The Interaction of Triiodothyroacetic Acid with Horse Liver Alcohol Dehydrogenase

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SUMMARY

Triiodothyroacetic acid is a competitive inhibitor of horse liver alcohol dehydrogenase with respect to the coenzyme. The $K_i$ value for triiodothyroacetic acid in this system is 1.3 to 1.9 $\mu M$. Triiodothyroacetic acid appears to interact with a hydrophobic portion of the protein since it is able to displace the hydrophobic compound, 8-anilino-1-naphthalene sulfonic acid from liver alcohol dehydrogenase. Triiodothyroacetic acid also displaces hydrophobic probes from human globin and bovine serum albumin. The difference spectrum of triiodothyroacetic acid bound to liver alcohol dehydrogenase is very similar to that observed for triiodothyroacetic acid in solvents less polar than water. Yonetani-Theorell plots show that triiodothyroacetic acid binds at the adenosine diphosphate ribose portion of the coenzyme binding site and that bound triiodothyroacetic acid restricts the binding of metal chelators to the zinc of the active center. It is therefore concluded that the adenosine diphosphate ribose portion of the active center resides in a hydrophobic area of the protein and that this area is near the zinc of the active center.

The binding of the coenzymes, NAD$^+$ and NADH, to horse liver alcohol dehydrogenase has been extensively studied. Each enzyme molecule is believed to have two identical coenzyme binding sites (1). Zinc chelators are competitive inhibitors of the enzyme with respect to the coenzyme, but can bind simultaneously with the enzyme analogue adenosine diphosphate ribose (2). It was therefore proposed (2) that each coenzyme binding site consisted of two areas, one which binds the ADP-ribose portion of the coenzyme and the other which contains zinc and binds the nicotinamide portion of the coenzyme. Our laboratory (3) recently reported that triiodothyronine and triiodothyroacetic acid are potent inhibitors of alcohol dehydrogenase. Although both triiodothyronine and I$_3$TA modify the binding of NADH to the enzyme only I$_3$TA is a competitive inhibitor with respect to NADH.

$^1$ The term alcohol dehydrogenase refers to horse liver alcohol dehydrogenase.

$^2$ The abbreviations used are: I$_3$TA, triiodothyroacetate; BSA, bovine serum albumin.

Brand, Gohlke, and Rao (4) and Turner and Brand (5) have recently shown that 8-anilino-1-naphthalene sulfonic acid and rose Bengal, dyes known to interact with hydrophobic regions of protein, are also competitive inhibitors of alcohol dehydrogenase with respect to the coenzyme. There exists a general similarity among I$_3$TA, 8-anilino-1-naphthalene sulfonic acid, and rose Bengal in that all three are molecules having large hydrophobic regions in addition to an anionic group. The current work provides evidence that I$_3$TA interacts with the protein primarily through a hydrophobic mechanism, and that the hydrophobic region in alcohol dehydrogenase is at the ADP-ribose site which is in close proximity to the zinc in each active center of the molecule.

EXPERIMENTAL PROCEDURE

Materials—Crystalline human hemoglobin, horse liver alcohol dehydrogenase (crystalline suspension in 0.02 M phosphate buffer, pH 7), crystalline bovine serum albumin, Chelex-100, ADP-ribose, NADH, and NAD$^+$ were purchased from Calbiochem. I$_3$TA was obtained from Aldrich Chemical Company. 1,10-Phenanthroline and 8-anilino-1-naphthalene sulfonic acid were obtained from Eastman. The magnesium salt of 8-anilino-1-naphthalene sulfonic acid was recrystallized several times from water after filtration of the hot solutions through charcoal (6). a,a-Dipyridyl was purchased from Fisher.

