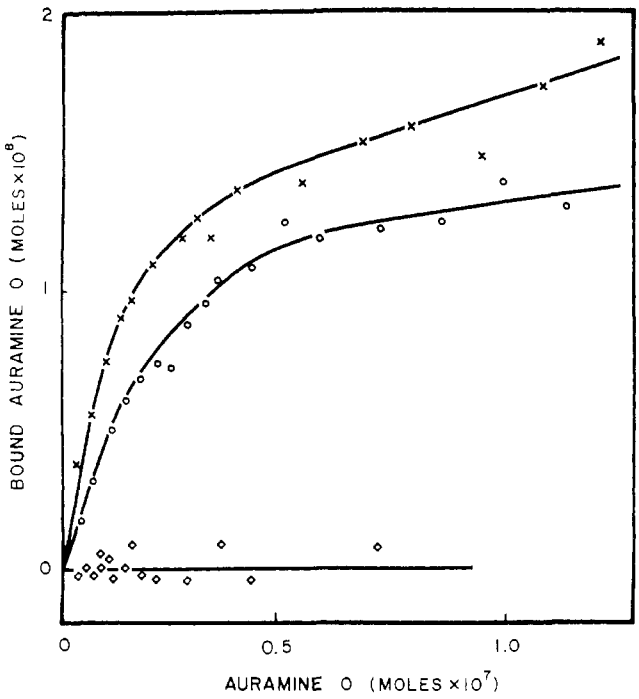

FIGURE 1: Equilibrium dialysis measurement of auramine O binding to three dehydrogenase complexes. Dialysis cells were constructed to contain 2.0-ml total volume on each side of the membrane. One side of the membrane contained 8.56×10^{-9} mole of dehydrogenase. A range from 6.8×10^{-9} mole to 2.8×10^{-7} mole of auramine O in 0.1 M sodium phosphate (pH 7.4) was added to the other side along with the following additions: middle line (○), none; top line (×), 1.10×10^{-6} mole of NAD; bottom line (◊), 1.10×10^{-6} mole of NAD plus 8.0×10^{-5} mole of pyrazole.

TABLE 1: Modifications of Auramine O Binding to Dehydrogenase Due to Presence of Various Additives.

Additive to Auramine O–Dehydrogenase	App $K_a \times 10^{-5}$	Concn of Additive (M)
None	0.90	None
NAD	2.54	6.9×10^{-5}
ADPR	1.05	2.0×10^{-4}
ADP	1.33	1.6×10^{-3}
AMP	2.28	7.1×10^{-4}
Isobutyramide	0.59	9.0×10^{-2}
Pyrazole	0.51	2.0×10^{-2}
N^1-Benzylnicotinamide chloride	0.37	1.0×10^{-2}
KCl	1.07	1.0×10^{-2}
NAD + pyrazole	No binding	6.9×10^{-5} + 1.0×10^{-2}

intensity of the bound dye but no shift in the maximum wavelength. This occurs in spite of the fact that more dye is bound in the presence of NAD. This is shown in Table I which summarizes the results of a series of equilibrium dialysis experiments aimed at studying the effect of NAD, its analogs, and substrate competitive inhibitors on the ability of dehydrogenase to interact with auramine O. The apparent association constant of the dye-enzyme complex is increased almost threefold in the presence of saturating concentrations of NAD. NADH causes only a slight decrease in the fluorescence emission but also causes a definite red shift (2 nm) of the maximum wavelength.

