The Mechanism of Action of 5'-Adenylyl Acid-activated Threonine Dehydrase

V. RELATION BETWEEN LIGAND-INDUCED ALLOSTERIC ACTIVATION AND THE PROTOMER-OLIGOMER INTERCONVERSION*

(Received for publication, July 17, 1973)

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SUMMARY

The relationship between the AMP-induced increases in molecular weight and the AMP-promoted decrease in $K_m$ for L-threonine for the biodegradative L-threonine dehydrase of Escherichia coli was investigated. The sedimentation velocity in sucrose gradients over a range of 0.07 to 22 $\mu$g of dehydrase in the presence of AMP showed little effect of protein concentration (ranging from 7.5 to 8.1 S). In the absence of AMP, the sedimentation velocity increased with protein concentration over the range of 0.61 to 366 $\mu$g from 3.7 to 6.4 S. Extrapolation of the line to zero protein concentration indicated a limiting $s$ value of 3.2 S.

Oligomers formed at high protein concentration (1 mg ml$^{-1}$) in the absence of AMP were shown by direct determination in stopped flow assays to have a $K_m$ for L-threonine significantly higher than observed in the absence of AMP at much lower protein concentration where monomer would be present. Therefore, oligomer formation alone is not a sufficient condition to cause the allosteric kinetic effect observed with AMP; namely, a decrease in $K_m$ for threonine.

Following dilution of the concentrated dehydrase in the absence of AMP, an activation was observed upon addition of AMP; the activation was shown to be second order in protein concentration as determined by integral, differential, and fractional time methods. Therefore, the rate limiting step for activation is a dimerization process.

These results show that oligomerization is a necessary, although not a sufficient, condition to cause the decrease in $K_m$ for threonine elicited by AMP. They also indicate that the sequential isomerization and concerted isomerization models of allosteric behavior are not well suited to the biodegradative threonine dehydrase.

Two fundamental models have been proposed to explain the kinetic and binding data for regulatory enzymes. The concerted isomerization model developed by Monod et al. (1) postulates that an equilibrium mixture of isomeric forms of an oligomeric enzyme with different affinities for a ligand may produce allosteric effects when the symmetry of the protein molecule is conserved at all times. Koshland and his co-workers (2-4) have demonstrated that the symmetry restrictions are not essential to the explanation of regulatory phenomena. Their sequential isomerization model is also based on isomeric forms of the regulatory enzyme. Ligand binding to a subunit induces a conformational change within that subunit which may influence to various degrees the ligand affinity of neighboring subunits. Neither a pre-existing equilibrium of isomeric molecules nor conservation of symmetry within the oligomeric protein molecule is assumed.

As the number of allosteric enzymes described in the literature has increased, it has become evident that models requiring the isomerization of an oligomeric protein may be too restrictive. Enzymes have been characterized which display dissociation-association transitions under the influence of an allosteric effector. The models advanced by Nichol et al. (5) and by Frieden (6) to mathematically describe this phenomenon assume a special case of the concerted isomerization model in which the macromolecular species which bind ligand are of different states of oligomerization coexisting in equilibrium. The binding of a ligand preferentially to one species causes a displacement of the equilibrium which favors the higher affinity species. It is this displacement of the equilibrium which produces the allosteric effects.

Levitaki and Koshland (7, 8) have recently presented evidence that cytosine triphosphate synthetase from E. coli undergoes ligand-induced changes in state of association and in activity which may be best described by the sequential isomerization model.

The biodegradative L-threonine dehydrase from Escherichia coli has been shown to be activated by AMP (9). The activation results from an AMP-induced large decrease in the $K_m$ for L-threonine with little or no increase in $V_{max}$ (10, 11). Dunne et al. (11) have shown small homotropic effects for threonine in the absence of AMP and also for AMP itself with values for Hill $n$ ranging from 1.0 to 1.4. Further, a heterotropic interaction between AMP and threonine has also been reported (12).

