Purification and Regulatory Properties of the Adenosine Diphosphate-activated Threonine Dehydratase*

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SUMMARY

The ADP-activated threonine dehydratase of Clostridium tetanomorphum has been purified approximately 1100-fold in 20% yield and crystallized. Such preparations are homogeneous when examined by sedimentation, electrophoretic, and immunological techniques. The pure enzyme displays an absorption maximum at 415 nm in neutral solutions, which is consistent with a Schiff base linkage between pyridoxal phosphate and an amino group donor on the protein.

Kinetic analyses performed at pH 8.0 in phosphate buffer gave evidence only for normal Michaelis behavior, even in the absence of the allosteric activator ADP. Cooperative effects were observed between ADP and L-threonine. Assays conducted in the absence of phosphate and ADP, however, exhibited an abnormal kinetic behavior which normalized as reaction time increased. Under similar conditions, the enzyme was observed to dissociate into subunits which reaggregated when ADP or L-threonine was present. These findings suggested that the abnormal kinetics was due to a dissociation-association reaction which occurred under conditions of the enzymatic assay. ADP, threonine, and phosphate were capable of preventing the dissociation to varying extents, but only ADP was observed to have the additional effect of altering the binding of substrate to enzyme.

Previous investigations on the inducible threonine dehydratase (l-threonine hydrolyase (deaminating), E.C. 4.2.1.16) from Clostridium tetanomorphum have established a regulatory role for this enzyme, based on its activation by nucleoside diphosphates, particularly ADP (1–3). The partially purified enzyme was shown to have an approximate molecular weight of 160,000 and could be dissociated into fragments of 120,000, 80,000, and 40,000 when treated with sodium dodecyl sulfate, heat, high pH, or simply stored in the frozen state (4). ADP could prevent this dissociation and could produce some degree of reassociation of the small molecular weight components.

Kinetically, threonine dehydratase has been reported to be a typical allosteric protein, which exhibits a sigmoidal relationship of threonine concentration to reaction velocity when assayed in the absence of ADP (5, 6). Normalized kinetics was observed when ADP was added. In this respect, the enzyme appears to differ from the inducible threonine dehydratase of Escherichia coli, which has a relatively similar subunit structure, but which exhibits typical Michaelis kinetics both in the presence and absence of AMP, the allosteric modifier (7).

The present paper describes experiments which were carried out with two aims. First, a purification procedure was sought which would provide relatively large amounts of pure threonine dehydratase for chemical and physical studies on the nature of the binding sites for ADP and substrate. Secondly, an elucidation of the relationship between ADP activation observed by kinetic measurements and the changes in quaternary structure promoted by ADP was desirable, since previous investigations had not attempted to interpret either phenomenon in terms of the other.

The results presented here describe the total purification and crystallization of the enzyme by a new procedure. Criteria of purity and properties of the pure enzyme are given. In addition, data are presented on the kinetic behavior and subunit structure of threonine dehydratase. These data are consistent with a model in which both ADP and threonine promote a closer association of enzyme subunits, and ADP functions additionally to increase the affinity of enzyme for substrate. This latter effect is dependent on the prior conversion of any dissociated forms to the native oligomer.

MATERIALS AND METHODS

C. tetanomorphum, ATCC 3000, was grown anaerobically at 37° on a complex glutamate medium (2) supplemented with 0.2% L-threonine. For enzyme isolation, six 13-liter carboys were started with a 10% inoculum and harvested in a Sharples centrifuge after growth for 20 hours.

Enzyme activity was determined with a Gilford model 2000 spectrophotometer at 340 μm and 25° by the use of an NADH-
lacate dehydrogenase coupled assay. A cuvette with a 1-cm light path and total reaction volume of 0.2 ml normally contained enzyme; 20 μmoles of potassium phosphate buffer, pH 8.0; 1 μmole of ADP; 0.1 μmole of NADH; 1 μmole of dithiothreitol; 25 μg of lactate dehydrogenase (Sigma Type II); and 25 μmole of l-threonine. After preliminary incubation for 5 min, reactions were initiated by the addition of substrate. One unit of activity is defined as that amount of enzyme which produces a change of 1.0 A per min or 0.032 μmole of α-ketobutyrate per min. The specific activity represents enzyme units per mg of protein. Protein was determined by the method of Lowry et al. (8).

