The effect that a modifier or allosteric regulator may have on an enzyme has been examined in several ways (1, 2). In general, the greatest emphasis has been placed on a kinetic approach (3–6) but protection against inactivation (7–12) and changes in the sedimentation properties (13–17) have also been measured. Association of isolated lower molecular weight materials, obtained in partially purified form by gel filtration, is promoted by ADP and threonine. The ability to be aggregated by ADP may be lost by prolonged exposure to dissociating agents. ADP is more efficient than threonine in preventing dissociation or promoting association.

At least eight molecular species, detected by a sucrose gradient centrifugation and gel filtration, have been derived from purified preparations of the enzyme. Seven of these are enzymatically active; the smallest forms, produced by alkylation following treatment with urea and β-mercaptoethanol, are enzymatically inactive. The kinetic properties and heat stability of the fully polymerized enzyme and three of the dissociated forms have been compared.

Threonine deamination occurs upon exposure to heat, to alkaline pH, or to denaturing agents, or when preparations are diluted or allowed to age at $-20^\circ$. Dissociation in the presence of urea, detergents, or an alkaline pH may be prevented by addition of adenine diphosphate, threonine, phosphate buffer at pH 7.0, or monovalent or divalent cations. Association of isolated lower molecular weight materials, obtained in partially purified form by gel filtration, is promoted by ADP and threonine. The ability to be aggregated by ADP may be lost by prolonged exposure to dissociating agents. ADP is more efficient than threonine in preventing dissociation or promoting association.

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**SUMMARY**

Dissociation of the threonine deaminase of *Clostridium tetanomorphum* occurs upon exposure to heat, to alkaline pH, or to denaturing agents, or when preparations are diluted or allowed to age at $-20^\circ$. Dissociation in the presence of urea, detergents, or an alkaline pH may be prevented by addition of adenine diphosphate, threonine, phosphate buffer at pH 7.0, or monovalent or divalent cations. Association of isolated lower molecular weight materials, obtained in partially purified form by gel filtration, is promoted by ADP and threonine. The ability to be aggregated by ADP may be lost by prolonged exposure to dissociating agents. ADP is more efficient than threonine in preventing dissociation or promoting association.

At least eight molecular species, detected by a sucrose gradient centrifugation and gel filtration, have been derived from purified preparations of the enzyme. Seven of these are enzymatically active; the smallest forms, produced by alkylation following treatment with urea and β-mercaptoethanol, are enzymatically inactive. The kinetic properties and heat stability of the fully polymerized enzyme and three of the dissociated forms have been compared.

The possible deamination of other amino acids was examined by incubating 0.1 to 0.5 mg of protein from partially purified fractions in stoppered test tubes with 50 μmoles of the amino acid, 50 μmoles of Tris buffer (pH 7.0), 20 μmoles of β-mercaptoethanol, and 10 μg of pyridoxal phosphate in a total volume of 0.5 ml. The reaction mixtures were incubated for $\frac{1}{2}$ to 6 hours and ammonia was determined with Nessler’s reagent (24).

Estimation of Molecular Weight by Gel Filtration—Enzyme preparations were passed through columns (2.5 × 50 cm) of Sephadex G-100 or G-150 prepared and eluted according to the method of Andrews (25). Columns were standardized by determining the elution pattern for each of the following: catalase, β-globulin, glucose 6-phosphate dehydrogenase, hemoglobin, calf alkaline phosphatase, bovine heart cytochrome c, bovine serum albumin, vitamin B₁₂, and FMN. Hemoglobin, cytochrome c, vitamin B₁₂, and FMN were determined from measurements of optical density at 410, 408, 360, and 450 nm, respectively. Catalase, β-globulin, and serum albumin were detected by absorbance at 280 nm. Alkaline phosphatase was assayed by measuring the hydrolysis of p-nitrophenyl phosphate in a reaction mixture containing 0.6 μ Tris buffer at pH 9.4, 10⁻⁴ M MgCl₂, and 2 × 10⁻³ M p-nitrophenyl phosphate. The reaction was stopped by the addition of 1 ml of 2 M phosphate buffer at pH 8.5 and the optical density at 410 nm was measured. Glucose 6-phosphate dehydrogenase was assayed according to the method of Kornberg and Horecker (26).

**EXPERIMENTAL PROCEDURE**

**Assays**—The assay of threonine deaminase, the methods used for enzyme purification, and the binding of ¹⁴C-ADP have been described (23). In experiments with preparations of low specific activity, the assay incubation period was extended to 1 to 12 hours, depending on the preparation used. Specific activity is expressed as micromoles of α-ketobutyrate formed per mg of protein per 20 min in the standard assay.