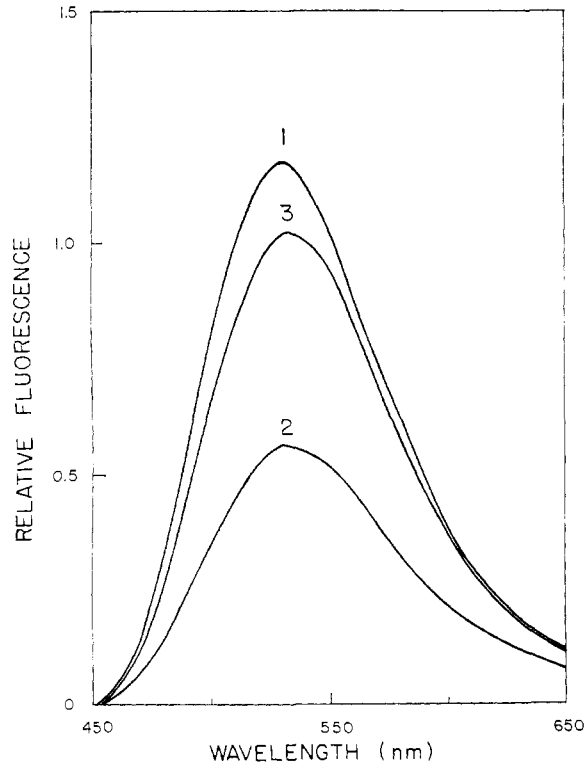

FIGURE 2: Corrected emission spectra of auramine O bound to several enzyme complexes. The curves show the fluorescence of 2.08×10^{-5} M auramine O in the presence of 2.02×10^{-6} M dehydrogenase in 0.1 M sodium phosphate (pH 7.4) and the following additions: curve 1, none; curve 2, 1.48×10^{-4} M NAD; curve 3, 2.56×10^{-5} M NADH. Excitation was at 430 nm.

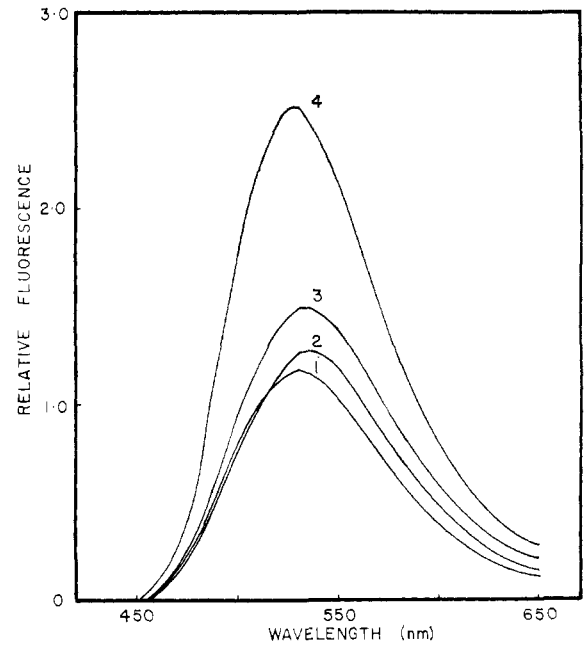

FIGURE 3: Enhancement of the corrected fluorescence emission spectra of the auramine O–dehydrogenase complex in the presence of various adenine-containing compounds. Each curve represents 5.06×10^{-6} M auramine O and 1.80×10^{-6} M dehydrogenase in 0.1 M sodium phosphate (pH 7.4) plus the following additions: curve 1, none; curve 2, 1.84×10^{-4} M ADPR; curve 3, 3.46×10^{-4} M ADP; curve 4, 1.90×10^{-4} M AMP. Excitation was at 430 nm.