All standard chemicals were reagent grade and were used without further purification. Ion exchange celluloses were obtained from Bio-Rad Laboratories. Dithiothreitol was purchased from Calbiochem, and ADP from Schwarz BioResearch, Inc. All other biochemicals, unless stated otherwise, were obtained from Sigma Chemical Company. Hydroxylapatite was prepared as described by Levin (9).

Measurements of pH were performed at 25° and are otherwise uncorrected. A Beckman expandomatic meter equipped with a combination electrode was used to determine the pH of reaction mixtures. The normal assay conducted in phosphate buffer at pH 8.0 decreased in pH to approximately 7.8 after reaction for 30 min. In all kinetic results presented, the pH stated is that determined after reaction had proceeded for 30 min.

Sucrose density gradient centrifugation was performed essentially as described by Martin and Ames (10). The SW-39 rotor and a Spinco model L2 preparative ultracentrifuge were used for all experiments. Rotor temperature was maintained at 4° throughout the 16-hour centrifugation. Approximately 30 fractions, of 0.15 ml each, were collected by bottom puncture and assayed for dehydratase activity as described above. Yeast alcohol dehydrogenase assays were performed by the procedure of Vallee and Hoch (11), but were scaled down 15-fold from the published procedures.

RESULTS

Throneine Dehydratase Purification

All operations were performed at 0-4° unless otherwise specified. The term, phosphate-mercaptoethanol buffer, will be used to refer to 0.05 M potassium phosphate, pH 7.2, containing 0.01 M 2-mercaptoethanol.

Step 1—Frozen cell paste, 140 g, was thawed in 300 ml of phosphate-mercaptoethanol buffer and subjected to 12 15-sec treatments of sonic oscillation with a Branson LS-75 Sonifier operated at maximum power. The supernatant obtained after centrifugation for 30 min at 27,000 × g was decanted. The same procedure was followed for the next (NH₄)₂SO₄ precipitation between 43 and 53% saturation. The precipitate was dissolved in a minimal amount of 0.4 M potassium phosphate buffer, pH 7.0, frozen rapidly, and kept overnight at -20°.

The combined supernatants from the heat treatment were concentrated by the addition of solid (NH₄)₂SO₄ to 60% saturation. The precipitate was dissolved in a minimal amount of phosphate-mercaptoethanol buffer (approximately 75 ml). This solution was passed through a Sephadex G 25 fine column (4.8 × 48 cm) to remove sulfate and phosphate ions. The Sephadex was in equilibrium with a 0.1 M Tris-HCl buffer, pH 7.6, containing 1 M KCl. The enzyme was eluted with this same solution.

Step 5—A hydroxylapatite column (3.4 × 22 cm) was equilibrated with 0.1 M Tris-HCl buffer, pH 7.6, plus 1 M KCl. The enzyme from Step 4 was applied to the column, and then the column was washed with the equilibration buffer until the effluent was colorless. The column was then eluted by a concave gradient consisting of 100 ml each of the following molarity potassium phosphate buffers, pH 7.2, made 0.01 M with respect to 2-mercaptoethanol, and delivered from a Buchler Varigrad: 0.04, 0.05, 0.12, 0.16, 0.20, 0.25, 0.30, and 0.40 M. Fractions of 10 ml were collected. Enzyme-containing fractions were pooled, and the salt concentration was determined with a conductivity meter (Barnstead purity meter) and diluted to a salt concentration of 0.07 M or below.

Step 6—The diluted enzyme solution from above was applied to a DEAE-cellulose column (2.2 × 40 cm) which had been equilibrated with phosphate-mercaptoethanol buffer. Gradient elution was achieved as in the previous step but consisted of 100 ml each of the following molarities of potassium phosphate buffer, pH 7.2, plus 0.01 M 2-mercaptoethanol: 0.08, 0.10, 0.12, 0.16, 0.20, 0.25, 0.30, and 0.30 M. The active fractions were pooled and diluted until the salt concentration was below 0.07 M.