The possible deamination of other amino acids was examined by incubating 0.1 to 0.5 mg of protein from partially purified fractions in stoppered test tubes with 50 μmoles of the amino acid, 50 μmoles of Tris buffer (pH 7.0), 20 μmoles of β-mercaptoethanol, and 10 μg of pyridoxal phosphate in a total volume of 0.5 ml. The reaction mixtures were incubated for $\frac{1}{2}$ to 6 hours and ammonia was determined with Nessler’s reagent (24).

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†Recipient of Research Career Award GM-K6-422.
in the text. The tubes were centrifuged at 38,000 rpm in an SW 39 rotor of a Spinco model L centrifuge for 15 hours at 2°C, then punctured, and 46 to 48 fractions were collected and assayed immediately for threonine deaminase activity and protein content. Protein was determined by using one-fifth the volumes of all of the reactants in the standard protein assay described previously (23). β-Mercaptoethanol was omitted from the sucrose solutions in experiments in which protein was determined. Hemoglobin, catalase, and alcohol dehydrogenase were used as standards in estimating the sedimentation constants of enzyme preparations. Hemoglobin and catalase were determined as described; alcohol dehydrogenase was assayed according to Racker (28) in the presence of 0.1 M β-mercaptoethanol.

**Dissociation of Threonine Deaminase**—Three methods were used to obtain quantities of disaggregated enzyme: aging by storage at -20°C, dialysis for varying periods of time against 0.05 M Tris buffer 0.005 M β-mercaptoethanol, pH 9.5, and treatment with 2 M urea-0.05 M β-mercaptoethanol. Urea was recrystallized and prepared immediately before use (29).

To separate the desired molecular species, dissociated fractions were subjected to gel filtration by using a column (3 X 82 cm) of Sephadex G-100 calibrated as described above. The S value of each isolated fraction was estimated by sucrose density centrifugation. In some experiments, preparative sucrose gradient centrifugation was used to obtain the partially dissociated forms of the enzyme. In the latter method, either the SW 25 rotor or the 40 rotor (angle head) yielded fairly good separation of proteins. Amounts of 1 to 3 ml of dissociated fraction were layered on 22.5 ml of the above 5 to 20% sucrose gradients and centrifuged for 36 to 40 hours at 25,000 rpm in an SW 25 rotor of a Spinco model L centrifuge. Alternatively, 0.2- to 0.5-ml amounts of preparations were layered on 10.5 ml of the same gradient and centrifuged for 15 hours in the 40 rotor of a Spinco model L centrifuge. When either the SW 25 or 40 rotor was used, 60 to 65 fractions were collected and assayed. Urea, 0.5 M, and β-mercaptoethanol, 0.05 M, were included in the sucrose gradients and the eluting buffer used for gel filtration when urea-treated fractions were the source of the dissociated forms of the enzyme.

**Reductive Dissociation and Alkylation**—Concentrated purified fractions (300- to 700-fold purification) containing 0.5 to 4 mg per ml of protein were dialyzed at room temperature against either 0.5% sodium lauryl sulfate, 8 M recrystallized urea, or 4 M recrystallized guanidine in the presence of 0.1 M β-mercaptoethanol for 24 to 48 hours. Moniodoacetate (2 M) in 2 M Tris buffer, pH 8.5, was added to give a concentration of 0.3 M moniodoacetate, the preparation was left at room temperature for 1 hour, β-mercaptoethanol was added to give a concentration of 0.3 M, and the preparation was dialyzed for 12 hours against 500 volumes of 0.1% sodium lauryl sulfate. A small amount of precipitate was removed by centrifugation. Alternatively, the method of Anfinsen and Haber (30) was followed.

**RESULTS**

**Dissociation and Association Reactions**

1. Detection of Dissociated Forms—A single peak of enzyme activity corresponding to an S value of approximately 7.9 S (27)

1 Sufficient quantities of purified enzyme preparations have not been available to permit accurate determinations of molecular weights by analytical centrifugation. The present estimates are uncorrected values, based largely on detection of enzymatic activity, and the assumption that the proteins are spherical.
The sedimentation pattern obtained following dialysis in the absence of mercaptoethanol shows four peaks corresponding to those found in Curve B, three intermediate peaks falling between these, and two peaks having S values greater than 7.9. The regularity of the pattern found in Curve C (molecular weights for the seven major peaks shown in this experiment are estimated to be: 160,000, 140,000, 116,000, 100,000, 79,000, 63,000, and 40,000) suggests a basic subunit of approximately 2.3 S. Traces of enzymatic activity have been detected in the 1.8 to 2.3 S regions of sucrose gradients (and have also been found in gel filtration experiments in the 10,000 to 25,000 molecular weight regions), but this activity is extremely low and is readily lost (half-life of approximately 8 hours). The smallest unit possessing measurable and reasonably stable enzymatic activity is approximately 3.1 S (or approximately 40,000 molecular weight) as shown in Curves B and C of Fig. 2.