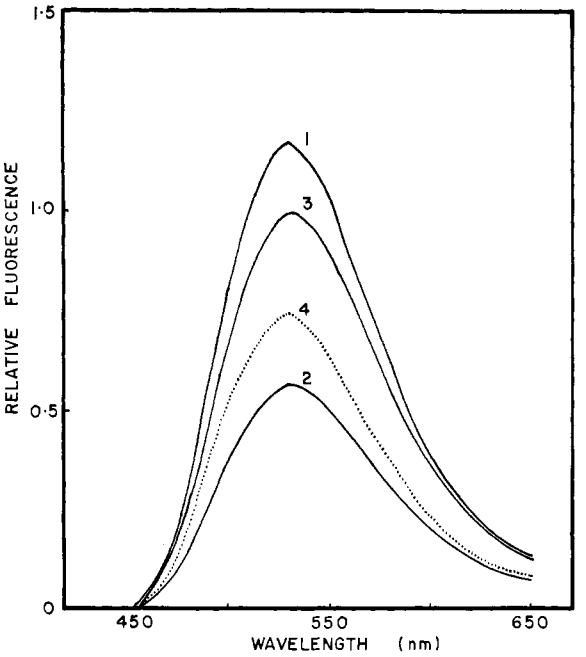

FIGURE 4: Corrected fluorescence emission spectra of the auramine O–dehydrogenase complex in the presence of N^1-benzylnicotinamide chloride. Curve 1 contains 1.80×10^{-6} M dehydrogenase and 5.06×10^{-6} M auramine O in 0.1 M sodium phosphate (pH 7.4). Curve 2 contains an addition of 1.13×10^{-2} M N^1-benzylnicotinamide chloride. Curve 3 contains an addition of 1.13×10^{-2} M potassium chloride. When the effect of potassium chloride is subtracted from the effect of N^1-benzylnicotinamide chloride, the dotted line (curve 4) results. Excitation was at 430 nm.

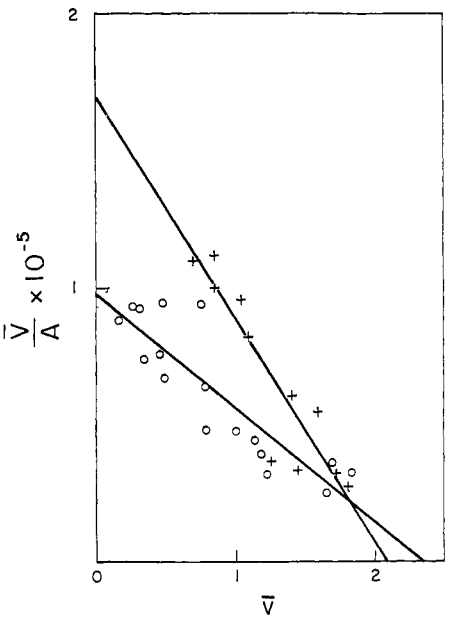

FIGURE 5: Equilibrium dialysis measurement of inhibition of auramine O binding to dehydrogenase by N^1-benzylnicotinamide chloride and potassium chloride. The Scatchard plots were generated in 0.1 M sodium phosphate (pH 7.4) with 3.15×10^{-6} M dehydrogenase–auramine O ranging from 2.5×10^{-6} to 7.5×10^{-5} M, and the following: top line (+), 0.1 M potassium chloride; bottom line (O), 0.1 M N^1-benzylnicotinamide chloride. Lines generated by least-squares analysis.

Since oxidized and reduced coenzyme had significantly different effects on the environment of the bound dye, the effect of coenzyme fragments on the dye fluorescence was also investigated. Figure 3 illustrates the effects of saturating concentrations of some adenine-containing compounds. Curve 1 shows the fluorescence of the auramine O–dehydrogenase complex. In contrast to the situation with NAD, ADPR (curve 2), ADP (curve 3), and AMP (curve 4) enhance the fluorescence.

Although AMP causes no shift in wavelength maximum, there is a 1-nm shift observed with ADP and a 2-nm shift caused by addition of ADPR. This latter effect is similar to the red shift caused by NADH. The effect of saturating concentrations of ADPR, ADP, and AMP on the binding of auramine O as measured by equilibrium dialysis is shown in Table I. The fluorescence enhancement can be explained by the corresponding changes in binding constants shown in Table I.

As is shown in Figure 4, N^1-benzylnicotinamide chloride decreases the fluorescence of a mixture of auramine O and dehydrogenase. Curve 1 is the emission spectrum of the enzyme–dye mixture alone. Curve 2 is the spectrum after addition of N^1-benzylnicotinamide chloride, chloride ion itself (curve 3) decreases the fluorescence. Curve 4 represents the subtraction of the chloride ion effect from that of N^1-benzylnicotinamide and may therefore reflect the effect of the coenzyme fragment.