Step 7—Enzyme from the previous step was absorbed onto a column of Ecteola-cellulose (Bio-Rad, Richmond, California), 2.2 × 40 cm, which was equilibrated with phosphate-mercaptoethanol buffer. The column was developed with a potassium phosphate gradient prepared from 100-m1 aliquots of the following molarities at pH 7.2 in the presence of 0.01 M 2-mercaptoethanol and 0.2 mM ATP: 0.06, 0.08, 0.10, 0.12, 0.16, 0.20, 0.25, and 0.30 M. The enzyme eluted with the ATP front.

Under most conditions, a single protein peak with an unsymmetrical trailing edge was observed eluting from the Ecteola-cellulose column. For maximum purification, the last tubes from the trailing edge were not combined with the major portion of the peak, since the fractions in these tubes had reduced specific
ADP-activated Threonine Dehydratase  
Vol. 243, No. 6  7.2) was slowly added until the bulk of the precipitate had dissolved. Following brief centrifugation, the clear yellow supernatant was placed at room temperature. Crystallization began within 3 hours. A photomicrograph of the crystals is shown in Fig. 1.

A tabulation of the results of the purification is presented in Table I. Although determination of the final specific activity is made difficult by the small amount of protein present, the best estimate is 15,800 ± 200 units per mg of protein, or approximately 500 i.u. per mg.

Criteria of Purity

Preparations having a specific activity of 15,000 or greater were examined for homogeneity by sedimentation velocity, immunological specificity, electrophoresis on cellulose acetate, and by polyacrylamide disc electrophoresis.

Sedimentation patterns illustrated in Fig. 2, a through c, revealed no detectable deviation from homogeneity. The enzyme migrated as a single component, with a calculated $s_{20, v}$ of 7.70 S. This value was determined for the stated concentration and has not been estimated for infinite dilution. Sucrose density gradient centrifugation conducted at a protein concentration of 30 μg/4.5 ml of gradient, with phosphate buffer, pH 7.0, and 1 mM ADP, gave a sedimentation coefficient of 7.6 ± 0.1 S.

Electrophoresis of 60 μg of protein on cellulose acetate in 0.03 M phosphate buffer, pH 7.6, or disc electrophoresis, with a pH of 7.6 in the separating gel and a protein sample of 20 μg, showed only a single band when stained with Amido black 10B or aniline blue-black. Some noncrystalline preparations did, however, contain a trace component, estimated to be less than 5% of the total, which migrated approximately twice as fast as the major protein band. This component was never observed in crystalline preparations and therefore has been regarded as a probable contaminant in solutions of specific activity 15,000 to 15,600.

Antiserum obtained from a rabbit which had received a subcutaneous injection of 3 mg of a preparation of specific activity 15,300, emulsified in Freund's complete adjuvant, was examined against purified enzyme fractions with the use of the Ouchterlony gel diffusion technique (12). The results indicated that a single antigenic component was present in all instances and further support the claim of homogeneity for the sample of enzyme used for immunization.

A partial absorption spectrum of the pure enzyme is presented in Fig. 3. This spectrum reveals a single absorption maximum

Table I  
Summary of l-threonine dehydratase purification

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Volume</th>
<th>Amount</th>
<th>Protein</th>
<th>Specific activity</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ml</td>
<td>units/ml</td>
<td>mg/ml</td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>Crude extract</td>
<td>800</td>
<td>261</td>
<td>18.0</td>
<td>14.5</td>
<td>100</td>
</tr>
<tr>
<td>Protamine sulfate</td>
<td>940</td>
<td>248</td>
<td>11.3</td>
<td>22</td>
<td>111</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ (43 to 53%)</td>
<td>119</td>
<td>1,760</td>
<td>26.7</td>
<td>66</td>
<td>100</td>
</tr>
<tr>
<td>Heat, 60°C</td>
<td>165</td>
<td>1,100</td>
<td>6.3</td>
<td>174</td>
<td>87</td>
</tr>
<tr>
<td>Hydroxyapatite</td>
<td>200</td>
<td>750</td>
<td>0.4</td>
<td>1,875</td>
<td>72</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>200</td>
<td>667</td>
<td>0.08</td>
<td>7,500</td>
<td>57</td>
</tr>
<tr>
<td>Crystallization</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crystals</td>
<td>0.6</td>
<td>26,100</td>
<td>1.66</td>
<td>15,700</td>
<td>7.5</td>
</tr>
<tr>
<td>Supernatant</td>
<td>0.9</td>
<td>33,300</td>
<td>2.08</td>
<td>16,000</td>
<td>14.4</td>
</tr>
</tbody>
</table>