No evidence has been obtained for the direct participation of AMP chemically in the catalytic mechanism (13). Rather,
AMP exerts its allosteric influence primarily by favoring formation of the nonequivalent enzyme-threonine complex, the first step in the dehydration mechanism (14, 15).

AMP also exerts profound effects on the structure of the dehydrase. The dehydrase exists as a monomer of molecular weight of about 40,000 at low enzyme concentrations in the absence of AMP (10). In the presence of AMP, an increase in sedimentation velocity consistent with the conversion of monomer to tetramer is observed (10, 16), even at very low enzyme concentrations used in the coupled spectrophotometric assay. The sedimentation velocity of the dehydrase in the absence of AMP depends strongly upon dehydrase concentration with oligomer formation being favored by high protein concentration (10, 16).

These observations concerning activation and oligomerization of the dehydrase have led to the supposition that changes in quaternary structure may be directly involved in the change in kinetic characteristics. There are several distinct ways in which activation and oligomerization may be related.

1. Oligomerization may be sufficient for activation, in which case, AMP functions by encouraging oligomerization, for instance, by binding preferentially to low Kₘ oligomers. AMP binding to the monomers, if it occurs, is not sufficient for activation.

2. The binding of AMP to the monomer may be sufficient for activation. Changes in quaternary structure would be an indirect and unimportant consequence of the activation of the monomer.

3. Both oligomerization and binding of AMP to the monomer are necessary for activation. Binding of AMP to monomer produces oligomerization which is a necessary second step in the activation process; however, oligomerization itself is insufficient for activation.

It is apparent that the first possibility is consistent with the concerted transition model extended to include an association-dissociation equilibrium between polymerizing species. However, the last two mechanisms are inconsistent with both the concerted and sequential isomerization models. The experiments described in this paper were performed to clarify the relationship between the allosteric activation and oligomerization of the dehydrase produced by AMP.

**EXPERIMENTAL PROCEDURE**

**Enzymatic**

**Threonine Dehydrase**—The enzyme was purified from E. coli ATCC 8739 according to the procedure described in the following paper (15). Although the specific activity of homogeneous dehydrase is about 480 μmoles min⁻¹ mg⁻¹, for these studies, dehydrase of specific activity 160 to 320 μmoles min⁻¹ mg⁻¹ was used.

**Coupled Spectrophotometric Assay**—For routine measurements of steady state velocities, the lactic dehydrogenase-coupled assay described previously was used (11).

When the velocity of threonine dehydration was to be accurately determined prior to attainment of steady state velocity (i.e. in determining protein order for activation), the amount of lactic dehydrogenase in the cuvette was increased from 0.050 mg ml⁻¹ to 0.5 mg ml⁻¹, and heart lactic dehydrogenase was substituted for the rabbit muscle form because of its 3-fold lower Kₘ and 15-fold higher V_max for α-ketobutyrate (11). At the lower concentration of coupling enzyme used in the steady state velocity measurements, a time-dependent increase in rate of DPNH oxidation of approximately 2 min duration was observed. At the increased lactic dehydrogenase concentration, a velocity lag of about 5 s would be expected (17). This lag may be attributed to the high Kₘ and low V_max relative to pyruvate which the lactic dehydrogenase exhibits for α-ketobutyrate.

At the 0.5 mg ml⁻¹ concentration of lactic dehydrogenase, the assays were 0.187 M in (NH₄)₂SO₄ associated with the commercial lactic dehydrogenase. Experiments were run both at this salt concentration and following removal of the ammonium sulfate by passage through Sephadex G-25. Kinetic measurements showed a 2- to 3-fold higher Kₘ for threonine, a 3-fold higher Kₘ for AMP, and a 10 to 20% lower V_max in ammonium sulfate. However, the dependence of rate of dehydrase activation on enzyme concentration and the protein order for activation were not altered by the high salt environment. Effects of ionic strength on sedimentation velocity have not been determined. However, based upon the observed protein order dependence, the same oligomerization is required for activation in both the presence and absence of ammonium sulfate.