Figure 5 shows the effect of N^1-benzylnicotinamide chloride on the binding of auramine O to dehydrogenase as measured by equilibrium dialysis. These experiments and similar experiments are summarized in Table I ahd show that the coenzyme fragment competes with the binding of the dye, thus explaining the decrease in fluorescence shown in Figure 4.

The effect of two substrate competitive inhibitors, pyrazole and isobutyramide, on the binding of auramine O to the enzyme is shown in Figure 6. From these binding curves one observes that both isobutyramide and pyrazole inhibit the binding of auramine O to dehydrogenase. Scatchard (1949) analysis of these curves indicates a decrease in the apparent binding constant of the dye in the presence of either inhibitor (Table I). Higher concentrations of either inhibitor completely release the dye from the enzyme. Likewise, increasing concentrations of either pyrazole or isobutyramide cause a decrease in the fluorescence emission to zero with no shift in the maximum wavelength.

Since the auramine O binding site seemed to be closer to the substrate binding sites than to the coenzyme binding sites, it was decided to look at auramine O inhibition of the enzyme with cyclohexanol as the substrate. Figure 7 is a Lineweaver–Burk plot (1934) of the observed inhibition. The K_I calculated from this figure for auramine O is 6.96×10^{-6} M. The K_m for cyclohexanol is 7.46×10^{-3} M. Figure 8 is a Dixon plot (Dixon, 1953) of the inhibition at three concentrations of cyclohexanol. The K_I of 7.05×10^{-6} M agrees well with the previous determination. The K_a calculated from the K_I values is 1.43×10^5 M^{-1} and compares with the K_a (1.54×10^5 M^{-1}) determined previously with ethanol as the substrate (Conrad *et al.*, 1970).

Discussion

The available research to date indicates that auramine O interacts with dehydrogenase in a most specific, as well as unique, manner. Previously, dehydrogenase was shown to bind 2 moles of auramine O with a K_a of 1.0×10^5 M^{-1} (Conrad

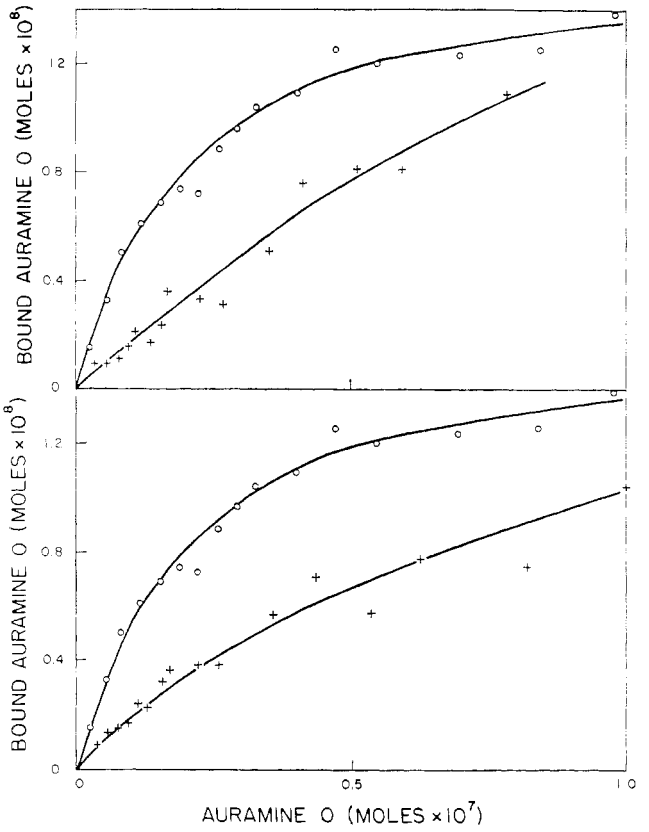

FIGURE 6: Effect of pyrazole and isobutyramide on the binding of auramine O to dehydrogenase measured by equilibrium dialysis. In both sections the binding curve of auramine O to dehydrogenase in 0.1 M sodium phosphate (pH 7.4) (○) is used for comparison. Upper section: bottom line (+) represents binding of 1.0×10^{-8} to 3.0×10^{-7} mole of auramine O to 1.21×10^{-8} mole of dehydrogenase in the presence of 8.0×10^{-5} mole of pyrazole. Lower section: bottom line (+) represents binding of 1.0×10^{-8} mole to 3.0×10^{-7} mole of auramine O to 1.21×10^{-8} mole of dehydrogenase in the presence of 4.0×10^{-4} mole of isobutyramide.