activities. The pooled fractions were concentrated by the following procedure. The enzyme solution from Step 7 was diluted to below 0.07 M phosphate concentration and absorbed onto a small (volume, about 5 ml) column of DEAE-cellulose prepared as in Step 6. Elution was accomplished with 0.4 M potassium phosphate buffer, pH 7.0. Under these conditions, the enzyme eluted as a yellow fraction in a total volume of 5 to 15 ml. Further concentration could be achieved by addition of solid (NH₄)₂SO₄ to 60% saturation, centrifugation, and dissolution of the yellow precipitate in a minimal amount of 0.4 M potassium phosphate buffer, pH 7.0. At this stage, activity could be preserved in the frozen state for periods of several months. Addition of dithiothreitol to 0.01 M concentration improved stability markedly, but interfered with determination of protein content.

Step 8—Crystallization of the enzyme was accomplished on samples which had been concentrated by the (NH₄)₂SO₄ precipitation step described above. The precipitate was then resuspended in 0.4 ml of a solution of 0.4 M potassium phosphate buffer, pH 7.2, containing (NH₄)₂SO₄ dissolved to 35% saturation (calculated as for water). Phosphate buffer (0.4 M, pH 7.2) was slowly added until the bulk of the precipitate had dissolved. Following brief centrifugation, the clear yellow supernatant was placed at room temperature. Crystallization began within 3 hours. A photomicrograph of the crystals is shown in Fig. 1.

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A partial absorption spectrum of the pure enzyme is presented in Fig. 3. This spectrum reveals a single absorption maximum
at 415 μm, pH 7.5, as expected for a phosphopyridoxal-containing protein having the coenzyme bound as a hydrogen-bonded Schiff base. Treatment of native dehydratase with sodium borohydride at pH 8.0 resulted in the loss of absorption at 415 μm and a concomitant loss in enzymatic activity. These findings suggest that the coenzyme binding is similar to that in the E. coli threonine dehydratase (13) and that the enzymatic reaction is initiated by a transaldimination between n-threonine and an enzyme-phosphopyridoxal Schiff base.

**Kinetic Behavior of Reaction**

Previous reports on clostridial threonine dehydratase have documented that while hyperbolic curves of substrate concentration versus velocity were obtained when ADP was included in the reaction mixture, sigmoidal curves were observed when ADP was omitted (6). The present experiments, however, revealed that the velocity dependence on n-threonine concentration was hyperbolic under the usual reaction conditions, regardless of whether ADP was present or absent. Representative double reciprocal plots illustrating the linearity expected for such a kinetic relationship are presented in Fig. 4. Michaelis constant estimates from these data were 54 μM without ADP and 3.5 μM with ADP; the initial pH was 8.0 and final pH was 7.8 in all cases. These values compare reasonably well with those of Nakazawa and Hayaishi (6), who reported 37 μM and 3.0 μM, respectively. The latter values were obtained at a slightly higher pH (8.4) and in Tris buffer. In all cases, maximum velocities were identical with and without ADP. Considerably different values were reported by Whiteley and Tahara (5), but their results were obtained at a much higher pH (9.6) and were dependent on the age of the enzyme preparation.