L-Threonine is known to favor dissociation (11). This dissociative effect is relatively small at the threonine concentration used in the assays. However, at the low dehydrase concentration of the assays, the threonine will insure that before AMP is added, the dehydrase is largely in the monomeric state.

**End Point Assay**—At enzyme concentrations greater than could be measured accurately in the coupled assay, an end point assay was substituted in which enzyme was incubated with substrate in 75 mM potassium phosphate, pH 8.0, and 5 mM dithiothreitol for a fixed period of time. A 20-μl aliquot was removed and added to 2.0 ml of boiling 100 mM potassium phosphate, pH 8.0, to terminate the enzymatic reaction. The amount of α-ketobutyrate produced was determined by reduction with DPNH and excess lactic dehydrogenase.

**Stopped Flow Assay**—At very high protein concentrations (1 mg ml⁻¹), the appearance of product was monitored accurately in the coupled assay, an end point assay was substituted in which enzyme was incubated with substrate in 75 mM potassium phosphate, pH 8.0, and 5 mM dithiothreitol for a fixed period of time. A 20-μl aliquot was removed and added to 2.0 ml of boiling 100 mM potassium phosphate, pH 8.0, to terminate the enzymatic reaction. The amount of α-ketobutyrate produced was determined by reduction with DPNH and excess lactic dehydrogenase.

**Data Collection and Analysis**—To quantitate the increase in velocity as a function of time, the voltage output from the Gilford 2000 spectrophotometer representing absorbance was converted to digital form with the Gilford 410 Digital Absorbance Meter and was punched on paper tape at 1-s intervals using the Gilford 402 Multiplexer and Gilford 4010 Paper Tape Punch. The paper tape served as a source of data for a computer program which calculated the first derivative (velocity) as a function of time by least squares analyses.

**Removal of AMP**—Since the concentrated dehydrase (10 to 12 mg of protein ml⁻¹) is stored in 1 mM AMP, its removal was accomplished as follows. (a) For assays of concentrated dehydrase, AMP was rigorously removed by chromatography on Sephadex G-25. The column (0.6 × 4.6 cm) was equilibrated in 100 mM potassium phosphate, pH 9.0, containing 1 mM dithiothreitol, and the sample was eluted with the same buffer. By adding [¹⁴C]AMP to the dehydrase, it was shown that after this treatment, less than 0.1 mole of [¹⁴C]AMP remained per mole of dehydrase of molecular weight 160,000. (b) For lower enzyme concentrations used in coupled assays, the AMP concentration was decreased by dilution by one of the procedures described in

1 C. P. Dunne, R. C. Mensen, and W. A. Wood, unpublished experiments, 1972.
the previous paper (11). Dehydrase diluted 1000-fold to a concentration of \(-10 \, \mu g \, ml^{-1}\) in the absence of AMP was used to study the kinetics of AMP activation. Aliquots were further diluted 200-fold by addition to cuvettes which contained 20 mM threonine but no AMP. After 5 min of incubation in the assay mixture at the residual AMP concentration of \(5 \times 10^{-8} \, m\) (carried over in the dilution), AMP was added to 5 mM concentration, and the slow time-dependent increase in velocity was observed as described under “Results.” This is called the double dilution without AMP.

**Kinetic Determinations and Statistical Analyses**—\(K_m\) and \(V_{\max}\) were routinely determined by the three linear transformations of the Michaelis-Menten equation, \(v/s\) versus \(1/v\), \(s/v\) versus \(s\), \(v/s\) versus \(v\) (18). \(K_i\) values for substrate analogs were determined kinetically by the method of Dixon and Webb (19). The data were analyzed by a least squares computer program using the method of orthogonal polynomials in an adaptation of the POLFIT program supplied by Applied Computer Time Share, Inc. (20).