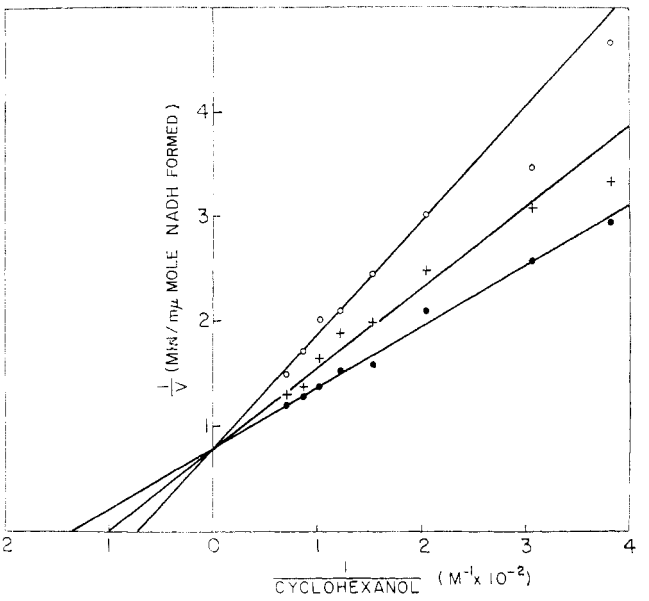

FIGURE 7: Lineweaver–Burk plot of auramine O inhibition of dehydrogenase with respect to cyclohexanol. Formation of NADH was followed fluorometrically with an Aminco-Bowman spectrophotofluorometer; excitation at 350 nm and emission at 460 nm. Reaction mixtures contained 1.64×10^{-4} M NAD, cyclohexanol from 2.62×10^{-3} to 1.64×10^{-2} M, and (●) no inhibitor, (+) 2.43×10^{-6} M auramine O, (○) 4.87×10^{-6} M auramine O, in a total volume of 3 ml of 0.1 M sodium phosphate at pH 7.4. The reactions were initiated by addition of 8.8×10^{-12} mole of enzyme.

et al., 1970). The data also indicated that the dye molecules do not bind at either the coenzyme or substrate binding sites. It was of interest to determine where on the enzyme the auramine O binding site lay with respect to other known binding sites; and also, whether changes in the dye binding site could be caused by molecules binding at regions of the active site.

Figure 9 is a schematic representation of the interrelationships between the various binding sites on the enzyme. For the most part, NAD and NADH are shown occupying the same site. That they compete for the same site has been known for some time (Theorell and Winer, 1959).

The coenzyme binding site is depicted in an open conformation (Velick, 1961) rather than in a closed conformation. Fluorescence spectra show a doubling of the auramine O intensity in the presence of saturating AMP. As larger adenine compounds were added, the auramine O fluorescence enhancement becomes correspondingly smaller. The equilibrium dialysis experiments show that the binding constant for auramine O is increased in the presence of adenine compounds to the same degree that the fluorescence intensity is enhanced. Addition of either oxidized or reduced coenzyme cause a reduction in the dye fluorescence.

The available data are consistent with the following map of the active-site region. There is an adenine recognition site on the enzyme which is at some distance from the auramine O binding site. This site, when occupied, causes a modification in the auramine O–dehydrogenase interaction which doubles the affinity as well as the fluorescence intensity. As the adenine compounds used become larger they extend closer to the dye binding site with a concomitant lowering of the enhancement and slight red shift of the emission maximum.

