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 active center 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 coenzyme 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 inhibit 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.

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 (5). 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 $\times 10^{4}$ M$^{-1}$ cm$^{-1}$ at 250 m$\mu$; 1,10-phenan throline, 31.0 $\times 10^{4}$ M$^{-1}$ cm$^{-1}$ at 246 m$\mu$ (6); alcohol dehydrogenase, 0.455 mg$^{-1}$ cm$^{-1}$ at 280 m$\mu$ (7); and apohemoglobin, 2.4 $\times 10^{4}$ M$^{-1}$ cm$^{-1}$ at 280 m$\mu$ (8).

Preparation and Characterization of Alcohol Dehydrogenase—In order to obtain enzyme solution free of exogenous zinc, alcohol dehydrogenase was dialyzed at 4°C 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-phenan throline 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
laeasured by the method of Dalziel (11). The concentration of active sites determined by use of the protein "Rose Bengal" absorption spectrometry (10) and was found to be 2.0 g content of alcohol dehydrogenase was determined by gravimetrically, or titrating a known amount of NADH with alcohol dehydrogenase in the presence of a large molar excess of iso- enzyme. The reaction mixture was added either ITA (5.5 μM), 1,10-phenanthroline (1.4 μM), or a,a-dipyridyl (960 μM). After 24 hours the absorbance at 350 nm was added. The initial rate of change in fluorescence at 350 nm, which accompanies the conversion of NADH to NAD+.

**Graphical Representation of Binding Data**—Alcohol dehydrogenase is known to bind 2 moles of the metal chelators 1,10-phenanthroline or a,a-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)

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

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-}

![Graphical Representation of Binding Data](http://www.jbc.org/)

### Table I

<table>
<thead>
<tr>
<th>Compound</th>
<th>( K_i ) or ( K_d )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,4-Dicarboxylic</td>
<td>3.0 (17)</td>
</tr>
<tr>
<td>Phenanthroline</td>
<td>3.3 (13)</td>
</tr>
<tr>
<td>a,a-Dipyridyl</td>
<td>4.0 (2, 6, 24)</td>
</tr>
<tr>
<td>DMP</td>
<td>4.0 (6)</td>
</tr>
<tr>
<td>BSA</td>
<td>1.3 (4)</td>
</tr>
<tr>
<td>Alcohol dehydrogenase</td>
<td>2.0 (4)</td>
</tr>
</tbody>
</table>

### Table II

<table>
<thead>
<tr>
<th>Absorbent and protein bound to 8-anilino-1-naphthalene sulfonic acid</th>
<th>System of measuring displacement</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Reduction in fluorescence(a)</td>
</tr>
<tr>
<td>Alcohol dehydrogenase</td>
<td>60</td>
</tr>
<tr>
<td>BSA</td>
<td>43</td>
</tr>
<tr>
<td>Globin</td>
<td>65</td>
</tr>
<tr>
<td>1,10-Phenanthroline</td>
<td>33</td>
</tr>
<tr>
<td>Alcohol dehydrogenase</td>
<td>8</td>
</tr>
<tr>
<td>BSA</td>
<td>5</td>
</tr>
<tr>
<td>Globin</td>
<td>6</td>
</tr>
</tbody>
</table>

\(a\) For the fluorescent experiments the concentration of the sulfonic acid was 30 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 a,a-dipyridyl (960 μM) was added to the cuvette and a new fluorescent reading was taken.

\(b\) For the equilibrium dialysis experiments approximately 2 mg of the appropriate protein in 1 ml of solution were 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 (95 μM), 1,10-phenanthroline (430 μM), or a,a-dipyridyl (1.53 mM). 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.
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 \mu M alcohol dehydrogenase and 108 \mu M I3TA, ■ ■ ■ ; 156 \mu M 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 \Delta \varepsilon is explained under "Results." Spectra were recorded with a Zeiss PMQII spectrophotometer.

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 \mu M alcohol dehydrogenase (----), 35% dimethyl sulfoxide (— — — —), and 50% 1-propanol (---). The concentrations of I3TA used were 27 \mu M for the alcohol dehydrogenase experiment and 116 \mu M for the solvent experiments. The spectra were taken on a Cary model 15 recording spectrophotometer.
by use of double difference spectrophotometry that the further evidence that the I3TA binding site of alcohol dehydrogenase was slightly more intense indicating that on the average slightly more than 1 molecule of I3TA might be bound per active site. 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 binding of the sulfonic acid to a hydrophobic region of the protein. As seen in Table II in each case the presence of I3TA reduces 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 of 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.

The reaction mixture contained 0.1 M potassium phosphate buffer, pH 7.0, in a total volume of 2.25 ml.