**Other Methods**

**Sucrose Density Gradient Centrifugation**—Density gradient centrifugations, performed essentially as described by Martin and Ames (21), were carried out in either the SW 39 rotor (Spinco Division, Beckman Instrument Company), or the SB-283 rotor (International Equipment Company). Runs in the SW 39 rotor were at 4° for 17 hours, while those in the SB 283 rotor were conducted at the same temperature, but for a period of 31 hours. The linear sucrose gradients of 5 to 20% contained 0.1 M potassium phosphate buffer, pH 8.0, 1 mM dithiothreitol; fructose diphosphate aldolase of rabbit muscle (\(s_{20,w} = 7.9\) S) (22), horseradish peroxidase (\(s_{20,w} = 3.5\) S) (23), and beef liver catalase (\(s_{20,w} = 11.4\) S) (24) were employed as markers in the gradients. Noll (25) has pointed out that sedimentation velocity determinations in 5 to 20% sucrose gradients may not be valid for any rotor other than the SW 39. Therefore, it was necessary to standardize the gradient run in the SB-283 rotor. Three enzymes of known \(s\) value (beef liver catalase, rabbit muscle aldolase, and horseradish peroxidase) were centrifuged together and each one was used in calculations of the \(s\) value for the other two. Extremely good agreement between known and calculated \(s\) value was obtained when the absolute difference between marker and unknown protein \(s\) value was less than \(\pm 0.5\) Svedberg units. As long as this limitation is met, sucrose density centrifugations in 5 to 20% sucrose are valid in the SB-283 rotor.

Protein measurements were routinely made by the method of Lowry et al. (26) using bovine serum albumin as standard.

**RESULTS**

**Effect of AMP and Protein Concentration on Quaternary Structure**—An earlier paper in this series presented data on the dependence of sedimentation coefficient on dehydrase concentration in the presence and absence of AMP (10). In those studies, AMP was removed only to the extent of the dilution involved. Since that time, the data presented in Fig. 1 have been obtained with highly purified enzyme, wherein AMP was removed by chromatography on Sephadex G-25, as described under “Experimental Procedure.” Fig. 1 shows that the \(s\) value varies over a wide range depending on the presence or absence of AMP. At low dehydrase concentrations, the lower limit \(s\) value in the absence of AMP was 3.2 S. An upper limit \(s\) value of about 8.1 S was obtained in the presence of AMP and at relatively high dehydrase concentrations. These sedimentation coefficients correspond to limit molecular weights of approximately 40,000 and 160,000, when an average partial specific volume for globular proteins is assumed (27). Since a single peak was observed in each gradient, it is assumed that there is a rapid equilibration between monomer and tetrameric forms of the dehydrase (28).

The dependence of the \(s\) value on dehydrase concentration was minimal in the presence of AMP, where a range of \(s\) from 7.5 to 8.1 S was observed over a dehydrase concentration range of 0.07 \(\mu g\) to 22 \(\mu g\) applied to the gradient. In the absence of AMP over the range of 0.61 \(\mu g\) to 366 \(\mu g\) applied to the gradient, the \(s\) value increased from 3.0 to 6.4 S. Hence, the more rigorous removal of AMP in these studies did not alter the fact that an oligomerization in the absence of AMP occurs. However, these studies did establish that approximately 10-fold higher dehydrase concentration was required than previously reported (10).

From the data in Fig. 1, it would be predicted that at dehydrase concentrations in excess of 1 \(mg\, ml^{-1}\) in the absence of AMP, the quaternary structure of the enzyme, as reflected by sedimentation coefficient, should be similar to that of enzyme in 5 mM AMP. This prediction has been confirmed by showing at protein concentration greater than 1 \(mg\, ml^{-1}\) that the same \(s\) value was obtained with or without AMP (29).\(^1\)

\(^1\) The authors wish to acknowledge Dr. Robert A. Niederman for his assistance with the sucrose density gradient experiments.