Concentrations of the various substances were determined by use of the following extinction coefficients: NAD$^+$ and ADP-ribose, $15.4 \times 10^4$ $\text{cm}^{-1} \text{M}^{-1}$ at 250 $\mu M$; 1,10-phenanthroline, $31.0 \times 10^4$ $\text{cm}^{-1} \text{M}^{-1}$ at 246 $\mu M$ (6); alcohol dehydrogenase, 0.455 $\text{mg}^{-1} \text{cm}^{-1}$ at 280 $\mu M$ (7); and apohemoglobin, $2.4 \times 10^4$ $\text{M}^{-1} \text{cm}^{-1}$ at 280 $\mu M$ (8).

Preparation and Characterization of Alcohol Dehydrogenase—In order to obtain enzyme solution free of exogenous zinc, alcohol dehydrogenase was dialyzed at 4$^\circ$ for 76 hours against two 1-liter portions of 0.1 M potassium phosphate buffer, pH 7.0, which contained 5 to 10 mg of Chelex-100. Next it was dialyzed against two more 1-liter portions of 0.1 M potassium phosphate buffer, pH 7.0, which had been passed through a column of Chelex-100, 10 $\times$ 30 cm. At the end of 4 days of dialysis no zinc could be detected in the dialysate by use of the metal chelators 1,10-phenanthroline or a,a-dipyridyl. Any precipitate that had been formed in the dialysis sac during this time was removed by centrifugation of the alcohol dehydrogenase solution at 18,000 rpm for 1 hour. This procedure yielded...
hemoglobin by the acid-acetone procedure of Rossi-Fanelli, Antonini, and Caputo (12).

**Kinetic Studies**—All kinetic studies were performed in an Amino-Bowman spectrophotofluorometer by measuring the increase in fluorescence at 450 μm which accompanies the conversion of NAD+ to NADH. Assays were done at 23° in 1-cm cuvettes which contained 2.25 ml of 0.1 m potassium phosphate buffer, pH 7.0. The concentration of ethanol was always 6.2 mm and the concentration of dehydrogenase ranged from 0.008 to 0.010 mg/2.25 ml.

**Graphical Representation of Binding Data**—Alcohol dehydrogenase is known to bind 2 moles of the metal chelators 1,10-phenanthroline or α,α-dipyridyl per mole of enzyme (2, 13). The moles of chelator bound per mole of protein subunit (9) at a given chelator concentration is given by the equation (14)

\[
P = \frac{nK(D)}{1 + K(D)}
\]

where \( n \) = number of binding sites per subunit weight of 42,000, \( K \) = intrinsic binding constant for the independent binding sites, and \( D \) = total concentration of chelator minus concentration of chelator bound to the protein. The method for the determination of the concentration of bound chelator is ex-

![Graph](https://example.com/image.png)

**Figure 1.** Kinetics of the inhibition of alcohol dehydrogenase activity by triiodothyroacetate (T₃-AA) when NAD⁺ concentration varied in the reaction mixture. The reaction mixture contained 0.1 m potassium phosphate buffer, pH 7.0, in a total volume of 2.25 ml.

### Table I

<table>
<thead>
<tr>
<th>Compound</th>
<th>( K_i ) or ( K_d ) (pM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( K_d )</td>
</tr>
<tr>
<td>NADH</td>
<td>0.20 (17)</td>
</tr>
<tr>
<td>NAD⁺</td>
<td>4-9.0 (2, 6, 24)</td>
</tr>
<tr>
<td>1,10-Phenanthroline</td>
<td>265</td>
</tr>
<tr>
<td>α,α-Dipyridyl</td>
<td>16-35 (6)</td>
</tr>
<tr>
<td>DTP-ribose</td>
<td>8</td>
</tr>
<tr>
<td>BSA</td>
<td>1.9</td>
</tr>
<tr>
<td>Aminobenzal</td>
<td>120 (4)</td>
</tr>
<tr>
<td>α,α-Dipyridyl</td>
<td>140 (6)</td>
</tr>
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<td>1.9</td>
</tr>
<tr>
<td>Aminobenzal</td>
<td>120 (4)</td>
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</table>