The oxidized and reduced nicotinamide rings of NAD and NADH, respectively, are accorded separate binding sites due to their significantly different structures and the grossly different pH profiles of their binding affinities (Theorell and Winer, 1959). That there may be two separate binding sites for the oxidized and reduced nicotinamide rings of the coenzyme has been previously suggested by Anderson for yeast alcohol dehydrogenase (Anderson *et al.*, 1965; Fonda and Anderson, 1967) and rabbit muscle L-α-glycerophosphate dehydrogenase (Anderson *et al.*, 1970). The observation here supporting the theory of separate sites for the reduced and oxidized nicotinamide moieties of the coenzyme is the significant difference in auramine O fluorescence caused by addition of NAD or NADH. NADH resembles ADPR in causing a red shift of the emission maximum but NADH causes a slight decrease in the intensity. NAD causes a drastic decrease in the fluorescence intensity but causes no change in the maximum wavelength. It was also observed that N^1-benzylnicotinamide chloride releases auramine O from the enzyme and exhibits no evidence of ternary complex formation. Since NAD makes the dye bind more tightly to protein, it appears that the release of the dye brought about by the analog can be attributed to the benzyl moiety.

Ethanol and acetaldehyde have been shown previously to occupy separate sites on the enzyme (Winer and Theorell, 1960; Wratten and Cleland, 1963). Conrad *et al.* (1970) showed that the auramine O binding site does not overlap the

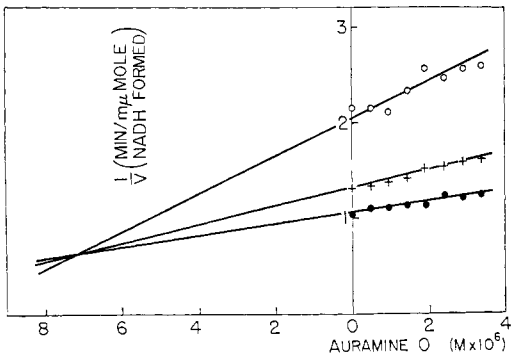

FIGURE 8: Dixon plot of auramine O inhibition of dehydrogenase. Reaction mixtures contained 1.64×10^{-4} M NAD, auramine O from 0 to 3.41×10^{-6} M and (●) 1.60×10^{-2} M cyclohexanol, (+) 9.61×10^{-3} M cyclohexanol, (○) 3.20×10^{-3} M cyclohexanol. Other conditions as described in the legend to Figure 7.

ethanol binding site. Both pyrazole, a competitive inhibitor of ethanol, and isobutyramide, a competitive inhibitor of acetaldehyde, have been shown to be competitive inhibitors of auramine O binding. These findings suggested that the auramine O binding site is adjacent to but distinct from the normal substrate binding site.

The kinetic studies using cyclohexanol as the substrate tied together all the binding sites. Auramine O exhibited competitive inhibition with respect to cyclohexanol. These results provide for an overlap between the auramine O binding site and the cyclohexanol binding site; although previously, it had been established that auramine O and ethanol do not share a mutual binding site on this enzyme. The site of these interactions is within an extensive hydrophobic region where the long-chain alcohols, aldehydes, fatty acids, and fatty acid amides, interact with the enzyme (Winer, 1958; Winer and Theorell, 1960).

The topographical map presented in this report is different from two previously presented active-site maps (Theorell and McKinely McKee, 1961; Wallenfels and Sund, 1957). Although the other maps incorporated functional groups of the enzyme as an integral part, this map does not include any residues of the protein. The purpose of this map is to elucidate the geometric relationship between the various binding sites within the active site. The coenzyme is pictured in an open conformation whereas the previous maps showed the coenzyme in a closed conformation. Separate binding sites for the oxidized and reduced nicotinamide ring systems should satisfy the recent microscopic reversibility arguments applied to the separate substrate binding site hypothesis (Sigman and Winer, 1970). In previous schematics separate oxidized and reduced substrate binding sites have not been utilized.