\(^2\) J. R. Piperno and W. A. Wood, unpublished observations, 1969.
the sucrose gradients all of the components of the catalytic assay with "L"-allo-threonine substituted for L-threonine. L-Allothreonine is dehydrated at 1% the rate of L-threonine and both D- and L-allothreonine rapidly form covalent adducts with dehydrase-bound pyridoxal phosphate, as evidenced by immediate loss of circular dichroism signal at 415 nm (10). These experimental conditions thus coincide for all parameters except time, temperature, and the presence of sucrose. Determination of s values (a) at short time intervals, (b) at 0.02 to 0.05 µM dehydrase, (c) in the absence of sucrose; and (d) during catalysis, was accomplished by active enzyme centrifugation in the ultraviolet scanning ultracentrifuge with essentially identical results. In the absence of AMP, the s value was 3.2 S, whereas in the presence of 5 mM AMP, the s value was 7.3 S.

On the basis of these experiments, it is concluded that in the coupled enzyme assays, the AMP-free species with the high Kₘ exists as a monomer and the low Kₘ species with AMP bound exists as an oligomer. However, these data do not distinguish between oligomer formation as a necessary or coincidental step in the allosteric activation by AMP.

Effect of Oligomer Formation in Absence of AMP on Kinetic Activation—The sedimentation velocity experiments revealed that oligomerization can be achieved in the absence of AMP by increasing the dehydrase concentration. In a reversible oligomerizing system, the relationship of monomer (M) and N-mer concentrations follow the equation, K = N/M⁴, in which K is an association constant.

From the sedimentation velocity experiments, it can be inferred that the value of this association constant for threonine dehydrase is highly dependent on the presence or absence of AMP. It has been shown in the previous paper that the mode of kinetic activation of threonine dehydrase by AMP is through a 23-fold decrease in the Kₘ for the substrate (11). To determine whether oligomerization alone is sufficient to produce such a large decrease in the Kₘ, measurement of threonine binding to AMP-free oligomers formed at high protein concentrations was undertaken. Because of the range of velocities involved, both end point and stopped flow assays at 28°C in the absence of AMP were used as described under “Experimental Procedure.” A Lineweaver-Burk plot of the data obtained at 1 mg of dehydrase ml⁻¹ in the stopped flow assay is shown in Fig. 2. Table I summarizes the values obtained in the three assay procedures and at various dehydrase concentrations. It is evident that in the absence of AMP and at dehydrase concentrations up to 1 mg ml⁻¹, the Kₘ for threonine is appreciably greater (225 mM versus 60 mM) than observed at the low concentration (0.025 µg ml⁻¹) and much higher than observed in the presence of AMP (bottom line, Table I). Therefore, oligomerization per se is not a sufficient condition to cause the change in kinetic parameters associated with AMP activation. It follows that there is little or no spontaneous formation of low Kₘ oligomers in the absence of AMP.