### Table II

<table>
<thead>
<tr>
<th>Compound</th>
<th>Absorbent and protein bound to 8-anilino-1-naphthalene sulfonic acid</th>
<th>System of measuring displacement</th>
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<tbody>
<tr>
<td></td>
<td>Reduction in fluorescence</td>
<td>Displaced by equilibrium dialysis</td>
</tr>
<tr>
<td></td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>ITA</td>
<td>Alcohol dehydrogenase</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>BSA</td>
<td>62</td>
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<tr>
<td></td>
<td>Globin</td>
<td>65</td>
</tr>
<tr>
<td>1,10-Phenanthroline</td>
<td>Alcohol dehydrogenase</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>BSA</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Globin</td>
<td>5</td>
</tr>
<tr>
<td>α,α-Dipyridyl</td>
<td>Alcohol dehydrogenase</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>BSA</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Globin</td>
<td>6</td>
</tr>
</tbody>
</table>

*For the fluorescent experiments the concentration of the sulfonic acid was 20 μM, alcohol dehydrogenase was 7 μM, BSA was 6.1 μM, and apohemoglobin was 7.25 μM. The fluorescence (excitation 405 μm, and emission 510 μm) of the protein-8-anilino-1-naphthalene sulfonic acid mixture was measured and then either ITA (5.5 μM), 1,10-phenanthroline (145 μM), or α,α-dipyridyl (960 μm) was added to the cuvette and a new fluorescent reading was taken.

*For the equilibrium dialysis experiments approximately 2 mg of the appropriate protein in 1 ml of solution was dialyzed against 100 ml of 0.1 m potassium phosphate, pH 7.0, buffer containing the sulfonic acid at a concentration of 95 μM. To the experimental was added either ITA (98 μM), 1,10-phenanthroline (430 μM), or α,α-dipyridyl (1.53 μM). After 24 hours the absorbance at 350 μm of the protein-8-anilino-1-naphthalene sulfonic acid solution minus the absorbance of the dialysate was compared for the control and experimental.
I₃TA Interaction with Alcohol Dehydrogenase

Fig. 2. Light absorption changes accompanying complex formation of I₃TA with alcohol dehydrogenase. Double difference spectra were recorded in 0.1 M potassium phosphate buffer at pH 7.0, at the following concentrations of reactants: 38 µM alcohol dehydrogenase and 108 µM I₃TA, ○—○; 156 µM alcohol dehydrogenase and 27 nM I₃TA, □—□. The arrangement of cuvettes was such that Cuvette I contained I₃TA, alcohol dehydrogenase, and buffer; Cuvette II contained buffer; Cuvette III contained alcohol dehydrogenase and buffer; Cuvette IV contained I₃TA and buffer. Each compartment contained 1 ml of fluid. The determination of Δε is explained under “Results.” Spectra were recorded with a Zeiss PMQII spectrophotometer.

plained under “Results.” A Klotz plot of 1/P versus 1/(D) yields a line which intersects the ordinate at 1/n and has a slope equal to 1/nK (14). Therefore if a Klotz plot of 1/P against 1/(D) is constructed from the data of equilibrium experiments performed in the presence and absence of I₃TA then the effect of I₃TA on the metal chelator binding parameters n and K can be evaluated.

Double Difference Spectra—Tandem cells were obtained from Precision Cells, Inc. These cells were 1 cm in width and contained two compartments that were each 0.3 cm in width. Absorbance differences between the first and second pair of cuvettes were measured at various wave lengths using a Zeiss PMQII spectrophotometer or a Cary model 15 recording spectrophotometer.