The worth of the present map will be enhanced as the location of more site specific compounds are precisely positioned. For instance, 2,2′-bipyridyl was shown by Sigman (1967) to interact with several enzyme-bound adenine and nicotinamide containing inhibitors in much the same fashion as does auramine O. Two differences observed are the competition of NAD with bipyridyl but not with auramine O as well as the metal-chelating ability of bipyridyl. From this one would position bipyridyl slightly nearer the coenzyme binding site than auramine O and possibly overlapping part of the nicotinamide binding sites. This would place a zinc atom somewhere in the region between the nicotinamide binding sites and the auramine O binding sites.

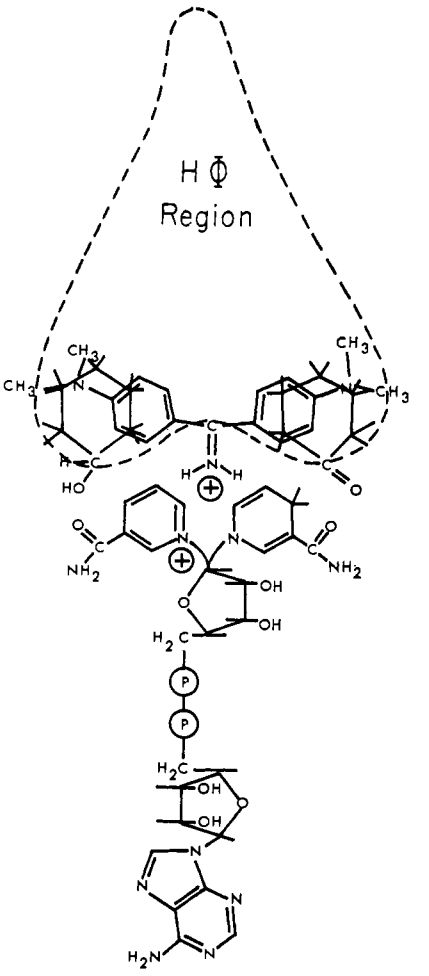

FIGURE 9: Topographical map of various binding regions at active site of dehydrogenase.

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References

Anderson, B. M., Kim, S. J., and Wang, C.-N. (1970), *Arch. Biochem. Biophys. 138*, 66.

Anderson, B. M., Reynolds, M. L., and Anderson, C. D. (1965), *Arch. Biochem. Biophys. 111*, 202.

Brand, L., Gohlke, J. R., and Rao, D. S. (1967), *Biochemistry 6*, 3510.

Conrad, R. H. (1968), Ph.D. Dissertation, The Johns Hopkins University.

Conrad, R. H., Heitz, J. R., and Brand, L. (1970), *Biochemistry 9*, 1540.

Dixon, M. (1953), *Biochem. J. 55*, 170.

Fonda, M. L., and Anderson, B. M. (1967), *Arch. Biochem. Biophys. 120*, 49.

Karrer, P., and Stare, F. J. (1937), *Helv. Chim. Acta 20*, 418.

Lineweaver, H., and Burk, D. (1934), *J. Amer. Chem. Soc. 56*, 658.

Scatchard, G. (1949), *Ann. N. Y. Acad. Sci. 51*, 660.

Sigman, D. S. (1967), *J. Biol. Chem. 242*, 3815.

Sigman, D. S., and Winer, A. D. (1970), *Biochim. Biophys. Acta 206*, 183.
Sund, H., and Theorell, H. (1963), *Enzymes 7*, 25.
Theorell, H., and McKinley McKee, J. S. (1961), *Acta Chem. Scand. 15*, 1834.
Theorell, H., and Winer, A. D. (1959), *Arch. Biochem. Biophys. 83*, 291.
Theorell, H., and Yonetani, T. (1963), *Biochem. Z. 338*, 537.
Velick, S. F. (1961), *in* Light and Life, McElroy, W. D., and Glass, B., Ed., Baltimore, Md., The Johns Hopkins Press, p 108.
Wallenfels, K., and Sund, H. (1957), *Biochem. Z. 329*, 59.
Winer, A. D. (1958), *Acta Chem. Scand. 12*, 1695.
Winer, A. D., and Theorell, H. (1960), *Acta Chem. Scand. 14*, 1729.
Witholt, B., and Brand, L. (1968), *Rev. Sci. Instrum. 39*, 1271.
Wratten, C. C., and Cleland, W. W. (1963), *Biochemistry 2*, 935.