Support for the proposal that a smaller unit may be produced from the 3.1 S form comes from attempts to dissociate completely the enzyme under reduced conditions by using monoiodoacetate to prevent reassociation. When concentrated preparations of purified fractions (specific activity of 400 to 5000) were sedimented in a gradient containing no dissociating agents, the major peak of protein corresponded to 7.9 S (Fig. 3, Curve A). When the same material was centrifuged in a gradient containing 4 M urea, 4 M guanidine, or 0.5% sodium lauryl sulfate, no enzymatic activity could be detected but essentially all of the protein was present in a single broad peak having an estimated S value of approximately 3.1 (Fig. 3, Curve B). These preparations could be dissociated by dialysis (0.1 M mercaptoethanol and 4 M urea or 0.5% sodium lauryl sulfate) followed by alkylation with monoiodoacetate. Sedimentation of such preparations revealed a shift in the pattern with one, or possibly two, different species present. An estimated uncorrected S value of 2.3 was obtained for the main component of Curve B but more accurate information concerning the size of the basic subunit will have to come from analytical centrifugation.

2. Conditions Promoting Dissociation—Although dissociation or disaggregation of the enzyme in any partially purified fraction occurred to some extent on dialysis or aging, a greater over-all conversion of the 7.9 S species to components of lower S value could be achieved by the addition of urea, guanidine, or sodium lauryl sulfate as described above. As illustrated in Fig. 4 (Curve A), enzymatic activity was retained by the dissociated forms when these agents were incorporated into the gradients or onto gel elution solvents. The extent of conversion and the recovery of enzymatic activity depended not only on the type and concentration of dissociating agent used but also on the enzyme preparation. In general, ammonium sulfate fractions were more easily dissociated by low concentrations of hydrogen bond-breaking agents and yielded poor over-all recovery of activity (30 to 60%). On the other hand, highly purified fractions were more difficult to dissociate and gave higher recoveries, probably because most of the enzyme remained in the fully associated form.

Disaggregation of the 7.9 S enzyme to a mixture of several partially dissociated forms occurred during sedimentation or gel filtration at an alkaline pH (8.5 to 10.5), in the presence of low concentrations of urea (0.5 M), guanidine (0.2 M), or sodium lauryl sulfate (0.5%) and following heat inactivation (10 min at 55°) or dialysis (preparation left at 0° for 3 hours before sedimentation) or repeated freezing and thawing.
Fig. 4 shows the elution pattern obtained by gel filtration of an ammonium sulfate fraction (Curve A) dialyzed for 6 hours against 0.05% sodium lauryl sulfate-0.05 M β-mercaptoethanol. Peaks of enzymatic activity were eluted at volumes corresponding to 160,000, 122,000, 63,000, and 40,000. A separate experiment performed with an untreated crude extract (Curve B) is also shown. Sedimentation or filtration in the presence of 10−3 M p-hydroxymercuribenzoate, 0.1 M d-serine, 0.05 mM EDTA, 0.1 M L-isoleucine, or 0.05 M hydroxylamine resulted in some loss of activity depending on the inhibitor used, but did not promote significant dissociation of the enzyme.

3. Conditions Preventing Dissociation—The dissociation of the 7.9 S form of the enzyme in the presence of 2 M urea (shown in Curve A of Fig. 5), 0.1% sodium lauryl sulfate, or at pH 9.0 could be prevented by the simultaneous addition of a variety of compounds. The most efficient of these were nucleotide diphosphates (Fig. 5, Curve B). Comparison of nucleotides which stimulate threonine deamination (23) with those capable of preventing dissociation (Fig. 5, Curve C) was the most effective buffer although arsenate (0.2 M at pH 7.0) or pyrophosphate (0.2 M at pH 7.0) was partially effective. Phosphate buffer at pH 8.5 did not prevent dissociation by urea.

Dissociation was also prevented to varying degrees by buffers, salts, and substrates. Phosphate (0.2 M at pH 7.0) was the most effective buffer although arsenate (0.2 M at pH 7.0) or pyrophosphate (0.2 M at pH 7.0) was partially effective. Phosphate buffer at pH 8.5 did not prevent dissociation by urea.

The most efficient of these were nucleotide diphosphates (Fig. 5, Curve B). Comparison of nucleotides which stimulate threonine deamination (23) with those capable of preventing dissociation did not show any significant differences and all nucleotides tested (ADP, GTP, IDP, CDP, UDP, TDP, and dADP) reduced dissociation when present at a concentration of 2.5 × 10−3 M. At a concentration of 2.5 × 10−4 M, ADP, IDP, and GDP were perhaps slightly more effective than the other nucleotides, but this point was not tested exhaustively.

Dissociation was also prevented to varying degrees by buffers, salts, and substrates. Phosphate (0.2 M at pH 7.0) was the most effective buffer although arsenate (0.2 M at pH 7.0) or pyrophosphate (0.2 M at pH 7.0) was partially effective. Phosphate buffer at pH 8.5 did not prevent dissociation by urea.