RESULTS

Coenzyme Competitive Inhibitors of Alcohol Dehydrogenase—
I₃TA is a potent inhibitor of alcohol dehydrogenase. As seen in Fig. 1 the inhibition is competitive with respect to the coenzyme NAD⁺. A Kᵢ value of 1.9 µM was calculated for I₃TA by use of the formula

\[
\text{Slope I} = \frac{(I)}{C} = 1 + \frac{(I)}{K_i}
\]

I₃TA is also a competitive inhibitor with respect to the reduced coenzyme, NADH. A Kᵢ value in the NADH → NAD⁺ system was determined to be 1.3 µM. Dissociation constants and Kᵢ values for several NAD⁺ competitive inhibitors of alcohol dehydrogenase are presented in Table I. It would appear that I₃TA is the most potent competitive inhibitor of alcohol dehydrogenase with respect to NAD⁺ known. I₃TA apparently binds one-sixth to one-tenth as tightly to the coenzyme binding site of alcohol dehydrogenase as does NADH and approximately 50 times more tightly than the coenzyme NAD⁺. The competitive inhibitors in Table I might be divided into three classes. The metal chelators, 1,10-phenanthroline and α,α-dipyridyl, constitute the first class. The second class of competitive inhibitors is composed of the adenine derivatives, ADP-ribose and AMP. Tentatively, the third class of inhibitors that are competitive with NAD⁺ includes I₃TA, rose Bengal, and 8-anilino-1-naphthalene sulfonic acid. These three compounds are all large aromatic molecules containing hydrophobic regions as well as anionic groups. Furthermore, they all interact with a variety of proteins such as albumin, globin, and dehydrogenases other than alcohol dehydrogenase (4, 5, 8, 15). It follows from the work of Brand et al. (4) that a hydrophobic area at the active center of alcohol dehydrogenase may be important for the binding of 8-anilino-1-naphthalene sulfonic acid and rose Bengal to the enzyme. It was therefore of interest to examine the possibility that I₃TA also binds to this hydrophobic site.

Evidence for Binding of I₃TA to Hydrophobic Protein Surface Environments

An enhancement of fluorescence was first shown to accompany the binding of 8-anilino-1-naphthalene sulfonic acid to certain

Fig. 3. The difference spectra of I₃TA in buffer and more apolar environments. The control cuvette contained I₃TA in 0.1 M potassium phosphate buffer, pH 7.3. The experimental cuvette contained an equivalent amount of I₃TA in 0.1 M potassium phosphate, pH 7.3, and contained, in addition, 156 µM alcohol dehydrogenase (——), 35% dimethyl sulfoxide (———), and 50% 1-propanol (——). The concentrations of I₃TA used were 27 µM for the alcohol dehydrogenase experiment and 116 µM for the solvent experiments. The spectra were taken on a Cary model 15 recording spectrophotometer.
that the I3TA binding site of alcohol dehydrogenase (Fig. 3). This is due to the binding of the sulfonic acid to a hydrophobic region of the protein. As seen in Table II in each case the presence of I3TA enhances the protein enhanced fluorescence of 8-anilino-1-naphthalene sulfonic acid. It was furthermore established by equilibrium dialysis that the loss of fluorescence accompanying the addition of I3TA was due to the dissociation of the sulfonic acid-protein complex (Table II). Since 8-anilino-1-naphthalene sulfonic acid binds at hydrophobic sites it would appear that I3TA can effectively compete with the sulfonic acid for nonpolar regions of the various proteins. The metal chelators, 1,10-phenanthroline and α,α-dipyridyl, also reduce the fluorescence if the sulfonic acid bound to alcohol dehydrogenase but not to globin or BSA (Table II). This might indicate that in alcohol dehydrogenase the zinc is in close proximity to the 8-anilino-1-naphthalene sulfonic acid binding site.

**Difference Spectra**—Light absorption changes caused by the interaction of alcohol dehydrogenase and I3TA are shown in Fig. 2. The difference extinction coefficient, Δε, was calculated by two methods. First, I3TA was mixed with a large excess of alcohol dehydrogenase so that essentially all the I3TA was bound to the protein. The difference extinction coefficient was then calculated from the known concentration of I3TA. The alternate method was to mix alcohol dehydrogenase with a large excess of I3TA and calculate Δε from the concentration of active sites. The spectra obtained by both methods were qualitatively similar, however, the spectrum calculated by the latter method was slightly more intense indicating that on the average slightly more than 1 molecule of I3TA might be bound per active site. An effort was made to identify the light absorption changes that occur in enzyme-bound I3TA by examining the difference spectrum of I3TA in various apolar solvents. As seen in Fig. 3 the difference spectrum for I3TA in 35% dimethyl sulfoxide or 50% propanol compared to buffer resembles the difference spectrum of I3TA bound to alcohol dehydrogenase (Fig. 3). This is further evidence that the I3TA binding site of alcohol dehydrogenase is a hydrophobic region of the protein.