Requirement of Both Oligomer Formation and AMP Binding for Kinetic Activation—As described previously (11), after the double dilution in the absence of AMP, activation by AMP is slow enough to be observed in the coupled enzyme assay (Fig. 3, left, upper line). That is, in the absence of AMP, there is a very slow rate of dehydration. Following addition of AMP, this velocity decreases to zero for a short time (Part A of curve), following which there is a time-dependent increase in velocity (Part B of curve) until a steady state value (C) is obtained. The steady state rate is much faster than originally observed in the absence of AMP. When AMP was always present in the dilutions and assay mixture, no such activation process was observed. Instead, a linear velocity was immediately initiated upon addition of threonine (Fig. 3, left, lower line). Since the dehydrase has a high Kₘ for L-threonine before AMP is added (11) and has a low Kₘ in the presence of AMP when steady state is reached (11), it is concluded that the activation displayed under these conditions is, in fact, a conversion of the high Kₘ to the low Kₘ form via...
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Fig. 3. Demonstration of effects of order of addition of assay components on threonine dehydrase kinetics. In the panel on the left, the lower line represents an assay by the normal procedure, wherein the dehydrase was diluted 1000-fold to a concentration of ~10 ng ml⁻¹ in 0.1 M KPO₄ buffer, pH 8.0, and was also 1 mM in dithiothreitol and AMP. An aliquot was added to a cuvette containing 5 mM AMP and the assay components with the exception of threonine. At the time indicated by the arrow, L-threonine was added to obtain a concentration of 20 mM. The concentration of dehydrase in the cuvettes was 0.10 µg ml⁻¹ in 0.1 M KPO₄ buffer, pH 8.0; also, 1 mM in dithiothreitol. An aliquot was added to an assay mixture which was 20 mM in L-threonine. At the time indicated by the arrow, sufficient AMP was added to make the AMP concentration 5 mM. In the panel on the right, a variation of the single dilution procedure (11) is represented. The dehydrase was diluted 1000-fold in 0.1 M KPO₄ buffer, pH 8.0; also, 1 mM in dithiothreitol. Aliquots were added to cuvettes containing 5 mM AMP and the assay components with the exception of L-threonine. At the times indicated by the arrows, the reaction was initiated in the cuvette by the addition of L-threonine to obtain a concentration of 20 mM. The concentration of dehydrase in the cuvettes was 0.10 µg ml⁻¹. The dehydrase had a specific activity of 310 units mg⁻¹.

some kind of catalytically inactive intermediate. As shown in the right panel of Fig. 3, this activation does not require the presence of threonine. Preincubation with AMP for increasing time intervals before addition of threonine (indicated by arrows) resulted in a progressive decrease in the lag period and an earlier onset of the higher steady state rate.

Since there was an immediate cessation of activity induced by addition of AMP (Part A, Fig. 3), there must have been an immediate binding of AMP. The instantaneous rate of dehydrogenase at any time after the addition of AMP (Part B) is a direct measure of the concentration of newly activated dehydrase, presumably the low Kₘ form. That is, it may be assumed that at any time after the addition of AMP:

\[ (A) = \frac{\text{d}(\text{DPNH})}{\text{dt}} \]  

where \( (A) \) is the concentration of the low Kₘ dehydrase produced and Q is a proportionality constant. The activation process expressed in terms of the conversion of high Kₘ form to the low Kₘ form is:

\[ nB \xrightarrow{k_a} A \]  

where B is the inactive dehydrase, \( A \) is the low Kₘ dehydrase, \( k_a \) is the rate constant for activation, and \( n \) is a stoichiometric coefficient. If the activation depends only upon an isomerization process, \( n \) (and the enzyme concentration order) will be 1. If the activation depends on oligomerization of \( B \), \( n \) (and the enzyme concentration order) will then be greater than 1. From Equations 1 and 2, it is possible to derive a differential equation for the activation:

\[ \log \frac{\text{d}(\text{DPNH})}{\text{dt}} = \log k_a/Q + n \log (B_a) \]  

which is in a convenient form for plotting.

Values of \( \frac{\text{d}(\text{DPNH})}{\text{dt}} \) were determined as described under “Experimental Procedure” with data collection being initiated upon addition of AMP to the cuvette. Values of the second derivatives were determined from the initial slopes of plots of computer-generated values of \( \frac{\text{d}(\text{DPNH})}{\text{dt}} \) versus time. The values of the second derivative were determined at several enzyme concentrations and plotted according to Equation 3. As shown in Fig. 4 (lower part), a slope of approximately 1.9 was observed.

By integration of Equation 3 and other manipulations, it is possible to derive a half-time relationship which is valid for orders other than zero order:

\[ \log ti = (1 - n) \log (B_a) \]  

Plots of the log of the half-activation time versus the log of the initial nonactivated enzyme concentration should have a slope of \((1 - n)\) (30). The times at which the concentration of the activated dehydrase was one-half the steady state concentration were determined at several enzyme concentrations by the com-
puter analysis and plotted according to Equation 4 (Fig. 4, upper part). A slope of $-1.1$ was obtained; hence the protein concentration order is 2.1.