Threonine-Sensitive Aspartokinase–Homoserine Dehydrogenase of *Escherichia coli* K 12. Reaction with 6-Mercapto-9-β-D-ribofuranosylpurine 5′-Triphosphate*

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ABSTRACT: An adenosine triphosphate (ATP) analog, 6-mercapto-9-β-D-ribofuranosylpurine 5′-triphosphate (SH-TP), is a substrate for the aspartokinase activity of aspartokinase I–homoserine dehydrogenase I of *Escherichia coli* K 12. Incubation of the enzyme with SH-TP in the absence of L-threonine leads to a labeled enzyme of essentially the same molecular weight as the native protein which has specifically lost its aspartokinase activity while retaining in full its homoserine dehydrogenase activity. The dehydrogenase activity of the modified enzyme is desensitized to threonine, but is inhibited both by aspartate and by ATP. Reaction of the modified enzyme with mercaptoethanol releases three molecules of SH-TP per molecule of protein. Saturating levels of threonine protect the enzyme completely against SH-TP. Inactivation of the aspartokinase function by SH-TP probably does not occur by affinity labeling of the aspartokinase site(s) since (1) ATP and adenosine diphosphate (ADP) protect against SH-TP only at concentrations much greater than that of SH-TP; (2) ATP and aspartate bind to the native and modified enzymes with virtually unaltered affinities; and (3) the kinetics of reaction with SH-TP is not influenced by the presence of aspartic acid. Since reaction of the enzyme with SH-TP does not prevent binding of aspartokinase substrates, it is proposed that inactivation occurs by an interference with aspartokinase catalysis. The relevance of the SH-TP reaction to the subunit structure of the enzyme is discussed.

Physical and chemical studies of the bifunctional enzyme, aspartokinase I–homoserine dehydrogenase I (aspartokinase or ATP:L-aspartate 4-phosphotransferase, EC 2.7.2.4; homoserine dehydrogenase or L-homoserine:NADP oxidoreductase, EC 1.1.1.3) of *Escherichia coli* K 12, have shown that the protein is composed of six identical, or nearly identical, subunits (Truffa-Bachi *et al.*, 1969). The enzyme possesses six binding sites for the inhibitor, L-threonine, and the attachment of this ligand is cooperative (Janin *et al.*, 1969). On the other hand, a variety of evidence points to the existence of only three active sites for the homoserine dehydrogenase function (Janin *et al.*, 1969; Heck and Truffa-Bachi, 1970).

The recent demonstration that 6-mercapto-9-β-D-ribofuranosylpurine 5′-triphosphate (SH-TP)[1] reacts specifically with sulfhydryl groups at the adenosine triphosphatase sites of myosin (Murphy and Morales, 1970) suggested the possibility that this reagent might react selectively with the particular sulfhydryl groups necessary for the aspartokinase activity of aspartokinase I–homoserine dehydrogenase I (Truffa-Bachi *et al.*, 1966, 1968). Since the selective reaction of SH-TP with the enzyme could conceivably provide information relevant to the number of aspartokinase sites, which is a question of considerable interest, the experiments described in the present paper were undertaken.

Experimental Section

Materials. E. coli K 12, strain Tir 8 (Szentirmai *et al.*, 1968), was grown on a minimal medium containing 1% glucose as the carbon source. The cells were harvested in the late-log phase and stored at −15°. The homogeneous protein was isolated from bacterial extracts and purified as described by

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[1] Abbreviations used are: SH-TP, 6-mercapto-9-β-D-ribofuranosylpurine 5′-triphosphate; PMB, *p*-mercuribenzoate; DTNB, 5,5′-dithiobis-2-nitrobenzoate.