KCl, NH4Cl, and NaCl (all at 0.2 M) were partially effective with many preparations but prevented dissociation more efficiently when freshly isolated fractions were used. Divalent cations (0.2 M) appeared to be more effective than monovalent cations. MgCl2, MnCl2, CaCl2, and BaCl2 were all approximately equal in preventing dissociation by urea.

Threonine (0.25 M) prevented dissociation only at low concentrations of urea or sodium lauryl sulfate and only when freshly isolated active fractions were employed. L-Serine (0.25 M), another substrate for the enzyme, was only partially effective under any conditions. Lower concentrations of threonine and serine (0.05 to 0.15 M) in the presence of urea, sodium lauryl sulfate, or an alkaline pH led to varying degrees of dissociation and to low recoveries suggesting inactivation of the enzyme.

Maximum recovery of total activity in sucrose gradient centrifugation experiments was approximately 90 to 100% and was observed consistently only in the presence of either nucleotide diphosphate, phosphate buffer, or MgCl2. As shown earlier (23), these compounds are also the most effective in protecting the enzyme from inactivation by heat or dilution. Data presented in a later section indicate that the low molecular weight forms may be inactivated more readily than the high molecular weight species. Hence, it seems probable that poor recovery in sucrose gradient centrifugation experiments in the absence of these agents may be due to dissociation followed by inactivation of the lower molecular weight forms.

4. Association of Lower Molecular Weight Forms—If preparations of 3.1 S and 4.7 S materials (obtained by large scale gel filtration or preparative gradient centrifugation) are sedimented in sucrose gradients containing various additions, association or aggregation can be detected. Formation of the 7.9 S material was observed in the presence of nucleotide diphosphates and with either threonine or serine but not with phosphate, KCl, or MgCl2. These observations are illustrated in Fig. 6. In this experiment, a fraction eluted from a column of Sephadex G-100 and estimated to have a sedimentation coefficient of 3.1 was sedimented in a sucrose gradient. As seen from Curve A, the preparation contained traces of activity in the 3.9 S and 4.7 S regions as well as a major peak in the 3.1 S region. Inclusion of either phosphate or MgCl2 (Curve B) gave slightly higher...
FIG. 6. Effect of ADP, threonine, and other compounds on the sedimentation of a low molecular weight form of threonine deaminase. Sucrose gradients contained: A, no additions; B, either 0.2 M phosphate buffer at pH 7.0 or 0.02 M MgCl$_2$; C, 0.25 M L-threonine; D, 2.5 $\times$ 10$^{-3}$ M ADP. Gradients were layered with 0.2 ml of a fraction (0.17 mg of protein) having an estimated molecular weight of 40,000 and obtained by gel filtration of an ammonium sulfate fraction aged by storage at $-20^\circ$C. Gradients were prepared, fractionated, and assayed as described in "Experimental Procedure" with an assay incubation time of 2 hours. Threonine deaminase activity = micromoles of $\alpha$-ketobutyrate produced in the standard assay by 0.1 ml of fraction.

activities in the 3.1 S region but did not alter the pattern of sedimentation. Addition of threonine (Curve C) or ADP (Curve D) yielded activity in the 6.3 S and 7.9 S regions. Gradients containing ADP always yielded higher activity and more complete conversion to the 7.9 S or fully associated form than gradients containing threonine.

Complete conversion of smaller species to the fully associated form was most frequently observed with freshly isolated fractions following addition of nucleotides. However, in some experiments, addition of ADP to small molecular weight forms resulted in peaks corresponding to 7.1 S, 5.5 S, and 3.9 S components in addition to the 7.9 S form. Incomplete aggregation (frequent conversion to the 4.7 S form) was also noted when some preparations of the 3.1 S units were sedimented with threonine or serine in the gradient. It is not known whether the formation of components with S values lower than 7.9 was due to suboptimal conditions of association (and sedimentation) or to partial inactivation of some combining site or sites required for the formation of the fully associated enzyme. That the latter may be possible is suggested by the observations discussed below that loss of aggregative ability occurs under certain conditions.

Concentration of solutions of the lower molecular weight materials also promoted association. If a preparation of 4.7 S material containing 0.2 mg of protein per ml (obtained by gel filtration) was concentrated to one-tenth the original volume and centrifuged in a sucrose gradient, peaks corresponding to 6.3 S and 7.9 S were found as well as 4.7 S. In addition, if a relatively impure preparation of 4.7 S materials was used, traces of enzymatically active proteins having S values greater than 7.9 were detected. It will be recalled that aggregates greater than 7.9 S were also found following dialysis of ammonium sulfate fractions in the absence of mercaptoethanol (Fig. 2, Curve C). Addition of ADP to the gradient containing a concentrated preparation of threonine deaminase resulted in an almost complete conversion of 4.7 S and 6.3 S materials to the 7.9 S form but did not increase the amount of enzyme present in aggregates larger than 7.9 S.