**Yonetani-Theorell Graphs**—Theorell and Yonetani (1, 2) have shown by use of double difference spectrophotometry that the

\[
\frac{1}{v_1} = \frac{1}{V_{\text{max}}} + \frac{K_m}{S V_{\text{max}}} \left(1 + \frac{i_1}{K_{i_1}} \right) + \frac{K_m}{S V_{\text{max}} K_{i_2}} \left(1 + \frac{i_1}{\alpha K_{i_1}} \right) i_2
\]

where \(K_{i_1}\) and \(K_{i_2}\) are the respective dissociation constants for \(i_1\) and \(i_2\) and \(S\) refers to the substrate concentration. The value \(\alpha\) may be visualized in the following manner

\[
\frac{[E][i_1]}{[E] - i_1} = K_{i_1}
\]

\[
\frac{[E] - [i_1][i_2]}{[E] - [i_1] - [i_2]} = \alpha K_{i_1} = K_{i_1} i_2
\]

Therefore, if \(\alpha = 1\), then the \(i_1\) and \(i_2\) binding sites are independent. If \(\alpha > 1\) then the binding of one inhibitor decreases the affinity of the enzyme for the other inhibitor. If \(1 > \alpha > 0\) then the binding of one inhibitor increases the affinity of the enzyme for the other inhibitor. It can be seen in Equation 1 that if \(i_2\) is kept constant while \(i_1\) is varied then \(1/v_1\) is a direct function of the concentration of \(i_1\). A plot of \(1/v_1\) versus \(i_1\) will give a line with a slope

\[
\text{Slope} = (K_m/S V_{\text{max}} K_{i_1}) (1 + \frac{i_1}{\alpha K_{i_2}})
\]
The intercept on the Y axis is:

\[ \text{Intercept} = \frac{1}{V_{\text{max}}} + \left( \frac{K_m}{SV_{\text{max}}} \right) \left( 1 + \frac{i_s}{K_{R b}} \right) \]

If \( \alpha \) exists and several plots of \( 1/v_i \) as a function of \( i_s \) are constructed at different fixed concentrations of \( i_s \) then both the slope and \( Y \) intercept will increase with increasing concentrations of \( i_s \) and the resulting lines will converge at a common point. The \( abscissa \) value of this point equals \( \alpha K_{R b} \). However, if \( \alpha \) does not exist, that is if the two competitive inhibitors interact at the same site on the enzyme, only the \( Y \) intercept will increase with increasing concentrations of \( i_s \). The slope will remain constant and a series of parallel lines will be obtained.

With the use of this kinetic approach it is possible to determine whether I3TA binds at either the ADP-ribose or the zinc site of alcohol dehydrogenase. In Fig. 4 it can be seen that multiple inhibition by the inhibitor pairs I3TA and ADP-ribose results in a series of parallel lines indicating that I3TA binds at the ADP-ribose binding site of alcohol dehydrogenase. In Fig. 5 multiple inhibition by the inhibitor pair, 1,10-phenanthroline and I3TA, results in a converging line relationship. The intersection of these lines occurs at an \( abscissa \) value of \(-37.5 \mu M\). Hence an \( \alpha \) value of 10.8 has been calculated. This large positive value would indicate that by binding at the ADP-ribose site, I3TA restricts the binding of \( \alpha,\alpha \)-dipyridyl to the zinc of alcohol dehydrogenase.