In addition, theoretical plots for integrated rate equations for second order processes, $B + C \rightarrow A$ or $2B \rightarrow A$, were found to agree well with the data, whereas those for other protein orders of activation did not.

These results indicate that at a minimum, the state of aggregation of the kinetically activated species is a dimer. Thus, we conclude that the binding of AMP to the high $K_m$ monomer and oligomerization of the inactive monomer-AMP complex are both necessary to produce the observed 23-fold decrease in $K_m$.

**DISCUSSION**

From these studies and other data, it can be established that the kinetic and structural data for activation of threonine dehydrase by AMP are not completely consistent with either of the two basic models for the allosteric phenomenon, the concerted isomerization model of Monod et al. (1), or the sequential isomerization model of Koshland and co-workers (2-4). Although the kinetic data for substrate and activator permit classification of threonine dehydrase as a $K$ system enzyme (11) in the terminology of Monod et al. the dependence of the kinetic properties of the dehydrase on oligomerization of the monomer prevents interpretation of the allosteric activation on the basis of isomeric molecules of the same state of oligomerization. Further, application of the extension of the symmetry conserving model to associating-dissociating protein systems after Nichol et al. (5) or Frieden (6) is not possible for threonine dehydrase since, in these models, both AMP-free monomer and spontaneously formed oligomers would have similar kinetic properties; i.e. low affinities for the substrate. Similarly, interpretation of the data in terms of the sequential model is difficult because the dehydrase exists in different oligomeric states which are dependent on concentration of ligand and of enzyme. A precise description of the allosteric interaction mechanism in the case of threonine dehydrase requires measurements of ligand and substrate binding to the various monomeric and oligomeric forms of the enzyme as well as quantitation of the interactions between subunits. These studies are presently in progress in this laboratory.

The purpose of the experiments presented has been to define the relationship between AMP binding and oligomerization processes in the activation of threonine dehydrase. Therefore, it is necessary to determine whether both AMP binding and association of monomers are necessary to activate the enzyme, or if either one of these processes is sufficient.

The species of enzyme in the absence of AMP under assay conditions has a molecular weight of approximately 40,000 (10). Measurements of pyridoxal phosphate content and number of AMP binding sites by Shizuta et al. (29) indicate that this species possesses a single catalytic site and a single regulatory site for AMP binding. This monomeric species has catalytic activity, and it is this monomeric species that is activated by addition of AMP, as shown in Fig. 3. The molecular weight of the species produced by addition of AMP to AMP-free dehydrase at catalytic assay concentrations has not been measured definitively. However, at higher concentrations, the $s$ value observed in sucrose gradients ranged from 6.4 S at 0.61 $\mu$g on the gradient to 7.4 S at 61 $\mu$g on the gradient. These values are considerably higher than those for the same enzyme concentrations without AMP, but somewhat below those observed when AMP was never

removed. However, a direct relationship between AMP-induced $K_m$ changes observed in the coupled enzyme assay and molecular weight changes observed in the ultracentrifuge was not possible due to the large scale differences in time span of these experiments. The active-enzyme-centrifugation method developed by Cohen and Miro (31, 32) may eliminate this problem. This method has been recently employed by Schorr and Phillips (33) to show that threonine deaminase of *Clostridium tetanomorphum* undergoes a dimer-tetramer transition promoted by either the activator, ADP, or high levels of threonine. Nevertheless, the kinetic experiments described allow a determination of the fact that the increase in molecular weight is an essential step in the AMP-induced activation. The importance of such a steady state kinetic measurements for the determination of appropriate models for the allosteric transition has been pointed out by Hatfield et al. (34).

The previous assertion from this laboratory that AMP may not activate the monomer form directly but rather indirectly via promotion of oligomer formation (10) has been tested by measuring the $K_m$ of AMP-free oligomers produced at high protein concentrations. Both the direct determination of the $K_m$ for threonine at high dehydrogenase concentrations and the estimation of $K_D$ by circular dichroism titrations with competitive inhibitors of threonine which are known to undergo transaldimination (15) indicate that oligomerization alone is not sufficient to cause the AMP-induced allosteric activation (15).