The dissociation constants for ADP may be estimated with the use of the kinetic treatment described by Frieden (14). The applicable rapid equilibrium mechanism for this reaction would be as follows.

\[
E + S \rightleftharpoons ES \quad (K_1)
\]
\[
E + M \rightleftharpoons EM \quad (K_2)
\]
\[
ES + M \rightleftharpoons EMS \quad (K_3)
\]
\[
EM + S \rightleftharpoons EMS \quad (K_4)
\]

where \( K_1, K_2, K_3, \) and \( K_4 \) refer to the dissociation constants for the respective reaction and \( M \) represents the concentration of the allosteric modifier ADP. Furthermore,

\[
ES \rightarrow E + P \quad (V)
\]
\[
EMS \rightarrow EM + P \quad (V')
\]

where \( V \) and \( V' \) are the maximum velocities in the absence and presence of modifier, respectively.

The double reciprocal plots for threonine in the presence and absence of ADP indicate that the appropriate case for this enzyme is that of a competitive activator, where \( V = V' \) and \( K_2 > K_3 \) (14).

Evaluation of the respective dissociation constants for ADP is achieved from the ratio of Michaelis constants for threonine in the presence and absence of ADP, since this ratio is equal to \( K_1/K_3 \). The values previously given for these Michaelis constants yield a result of 0.065 for this ratio. \( K_1 \) can be obtained from measurements of the velocity of dehydration as a function of ADP concentration when substrate is totally saturating. In Fig. 5 the reciprocal of the difference in velocity with and without ADP (\( v - v_0 \)) versus the reciprocal of ADP concentration is plotted, with each curve being obtained at a different concentration of L-threonine. Calculation of an activator constant
As mentioned previously, double reciprocal plots with substrate and velocity as variables were essentially linear under the usual conditions of assay. Alterations in pH, however, produced considerable effect on the $K_m$ for threonine, both with and without ADP present. Furthermore, at pH values of 8.5 or higher, deviation from a simple linear relationship became marked when ADP was omitted from the reaction mixture. As illustrated in Fig. 6A, the $K_m$ for threonine undergoes an increase as the pH is increased or decreased from approximately pH 7.9 when ADP is included. In Fig. 6B, similar changes are noted in the absence of ADP, but at pH 8.6 a definitely bimodal curve is obtained. Reasonable values for the Michaelis constant can be calculated only if the high substrate points are used for slope determination.

Effects of pH on Reaction Kinetics

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Measurements of the $K_m$ for threonine as a function of reaction pH have been made with and without ADP present in the reaction mixture. When these data were plotted according to the rules outlined by Dixon and Webb (15), and straight line segments drawn with integral values for slope, several interesting points emerged (Fig. 7). The intersections of the straight line segments occur at nearly similar pH values on the two plots, with the only exceptions being a break at pH 8.6 which occurs when ADP is absent, and a shift in a break from 6.85 without ADP to 6.40 with ADP. Both breaks in the curves have positive changes in slope, an indication that these points are due to dissociations occurring in the enzyme-substrate complex and not to dissociations of free enzyme or free substrate. It can be speculated that the dissociation occurring at pH 8.6 is due to a tyrosyl residue which is located at or near the ADP-binding site. Such a group has recently been implicated in the allosteric center.
of threonine dehydratase and is protected by ADP against diazotization with diazobenzene sulfonic acid (16).

Confirmation for the change in the dissociation constant in the acid region was obtained from log $V_{\text{max}}$ versus pH plots. These results indicated a break at pH 6.4 in the presence of ADP, but in the absence of ADP, the dissociation occurred at pH 7.1 (as opposed to 6.85, calculated from the log $K_m$ versus pH plot). Thus, the shift seems significant. Other dissociation constants in the enzyme-substrate complex could not be determined from the $V_{\text{max}}$ data, since there appears to be a slight increase in $V_{\text{max}}$ associated with the change in buffer from phosphate to carbonate. Since an upward change in log $V_{\text{max}}$ versus pH plots is not interpretable, no significance can be attached to data in the pH range of 8 to 9. The results obtained from the $pK_m$ versus pH plot, however, are considered reliable because $K_m$ is not affected by buffer type.