**Spectrophotometric Studies on Chelation of Protein-bound Zinc by Metal Chelators in Presence and Absence of Triiodothyroacetate**

When either 1,10-phenanthroline or \( \alpha,\alpha \)-dipyridyl binds to the zinc of alcohol dehydrogenase, new absorption peaks appear in the difference spectrum of the chelator-enzyme complex. The \( \alpha,\alpha \)-dipyridyl-zinc-alcohol dehydrogenase has an absorption maximum at 308 \( \mu m \) with a difference extinction coefficient of \( 1.1 \times 10^4 M^{-1} cm^{-1} \) (13). The 1,10-phenanthroline-zinc-alcohol dehydrogenase complex has an absorption maximum at 297 \( \mu m \) with a difference extinction coefficient of \( 0.8 \times 10^4 M^{-1} cm^{-1} \) (2). Therefore if the enzyme-chelator complex is replaced by an enzyme-I3TA complex the extent of this displacement can be measured by absorbance changes at either 308 \( \mu m \) or 297 \( \mu m \).

However, because of light absorption changes caused by the combination between alcohol dehydrogenase and I3TA, new absorption difference coefficients had to be determined at the isobestic points of the alcohol dehydrogenase-I3TA complex. A new difference extinction coefficient for the \( \alpha,\alpha \)-dipyridyl-zinc-alcohol dehydrogenase complex was determined to be 0.96 \( \times 10^4 M^{-1} cm^{-1} \) at a wave length of 304 \( \mu m \). For the 1,10-phenanthroline-zinc-alcohol dehydrogenase complex a difference extinction coefficient of 0.82 \( \times 10^4 M^{-1} cm^{-1} \) at 274 \( \mu m \) was used.

As can be seen in Figs. 6A and 7A the presence of I3TA restricts the binding of \( \alpha,\alpha \)-dipyridyl and 1,10-phenanthroline to the zinc of alcohol dehydrogenase. The effect of I3TA on the chelator binding parameters, \( n \) and \( K \), was examined by use of Klotz plots (14). The amount of free chelator in the chelator enzyme solution was determined by the equation:

**Fig. 6.** Titration of alcohol dehydrogenase with \( \alpha,\alpha \)-dipyridyl (aaBP). The complex formation of \( \alpha,\alpha \)-dipyridyl with the zinc of alcohol dehydrogenase was measured by light absorption changes at 294 \( \mu m \). \( A_s \), light absorption changes as a function of the total \( \alpha,\alpha \)-dipyridyl concentration in the presence and absence of triiodothyroacetate (T3AA). The protein concentration was 20 \( \mu M \) and the \( \alpha,\alpha \)-dipyridyl solution was 21.6 \( \mu M \). The buffer was 0.1 \( \mu M \) potassium phosphate, pH 7.0, and the volume was 2 ml plus the \( abscissa \) value. The reference cell contained the same reagents as the sample cell except for the enzyme. \( B_s \), plot of the reciprocal of the moles of \( \alpha,\alpha \)-dipyridyl bound per mole of active sites of alcohol dehydrogenase (1/\( i_s \)) versus the reciprocal of the concentration of free \( \alpha,\alpha \)-dipyridyl in the presence and absence of I3TA. The method of determination of \( i_s \) and free \( \alpha,\alpha \)-dipyridyl is explained under “Results.”