NAD+ coenzyme competitive inhibitors ADP-ribose and 1,10-phenanthroline can bind simultaneously to the coenzyme binding site of alcohol dehydrogenase. However in the presence of coenzyme neither can bind, indicating that the coenzyme-enzyme interactions occurs at more than one site on the enzyme molecule. These sites can be classified as an ADP-ribose binding site and a zinc site. A graphical method was derived by these authors to further analyze the "interaction" of the ADP-ribose binding site with the zinc site of alcohol dehydrogenase (6). In the presence of two coenzyme competitive inhibitors, i1 and i2, the reciprocal of the velocity of the reaction is expressed by the following equation

\[
\frac{1}{V_i} = \frac{1}{V_{max}} + \frac{K_{m1}}{SV_{max}} \left(1 + \frac{i_1}{K_{R_1}}\right) + \frac{K_{m2}}{SV_{max}K_{R_2}} \left(1 + \frac{i_1}{\alpha K_{R_1}}\right) i_2
\]

where \(K_{R_1}\) and \(K_{R_2}\) 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

\[
\frac{[E][i_1]}{[E] - i_1} = K_{R_1} i_2
\]

\[
\frac{[E] - i_2}{[E] - i_1 - i_2} = \alpha K_{R_1} = K_{R_1} i_2
\]

Therefore, if \(\alpha = 1\), then the \(i_1\) and \(i_2\) binding sites are independent. If \(\alpha > 1\) 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 \(1/v_i\) is a direct function of the concentration of \(i_1\). A plot of \(1/v_i\) versus \(i_1\) will give a line with a slope

\[
\text{Slope} = (K_{m}/SV_{max} K_{R_1}) (1 + i_2/\alpha K_{R_2})
\]
The intercept on the Y axis is:

\[ \text{Intercept} = \frac{1}{V_{max}} + \left( \frac{K_i}{V_{max}} \right) \left( 1 + \frac{[I]}{K_{i2}} \right) \]

If \( \alpha \) exists and several plots of \( 1/v_i \) as a function of \( i_2 \) are constructed at different fixed concentrations of \( i_2 \), then both the slope and Y intercept will increase with increasing concentrations of \( i_2 \) and the resulting lines will converge at a common point. The \( abscissa \) value of this point equals \( \alpha K_{i2} \). 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 \( i_2 \). 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 \(-37.5 \, \mu\text{M}\). 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 1,10-phenanthroline 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 \( \alpha,\alpha'-\text{dipyridyl} \) binds to the zinc of alcohol dehydrogenase, new absorption peaks appear in the difference spectrum of the chelator-enzyme complex. The \( \alpha,\alpha'-\text{dipyridyl}-\text{zinc-alcohol dehydrogenase} \) has an absorption maximum at 308 \( \mu\text{m} \) with a difference extinction coefficient of \( 1.1 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1} \) (13). The 1,10-phenanthroline-zinc-alcohol dehydrogenase complex has an absorption maximum at 297 \( \mu\text{m} \) with a difference extinction coefficient of \( 0.8 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1} \) (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 \( \mu\text{m} \) or 297 \( \mu\text{m} \).

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 \( \alpha,\alpha'-\text{dipyridyl} \) zine-alcohol dehydrogenase complex was determined to be \( 0.96 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1} \) at a wave length of 304 \( \mu\text{m} \). For the 1,10-phenanthroline-zinc-alcohol dehydrogenase complex a difference extinction coefficient of \( 0.82 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1} \) at 274 \( \mu\text{m} \) was used.

As can be seen in Figs. 6A and 7A the presence of I3TA restricts the binding of \( \alpha,\alpha'-\text{dipyridyl} \) 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:

\[
\text{Intercept} = \frac{1}{V_{max}} + \left( \frac{K_i}{V_{max}} \right) \left( 1 + \frac{[I]}{K_{i2}} \right)
\]
taken in apolar solvents such as dimethyl sulfoxide and 1-pro-
ol dehydrogenase as well as from apohemoglobin, and bovine
albumin. Secondly, the spectrum of hydrophobic residue in addition to an anionic group. It is be-
able to show that the zinc moiety of alcohol dehydro-
gase is located at the pyridine ring binding region of the enzyme
ent ions (22), and p-chloromercuribenzoate (23) strongly suggest
general protein denaturants as urea (19), low pH (20, 21), deter-
sists (22), and p-chloromercuribenzoate (23) strongly suggest
k the protein surface environment at the coenzyme binding site. Use of such
eral protein denaturants as urea (19), low pH (20, 21), deter-
ents (22), and p-chloromercuribenzoate (23) strongly suggest
duction of tertiary protein structure that is generally not reversible,
tics (22), and p-chloromercuribenzoate (23) strongly suggest
k the protein surface environment at the coenzyme binding site. Use of such
ential observation is that ADP-ribose is the minimal structure required for
ction of tertiary protein structure that is generally not reversible,
structurally related proteins 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**

The Interaction of Triiodothyroacetic Acid with Horse Liver Alcohol Dehydrogenase
Kenneth McCarthy and Walter Lovenberg

J. Biol. Chem. 1969, 244:3760-3765.

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