**Structural and Kinetic Alterations at High pH**

The biphasic curve illustrated in Fig. 6B suggested that increasing threonine concentration produced a radical change in the properties of this enzyme when ADP was absent and the pH was high. The results presented in that figure were taken from velocity measurements made during the first 5- to 10-min period of the reaction. The nature of the assay employed (i.e., semi-continuous monitoring of absorbance changes) permits rate estimation during any part of the reaction. Observations of reaction rates after the initial 10-min period revealed that the velocity of those reactions conducted at high pH in the absence of ADP steadily increased with reaction time. Fig. 8 illustrates the changes which are seen in the double reciprocal plot when velocities are calculated only over selected time intervals. As can be seen, the rates (and therefore the slopes of the plots) are considerably dependent upon the time interval over which the rates are measured. After reaction times of nearly 30 min, the rates had increased so that a nearly linear double reciprocal plot was obtained from what had previously been definitely bimodal. Conventional plots of velocity versus substrate concentration for the data presented in Fig. 8 illustrate the sigmoid character previously reported for this pH range without phosphate or ADP (5, 6). The extent of the sigmoidicity decreases markedly as reaction time increases. These observations would indicate that a time-dependent phenomenon involving substrate in the absence of ADP was being observed.

A replott of the data according to the Hill equation (17) shows two portions of linearity for each curve (Fig. 9). At high concentrations of L-threonine, the slope is unity regardless of the extent of reaction; low threonine levels yield a family of curves as a function of reaction time. In the latter case, the Hill coefficient...
(slope) decreases from about 2.6 to 1.7 as reaction extent increases.

The most reasonable explanation for these results is that L-threonine can participate in some activation process aside from its normal role as substrate. If it is assumed that high pH produces some conformational change when ADP is not present, then L-threonine could assist in restoring the native conformation, with the time required to bring about this change being inversely proportional to the threonine concentration.

Support for conformational changes occurring at high pH has been obtained from sucrose density gradient centrifugation experiments conducted at various pH values, in the presence and absence of ADP or L-threonine. For these experiments, sucrose solutions were prepared in the appropriate buffers, with or without modifier, and the pH was readjusted to the desired value.

When ADP was to be present in the gradient, the enzyme was preincubated with ADP at the same concentration to correspond to the brief preliminary incubation period present in all kinetic assays.

Table III presents the sedimentation coefficients observed under the different conditions of centrifugation. The results clearly show that phosphate buffer tends to promote the formation of the aggregated (native) form of the enzyme, but with decreasing efficiency as the pH is increased. Tris buffer, on the other hand, permits dissociation into subunits in the absence of ADP and threonine at all tested pH values, but this dissociation is reversed to a considerable extent by the addition of ADP at a final concentration of 1 mM or by L-threonine at a final concentration of 50 mM. Even at the lower pH values tested for Tris buffer, dissociation of native enzyme to a lower order of structure was always observed under these conditions. This is in keeping with the suggestion that the presence of ADP or threonine at high concentrations produces conformational changes leading toward aggregation and restoration of native enzyme structure, particularly when Tris buffers are used instead of phosphate.

**DISCUSSION**

Although several reports of purification schemes for threonine dehydratase from *C. tetanomorphum* have recently appeared (5, 6), the present paper describes the first successful complete purification and crystallization of this enzyme. The yield of pure enzyme obtained through this procedure is reasonably good, considering the large number of steps required for purification. The time necessary to complete the procedure is approximately 5 days, but loss in activity due to storage during purification is small. Equally good purification has been obtained on a smaller scale than that described here, but the total quantity of enzyme obtained would normally not justify smaller operations. No attempt to scale up the present procedure has been made.

Measurements of certain physical constants, particularly molecular weight and pyridoxal phosphate content, and total amino acid analysis are currently in progress in this laboratory.

The kinetic behavior exhibited by threonine dehydratase, under the optimal assay conditions used in the present investigation, differs somewhat from that reported by others (5, 6), in that Michaelis kinetics is followed at neutral pH values with or without ADP, in contrast to the allosteric (sigmoidal) kinetics previously reported. These differences, however, appear to lie in the nature of the experimental conditions of the enzyme assay, and possibly in the nature of the assays. Tokushige, Whiteley, and Hayaishi (2), Whiteley and Hayaishi (3), Whiteley (4), and Whiteley and Tahara (5) have used a fixed time estimate of α-ketobutyrate production by dinitrophenylhydrazine formation. The reaction buffer was Tris-HCl at pH 9.5. Nakazawa and Hayaishi (6) used a similar assay but with Tris-HCl buffer.
of pH 8.4. The present work has used a coupled assay with phosphate, or in some cases, carbonate buffers. In the reactions containing phosphate buffer, normal Michaelis kinetics was always observed, provided the pH was below 9.0. In contrast to this, the use of Tris buffer at pH 8.4 or 9.6, or carbonate buffer of pH 8.5 or higher, led to sigmoidal kinetics when ADP was absent from the assay.