It may be inferred that AMP binds immediately to the monomer since addition of AMP produces an immediate cessation of threonine dehydrogenation. The final AMP concentration (5 mM) should cause at least 90% of the regulatory sites to be filled because the $K_m$ for AMP is less than 0.4 mM (11). We currently have not determined whether this transient decrease in velocity is caused by a large decrease in the catalytic efficiency, a significant increase in the $K_m$, or changes in both kinetic parameters. However, this observation does suggest that monomer binds AMP directly and that the much slower oligomerization process induced by AMP is not the result of preferential AMP binding to pre-existing oligomeric species. Further, under assay conditions, the concentration of spontaneously formed oligomeric species in the absence of AMP is probably too low to be of any kinetic significance in AMP binding. In addition, a rate constant for an association of protein molecules to account for the immediate decrease in velocity would be probably larger than the diffusion controlled limit.

That the binding of AMP to monomer is not sufficient and that oligomerization of the enzyme is necessary for kinetic activation is demonstrated by our characterization of the time-dependent increase in velocity observed in the coupled assay after addition of AMP to AMP-free enzyme. The second order dependence on protein concentration measured for the production of low $K_m$ enzyme demonstrates that the slow step in the activation is a dimerization process. This indicates that under the assay conditions, the least associated enzyme species which has been kinetically activated is a dimer formed from the association of two kinetically inactive monomers. Thus, it is probable that even though the monomer has a regulatory site, the binding of ligand to this site is not sufficient to directly induce activation of the

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6 R. A. Niederman, K. W. Rabinowitz, and W. A. Wood, unpublished data, 1968.

6 Both monomer and oligomer (dimer) have the same $V_{max}$ (11). However, under the assay conditions, the monomer is kinetically of low activity due to the large difference in $K_m$, whereas the oligomer is activated in the sense that the decrease in $K_m$ allows the activity to be displayed at the threonine concentration used.
enzyme monomer. Both AMP binding to the monomer regulatory sites and oligomerization of monomer AMP complexes must be included as necessary steps in the allosteric activation. The dependence of the half-time for activation on dehydrase concentration illustrates that differential inactivation of the enzyme in the assay cannot be responsible for an artifactual second order dependence of the initial rate of activation on enzyme concentration.

From the initial rate of activation and the half-activation time data and assuming the association of identical monomers, it is possible to calculate that the bimolecular rate constant for the activation is approximately $5 \times 10^8$ M$^{-1}$ s$^{-1}$. It is apparent that the activation occurs with a large rate constant but the observed rate is slow due to the low concentration of monomer used in the coupled assay ($10^{-10}$ to $10^{-9}$ M in monomer).

At the enzyme concentrations used in coupled assays, a necessary change in state of aggregation to produce the high affinity enzyme has been demonstrated. The results of the sucrose density gradient centrifugations suggest that at dehydrase concentrations up to $5 \times 10^{-2}$ M (0.02 mg ml$^{-1}$), an AMP-activation based on dimerization of monomers would be expected. At this higher enzyme concentration, the half-time for activation would be on the order of 100 ms rather than the 100 s observed in our experiments. At still higher dehydrase concentrations, an activation occurring largely by AMP-induced isomerization of pre-existent oligomeric species would be the significant mechanism of allosteric interaction.

Frieden (35) has discussed the concept of the hysteretic enzyme and its function in metabolic regulation; a hysteretic enzyme is defined as an enzyme which responds slowly to rapid changes in ligand concentration. Clearly, the behavior of the biodegradative threonine dehydrase fulfills this definition, at least at low ligand concentration. Clearly, the behavior of the biodegradative threonine dehydrase fulfills this definition, at least at low ligand concentration.

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