**Fig. 7.** Titration of alcohol dehydrogenase with 1,10-phenanthroline (O.P.). The complex formation of 1,10-phenanthroline with the zinc of alcohol dehydrogenase was measured by light absorption changes at 274 \( \mu m \). \( A_s \), the light absorption changes as a function of the total 1,10-phenanthroline concentration in the presence and absence of triiodothyroacetate (T3AA). The protein concentration was 20 \( \mu M \) and the 1,10-phenanthroline solution was 1.72 \( \mu M \). The buffer was 0.1 \( \mu M \) potassium phosphate, pH 7.0, and the volume was 2 ml plus the \( abscissa \) value. The reference cell contained the same reagents as the sample cell except for enzyme. \( B_s \), plot of the reciprocal of the moles of 1,10-phenanthroline bound per mole of active site of alcohol dehydrogenase 1/\( i_s \) versus the reciprocal of the concentration of free 1,10-phenanthroline in the presence and absence of I3TA. The method of determination of \( i_s \) and free 1,10-phenanthroline is explained under “Results.”
Mbibitor is I3TA. This molecule contains a large aromatic area. Firstly, I3TA will displace the dye 8-anilino-1-naphroyphobic residue in addition to an anionic group. It is believed that the nicotinamide portion of the coenzyme interacts in some fashion with the zinc of alcohol dehydrogenase while it appears that ADP-ribose is the minimal structure required for firm binding of NADH analogues to the enzyme. Depending on their size and charge other adenosine derivatives are less tightly bound to the enzyme. Evidence has also been presented, that the 3-acetylpyridine analogue of NADH, that there is a cooperative action between the nicotinamide binding site and the ADP-ribose binding site of alcohol dehydrogenase (18). Therefore, it is likely that the coenzyme-enzyme interaction occurs at more than one site on the enzyme.

Although the use of adenine derivatives and coenzyme analogues as probes in the study of the coenzyme binding site clearly loss the specificity as well as the diffuse nature of the coenzyme binding site, this method yields little information on the protein surface environment at the coenzyme binding site. Use of such general protein denaturants as urea (19), low pH (20), detergent (22), and p-chloromercuribenzoate (23) strongly suggest that the protein surface environment at the coenzyme binding site of alcohol dehydrogenase is dependent upon the tertiary structure of the enzyme. Since these reagents result in a destruction of tertiary protein structure that is generally not reversible, it is difficult to characterize the protein surface at the active center by this approach.

An alternative approach in the examination of the protein surface environment at the coenzyme binding site is the use of reversible, coenzyme competitive inhibitors which are not structural analogues of the coenzyme, yet apparently bind at the enzyme binding site because they recognize certain surface environments or prosthetic groups of the protein. For example, with the use of the metal chelator, 1,10-phenanthroline, it has been possible to show that the zinc moiety of alcohol dehydrogenase is located at the pyridine ring binding region of the enzyme (5). Another structurally unrelated coenzyme competitive inhibitor is I3TA. This molecule contains a large aromatic hydrophobic residue in addition to an anionic group. It is believed that hydrophobic interactions are important in the firm binding of I3TA to alcohol dehydrogenase for the following reasons. Firstly, I3TA will displace the dye 8-anilino-1-naphthalene sulfonic acid from its hydrophobic binding site on alcohol dehydrogenase as well as from aaphemoglobin, and bovine serum albumin. Secondly, the spectrum of I3TA bound to alcohol dehydrogenase closely resembles the spectrum of I3TA chem in apolar solvents such as dimethyl sulfoxide and 1-propanol.

Due to the fact that the dissociation constant for the alcohol dehydrogenase-I3TA complex is small and the presence of I3TA does not interfere with the recording of fluorescent kinetic data, I3TA is an excellent probe to use in the differentiation of the active center of alcohol dehydrogenase. The relation of the I3TA binding site to the ADP-ribose binding site and the zinc of alcohol dehydrogenase was examined by use of Yonetani-Theorell graphs. It appears that I3TA binds at the ADP-ribose binding site of alcohol dehydrogenase and when bound at this site greatly restricts the binding of 1,10-phenanthroline to the zinc of the enzyme. Furthermore, it was shown spectrophotometrically that I3TA displaces 1,10-phenanthroline and α,α-diprydyl from the zinc of the active center of alcohol dehydrogenase.

If the interaction of I3TA with alcohol dehydrogenase is primarily hydrophobic, as is postulated, then part of the active center of alcohol dehydrogenase can be characterized as a non-polar region that is in close proximity to the protein-bound zinc. Thyroxine and related compounds are known to interact with a wide variety of proteins. While the mechanisms of these interactions are not known, it is entirely possible that these hormones and derivatives exert their action and exhibit their apparent nonspecificity by simply recognizing hydrophobic regions on their target protein molecules.

REFERENCES