These kinetic findings were clarified by the results of sucrose density gradient centrifugation experiments wherein it was observed that a lowering of the apparent sedimentation coefficient occurred whenever phosphate buffer or ADP was absent. This dissociation could be prevented by the presence of phosphate, ADP, or L-threonine, but with decreasing effectiveness as the pH was increased. Under conditions of Tris buffer at pH 8.6, nearly all of the enzyme sedimented as a component with an S20,w of 4.5 S, whereas phosphate buffer of the same pH yielded a sedimentation coefficient of 7.6 S.

Thus, it can be stated that considerable differences exist in the state of aggregation of threonine dehydratase when phosphate and Tris buffers are used. These differences become less noticeable as the pH is increased, since this favors dissociation regardless of buffer type. Inclusion of ADP or L-threonine tends to minimize the differences in aggregation by promoting association of subunits, even in Tris buffer. Although it would appear that phosphate is capable of promoting aggregation of subunits into the native enzyme of 7.7 S, phosphate ions apparently do not affect enzyme-substrate interaction since the Kᵦ for threonine is virtually identical in Tris and phosphate buffers, either in the presence or absence of ADP.

One explanation for the observed sigmoidal kinetics, based on the conformational changes observed at high pH as threonine concentration increases, can be put forth. If it is assumed that the dissociated forms are inactive or nearly so, then increasing the threonine concentration promotes aggregation of these subunits into an active oligomer. The observable result is a marked stimulation of enzyme activity above the threonine level at which aggregation begins to be significant. This stimulation would continue until association is complete, whereupon normalized kinetics would prevail. Because ADP and inorganic phosphate protect effectively against dissociation and are capable of reassociating subunits, no such sigmoidal behavior is observed when these components are present. This ability of threonine to promote aggregation may be due to the binding of substrate at a specific site not identical with the normal substrate site (active site), but such an "activator" site for substrate is not observed. Nakazawa and Hayashi (6), in explanation of their kinetic data, have postulated a second threonine-binding site. A direct approach to detect an additional threonine site is available, and experiments are planned to establish this point.

Of the three compounds that can produce association of dehydratase subunits, only ADP has been clearly shown to have an additional activating effect on the catalytic process, namely that of lowering the Kᵦ for threonine. On the other hand, for activation by threonine, the present data do not give evidence for homotropic effects analogous to ADP activation, since normal Michaelis kinetics is exhibited once the native dehydratase conformation has been assumed. This leads to the conclusion that threonine is incapable of homotropic activation that would affect binding at the active center, and therefore cannot replace ADP as an activator. Thus, it can be postulated that threonine is effective in promoting association of enzyme subunits and serves as substrate, while ADP possesses the ability to affect the Michaelis constant of the native enzyme for threonine, in addition to its role in aggregation of dehydratase subunits. A summary of these relationships is illustrated in Fig. 10.

The significance of the dissociation of native enzyme and the appearance of sigmoidal kinetics in the absence of ADP and phosphate is obscure. Presently no data are available to indicate whether the intracellular concentrations of phosphate and ADP are ever sufficiently low to permit dissociation and the observation of a sigmoidal relationship in vivo. The results presented here suggest only that, aside from phosphate concentration, the relative amounts of ADP and L-threonine are of major importance in regulating the rate of dehydratase action in vivo. The marked increases in reaction velocity with increasing ADP, when substrate is not at the saturating level, could provide an effective regulatory mechanism without the necessity of invoking a sigmoidal relationship of velocity to threonine concentration, dependent upon changes in enzyme aggregation state.

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Purification and Regulatory Properties of the Adenosine Diphosphate-activated Threonine Dehydratase
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