Conversely, many of the conditions promoting dissociation appear to interfere with aggregation caused by the factors discussed above. An alkaline pH (7.5 to 8.0), for example, not only increases the conversion of 7.9 S forms to 3.1 S forms but also prevents the association of 3.1 S units in the presence of either threonine or ADP. When ADP was used in this experiment, the enzyme was allowed to react with the nucleotide at pH 7.0 under the optimal conditions for binding (23) before being added to the gradient and a 20-fold higher concentration of ADP was used in the gradient to offset hydrolysis. In view of this observation, it seems plausible that ADP can maintain polymerization by preventing dissociation at an alkaline pH rather than by promoting re-association of dissociated molecules. As expected, the presence of urea, guanidine, or sodium lauryl sulfate also prevents association of the 3.1 S forms.

5. Loss of Ability to Undergo Association—Partially dissociated forms apparently lose the ability to undergo ADP- or threonine-promoted association after extensive dialysis in 2 M urea or at an alkaline pH in the absence of mercaptoethanol or after aging by storage at $-20^\circ$C. Such preparations were not, however, completely inactivated kinetically with respect to ADP since a measurable, albeit decreased, stimulation of enzymatic activity and ability to bind $^{14}C$-ADP remained. This behavior was observed most frequently with the 3.1 S form and to a lesser extent with the 3.9 and 4.7 S forms. However, virtually complete loss of ability to aggregate was also observed in mixtures of several species obtained after 48 to 96 hours of dialysis in the absence of mercaptoethanol. In the latter instance, seven to nine components of different S values (e.g. Fig. 2, Curve C) were found and addition of ADP did not change the sedimentation pattern appreciably. Neither threonine nor serine had any effect in promoting association of such aged or dialyzed preparations. These experiments suggest the possible destruction of sites required for polymerization.

Methods used for "desensitizing" the enzyme with regard to kinetic response to ADP and the binding of ADP (23) resulted in extensive conversion of the 7.9 S form to a mixture of smaller molecular weight materials. However, as stated above, the same desensitization was readily observed with isolated preparations of 3.1 S material on storage at 5" in air in the absence of mercaptoethanol. As seen from Table I, these preparations first lost the ability to undergo aggregation, as measured by changes in sedimentation. After 1 day of storage, ADP promoted aggregation of the 3.1 S material to 7.9 S. After 3 days of storage, 80% of the enzyme remained as a 3.1 S component despite the presence of ADP in the gradient. The same preparation, however, lost relatively little enzymatic activity (i.e. specific activity) and ability to bind ADP. The catalytic response to ADP (i.e. stimulation at a low concentration of threonine) was also largely retained. Upon continued storage the catalytic response to ADP and the ability to bind the nucleotide diminished greatly although the preparations retained partial ability to deaminate threonine.

Properties of Dissociated Forms

1. Kinetic Properties—The "fully associated" form and three units of different S value were examined with respect to $K_m$ values for threonine and $K_a$ values for ADP (Table II).
TABLE I

Effect of storage of 3.1 S form of threonine deaminase on enzymatic activity, stimulation by ADP, ability to bind 14C-ADP, and ability to aggregate

Preparation was obtained by gel filtration of an ammonium sulfate fraction (specific activity = 45) dissociated by treatment with urea as described in “Experimental Procedure.” Preparation was dialyzed for 12 hours against 500 volumes of 0.005 M β-mercaptoethanol prior to assay (1 day of storage). Experiment 1, assayed as described in “Experimental Procedure” by using 0.1 pC (0.8 mg of protein) incubated with 0.005 μC of 14C-ADP (Schwarz BioResearch, specific activity = 4,130 cpm μg) at 37°C for 5 min and passed through a column of Sephadex G-50 as described elsewhere (23). Counts per min refers to total radioactivity in the protein-containing fractions; Experiment 4, sucrose gradient sedimentation as described in “Experimental Procedure” by using 0.2 ml (0.8 mg of protein) on each gradient.

<table>
<thead>
<tr>
<th>Properties</th>
<th>1 day</th>
<th>3 days</th>
<th>8 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Specific activity with 0.1 M L-threonine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Without ADP</td>
<td>12.2</td>
<td>8.1</td>
<td>5.4</td>
</tr>
<tr>
<td>With ADP</td>
<td>20.8</td>
<td>17.3</td>
<td>7.2</td>
</tr>
<tr>
<td>2. Stimulation by ADP of the deamination of 0.01 M L-threonine</td>
<td>5-fold</td>
<td>4-fold</td>
<td>1.3-fold</td>
</tr>
<tr>
<td>3. Binding of ADP (counts per min of 14C-ADP per mg of protein)</td>
<td>290</td>
<td>220</td>
<td>50</td>
</tr>
<tr>
<td>4. Sedimentation in a sucrose gradient containing 2.5 × 10^{-4} M ADP</td>
<td>90% as 7.9 S, 10% as 6.3 S</td>
<td>20% as 4.7 S, 80% as 3.1 S</td>
<td>100% as 3.1 S</td>
</tr>
</tbody>
</table>

should be noted that the partially dissociated components were obtained by filtration of an ammonium sulfate fraction aged by storage, whereas the 7.9 S form came from a subsequent step of purification. The latter had a specific activity comparable to that found when freshly prepared ammonium sulfate fractions are subjected to gel filtration. However, the $K_m$ values shown in Table II have also been obtained (23) with 7.9 S preparations of greater purity (specific activities of 3000 to 5000).

The $K_m$ values for threonine obtained for the four preparations were similar but differences were found in the effect of ADP at both high and low substrate concentration. It will be recalled (23) that ADP has little or no effect on the activity of the associated enzyme at saturating substrate concentration. This is confirmed in Table II where the specific activities (determined at saturating substrate concentration) of the two larger forms were not altered by the addition of ADP. The specific activities of the two smaller forms, on the other hand, were significantly higher in the presence of ADP. It is not known at the present time if this effect of ADP on $V_{max}$ indicates a basic difference in the kinetic properties of the smaller molecular weight materials or results from inactivation of these forms in the absence of ADP. It is of interest that ADP also affects $V_{max}$ when mixtures of partially dissociated components are the source of the enzyme. This is illustrated in Fig. 7. In this experiment, a partially dissociated fraction containing approximately equal amounts of 6.3 S, 3.9 S, and 3.1 S materials (sucrose gradient analysis) was stimulated by ADP at a saturating substrate concentration. Preparations of 6.3 S and 3.1 S forms isolated from this mixture by gel filtration responded in accordance with the data given in Table II for the larger and smaller components. Other experiments (preceding paper (23), Figs. 1 and 2) had shown that aged preparations frequently showed a stimulation by ADP at saturating substrate concentrations. If such aging resulted in an appreciable disaggregation, the kinetic properties of the small molecular weight forms produced would explain the effect on $V_{max}$.

The partially dissociated preparations differ also in the response to ADP at low substrate concentrations. Since the maximum ADP effect is seen at low substrate concentration, the $K_{ADP}$ for ADP was measured at one-tenth the saturating concentration of threonine. As shown in Table II, the larger units have a lower $K_{ADP}$ for ADP at this substrate concentration than the two smaller forms.

The sigmoidal curves found in response to increasing substrate concentration (23) with purified enzyme fractions were found also with dissociated forms. Analysis of the kinetic data

<table>
<thead>
<tr>
<th>Approximate S value</th>
<th>Specific activity</th>
<th>$K_m$ of threonine</th>
<th>$K_{ADP}$ of ADP</th>
</tr>
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<tbody>
<tr>
<td>7.9</td>
<td>250</td>
<td>2.0 × 10^{-2}</td>
<td>1.1 × 10^{-2}</td>
</tr>
<tr>
<td>6.3</td>
<td>209</td>
<td>2.1 × 10^{-2}</td>
<td>1.1 × 10^{-3}</td>
</tr>
<tr>
<td>4.7</td>
<td>14</td>
<td>1.5 × 10^{-2}</td>
<td>8.0 × 10^{-3}</td>
</tr>
<tr>
<td>3.1</td>
<td>5</td>
<td>5.0 × 10^{-3}</td>
<td>7.6 × 10^{-3}</td>
</tr>
</tbody>
</table>

TABLE II

Kinetic properties of associated enzyme and three dissociated forms of threonine deaminase

A major peak DEAE-cellulose column fraction (23) was 7.9 S; 6.3 S, 4.7 S, and 3.1 S forms were obtained by gel filtration of an ammonium sulfate fraction aged by storage at −20°C. The specific activity of the latter fraction before gel filtration was 44.9. $K_{ADP}$ for ADP was determined with 0.01 M L-threonine; specific activities were determined with 0.1 M L-threonine.

<table>
<thead>
<tr>
<th>S value</th>
<th>Specific activity</th>
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<td>5.0 × 10^{-3}</td>
<td>7.6 × 10^{-3}</td>
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</tbody>
</table>
Fig. 7. Deamination of increasing concentrations of threonine by a partially dissociated preparation and by isolated dissociated components. A, mixture of dissociated forms containing (by sucrose gradient analysis) approximately equal quantities of 6.3 S, 3.9 S, and 3.1 S materials obtained by dialyzing an ammonium sulfate fraction (specific activity = 73) against 0.5 M urea-0.005 M \( \beta \)-mercaptoethanol for 24 hours; B, 6.3 S form (specific activity = 16) isolated from A by gel filtration. Gel filtration and assay as described in "Experimental Procedure."

according to the Hill equation (33) showed a lower degree of "cooperativity" with respect to catalytic sites with the smaller molecular weight forms than with the 7.9 S unit. It has been shown earlier (23) that freshly prepared, highly active fractions of the latter size yielded values of \( n \sim 4 \), but \( n \) approached 2 if the fractions had been aged or dialyzed. Maximum values of \( n = 2 \) were obtained for the 6.3 S unit and \( n = 1.5 \) for the 4.7 S and 3.1 S forms. It should be noted, however, that these preparations were derived from fractions which had been dissociated either by aging or by exposure to urea, so that inactivation may have occurred. Addition of ADP decreased the value of \( n \) to 1 for all forms.

The specific activities of the dissociated forms depended on the initial activity of the fraction from which they were derived. In six separate gel filtration experiments with ammonium sulfate fractions having specific activities of 20 to 75, specific activities of 150 to 200 were obtained for the 6.3 S unit. The specific activities of the smaller two units were more variable and were consistently lower suggesting that activity (or stability) was related to the degree of polymerization.

In view of the finding (33) that dissociated forms of an allosteric enzyme, glutamic dehydrogenase, possess a greater alanine dehydrogenase activity than the associated enzyme, a qualitative survey was made for the possible deamination by threonine deaminase of amino acids other than threonine and serine. Mixtures of partially dissociated enzymes and purified preparations of the 7.9 S, 6.3 S, 4.7 S, and 3.1 S components were incubated with various amino acids. No evidence was obtained for deamination of any amino acids other than threonine and serine.

It is assumed that the small molecular weight forms per se may possess some enzymatic activity, i.e. that activity may not require prior polymerization to higher molecular weight materials during incubation of the assay reaction mixture. This assumption is based on the following reasoning. (a) As stated earlier, the alkaline pH used in the assay (pH 9.5) prevents the threonine- or ADP-promoted association of 3.1 S materials.

(b) Preparations of 3.1 S (and larger materials) which have lost the ability to undergo aggregation in the presence of ADP or threonine or both still possess catalytic activity (Table I). (c) The rate of deamination by both associative and nonassociative 3.1 S forms is constant for 5 hours of incubation in the presence of threonine as well as threonine plus ADP. Providing that polymerization is not an extremely rapid process, a higher rate of deamination might be expected after considerable incubation with threonine if polymerization had occurred. (d) The rate of deamination is proportional to the amount of enzyme over a 50-fold range of dilution with both associative and nonassociative preparations. Since aggregation is favored by high enzyme concentration, a disproportionate increase in activity might be expected at high enzyme concentration if such aggregation had occurred. These observations indicate that aggregation probably does not occur in the course of the assay and that, hence, the 3.1 S component possesses enzymatic activity.

2. Inactivation of Dissociated Forms by Heat and by Storage at \(-20^\circ\) — Earlier work (23) had shown that ADP, phosphate, and mono- and divalent cations protect the enzyme from inactivation by heat. Fig. 8 compares four forms of the enzyme with respect to heat stability under three conditions. The two larger forms, 7.9 S and 6.3 S, were protected from inactivation by both phosphate and ADP. It is seen that the 4.7 S preparation was slightly less stable to heat and that it was protected less by phosphate than the 7.9 S and 6.3 S forms. The protection by ADP, however, was equal to that found for the two higher

![Fig. 8. Effect of ADP and phosphate on the heat inactivation of preparations of different S value. Preparations obtained by gel filtration of an ammonium sulfate fraction dialyzed for 6 hours against 2 M urea-0.05 M \( \beta \)-mercaptoethanol. Each preparation was diluted 1:1 in (a) water, (b) 0.4 M phosphate buffer at pH 7.0, or (c) 2.5 \( \times 10^{-3} \) M ADP in 0.005 M phosphate buffer at pH 7.0. The diluted preparations were heated at 54\(^\circ\) for the indicated time. The samples were then cooled in ice, diluted 1 to 100 in water and assayed immediately as described in "Experimental Procedure." Assays with 7.9 S and 6.3 S materials were incubated for 20 min, assays with 4.7 S and 3.1 S components, for 1 hour. Specific activities and other properties of the preparations used in this experiment are shown in Table II.](http://www.jbc.org/doi/10.1042/0021-9525/241/21/4896.supp)
molecular weight species. The 3.1 S material was more susceptible to inactivation by heat than the 4.7 S form and was less effectively protected by ADP. Phosphate inhibited the initial activity and gave only a very slight protection against heat. The results obtained with phosphate and ADP may be explained in terms of the effects of these compounds on dissociation (Fig. 4) and association (Fig. 6) reactions. According to this view, failure of phosphate to protect the 3.1 S unit against inactivation may be attributed to the fact that this component is not readily dissociable to still smaller subunits. The ability of ADP to protect this component against inactivation may be due to association of the enzyme under the conditions of the experiment. It should be noted, however, that a direct protective effect on the catalytic site cannot be ruled out since desensitized preparations have not been examined for the effect of ADP on heat inactivation.

The same relative order of inactivation was found with regard to stability to storage. The two larger forms lost approximately 10% of their catalytic activity after 1 to 2 weeks of storage in water at -20° but the 4.7 S form and the 3.1 S form had half-lives of 3 to 7 and 2 to 4 days, respectively, at -20°.

**DISCUSSION**

The present experiments have shown that ADP and threonine affect both the catalytic properties of threonine deaminase and its state of aggregation. Dissociation of the enzyme can occur upon aging, dilution, heat inactivation, exposure to denaturing agents, an alkaline pH, and the procedures used for the desensitization of the allosteric site. ADP, threonine, phosphate buffer, and monovalent or divalent cations all prevent dissociation to varying degrees. Association of small molecular weight forms, on the other hand, takes place only when ADP (or other nucleotides capable of enhancing threonine deamination) is added or, less efficiently, in the presence of threonine. While partial dissociation to mixtures of several enzymatically active forms occurs readily in certain preparations, complete dissociation to the smallest unit possessing enzymatic activity (approximately 3.1 S) requires fairly rigorous treatment.

Preliminary investigations of the kinetic properties of mixtures of dissociated forms and isolated preparations of different S value have disclosed that the smaller forms of the enzyme have significantly lower catalytic activity and are less stable than the larger forms. In addition, ADP enhances the deamination of threonine by the 3.1 S, 3.9 S, and 4.7 S units at high substrate concentration, whereas it has little effect at high substrate concentration on the higher molecular weight forms. This may be due to inactivation of low molecular weight forms in the absence of ADP or may reflect inherent differences in catalytic properties of the various dissociated forms.

At least eight molecular weight components have been derived from purified preparations of the enzyme. It is not known at the present time whether these forms consist of identical subunits. Seven of these forms possess enzymatic activity but the smallest unit (or units) corresponding to 1.9 to 2.3 S has essentially no activity. These observations suggest that the fully associated enzyme is a polymer, possibly an octomer. However, in terms of catalytic units, the enzyme may consist of four protomers, each 3.1 S protomer being made up of subunits. If such a tetrameric enzyme dissociated, 6.3 S, 4.7 S, and 3.1 S components could be produced. It could be proposed that the "intermediate forms" (i.e. 7.1 S, 5.5 S, and 3.9 S) which are most frequently produced in the absence of reducing agents, could arise by aggregation between a "parent" 6.3 S, 4.7 S, or 3.1 S form and smaller subunits. However, if such aggregations occur, it follows that catalytic activity is not impaired since the intermediate forms always have a higher activity than the parent dissociated component.

The regulation of threonine deaminase and role of this enzyme in the over-all degradation of threonine may be visualized as shown in Fig. 9. ADP regulates the first step in the series of reactions involved in the catabolism of threonine by C. tetanomorphum. The rapid and efficient utilization of this amino acid is favored by maintenance of the enzyme in its most reactive state. Since the more highly polymerized forms (7.9 S and 6.3 S) are more active (Table II) and more stable (Fig. 8) than the smaller forms, disaggregation or dissociation may be termed unfavorable for the production of energy for growth via threonine fermentation. Dissociation of the polymerized enzyme is prevented by ADP and threonine, and the same compounds also promote association of dissociated forms (providing the dissociated forms have not been inactivated with respect to their aggregative ability). Monovalent cations, including ammonium ion, a competitive inhibitor of the reaction (23), also functions to prevent dissociation. In the absence of ADP, phosphate (produced by hydrolysis of propionyl phosphate) could also serve to stabilize the enzyme in the associated form.

The effects of ADP and threonine on the aggregated form of threonine deaminase fit the model proposed by Monod, Wyman, and Changeux (3) and Changeux (34) for an enzyme of the "K system." Thus, both substrate and allosteric effector alter the reactivity of the enzyme with respect to substrate. The cooperative effect of increasing substrate concentration assessed from a Hill plot yielded values of n ~ 4 (23). In the presence of ADP, this value is decreased to 1, indicating that ADP promotes the complete independence of substrate sites.

Monod, Wyman, and Changeux (3) postulate that allosteric enzymes are polymeric proteins which can exist in two states having different affinities with regard to substrate. According to this model, the effect of ADP on the deamination of threonine is to promote the transition of threonine deaminase from a less reactive to a more reactive form (Fig. 9). Threonine would also act in the same way by displacing the equilibrium between the two states in favor of the state having a higher affinity for threonine.

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REFERENCES

Threonine Deaminase of *Clostridium tetanomorphum*: II. DISSOCIATION TO SUBUNITS

H. R. Whiteley


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