ALLOSTERIC CONTROL OF BIODEGRADATIVE L-THREONINE DEHYDRASE: EFFECT OF AMP ON AN EARLY STEP IN THE REACTION MECHANISM[1]

R. A. Niederman[2], K. W. Rabinowitz, and W. A. Wood

Department of Biochemistry, Michigan State University, East Lansing, Michigan 48823

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SUMMARY

In highly purified L-threonine dehydrase preparations, allosteric activation by AMP was accompanied by: 1) a marked enhancement in the accumulation of an enzyme-substrate intermediary which absorbs at 455 mμ; 2) a marked increase in substrate-induced loss of circular dichroism at 415 mμ; 3) an increase in the affinity of the dehydrase for competitive inhibitors which are unable to undergo dehydration. These findings suggest that the effect of AMP is exerted during the first reactions of the overall dehydration process.

The AMP activation (1) of the biodegradative L-threonine dehydrase of Escherichia coli has been shown to result from a 30-fold decrease in K_m for L-threonine (2,3). AMP also causes a shift in the equilibrium between a monomer of molecular weight 40,000 and a tetramer of molecular weight 160,000 (2,3). The evidence of Phillips and Wood (4) indicates that the dehydration reaction involves an α,β-elimination mechanism typical of pyridoxal phosphate (PLP) catalysis; no evidence was found for a role for AMP in the reaction mechanism. Thus, AMP appears to function as an allosteric effector. Since appreciable spectral changes accompany PLP enzymatic catalysis, the biodegradative

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[2]Postdoctoral Fellow of the United States Atomic Energy Commission. Present address: Roche Institute of Molecular Biology, Nutley, New Jersey 07110.

L-threonine dehydrase presents a unique model for examination of the effect of an allosteric activator such as AMP on the reaction mechanism. In this communication, we present evidence that AMP stimulates an early reaction in the catalytic mechanism.

Experimental Procedure -- L-Threonine dehydrase was prepared from an isoleucine-requiring mutant isolated from E. coli (ATCC 8739) by Dr. A. T. Phillips. The organism was grown at 37° for 24 hours without aeration in a 120-liter fermentor on a medium consisting of 2% N-Z amine NAK (Sheffield Co.), 1% yeast extract (Difco), and 0.5% K_2HPO_4. The enzyme assay was the same as previously described (4), except that dithiothreitol was substituted for GSH. The purification was performed in the presence of 1 mM AMP and 1 mM dithiothreitol on extracts obtained by sonic oscillation and involved the following steps: (a) treatment with protamine sulfate and precipitation with 2.0 M ammonium sulfate; (b) chromatography on DEAE Sephadex as described by Tokushige (5); (c) calcium phosphate gel adsorption and elution by a procedure developed in this laboratory by Dr. J. R. Piperno; (d) chromatography on hydroxylapatite by the method of Levin (6); and (e) precipitation with 2.6 M ammonium sulfate. This procedure resulted in purification to near homogeneity with a 33% yield, and a specific activity of 450 μmoles/min/mg protein at 28°. In order to carry out activation experiments, AMP was removed from enzyme preparations on a Sephadex G-25 column.

Results and Discussion -- The following experiments were performed to determine the rôle of AMP on partial reactions of the overall catalytic dehydration mechanism of L-threonine dehydrase. Tokushige et al. (7) have shown that the 415 mμ absorption maximum of the internal aldimine of the dehydrase is shifted by addition of L-threonine to about 450 mμ; the peak subsequently returns to 415 mμ as the substrate becomes exhausted. From the following lines of evidence to be published in detail later, we suggest that this absorbancy band reflects the accumulation of a PLP-bound dehydrated intermediate, and that this intermediate is probably the PLP-Schiff base of

α-aminocrotonate: (a) changes in the rate of α-ketobutyrate formation and the decrease in the 455 mμ peak have the same time sequence; (b) the competitive inhibitors D-threonine, L-allothreonine and L-α-amino-n-butyrate are not dehydrated but do cause a loss in circular dichroism (see below); these do not cause the appearance of any absorption at 455 mμ; (c) an absorbance shift to longer wavelength would be expected from extension of the conjugated double bond structure of the Schiff base by dehydration; and (d) Goldberg and Baldwin (8) have shown that the intermediate absorbing at 468 mμ in the tryptophan synthetase reaction is probably the PLP-Schiff base of α-aminoacrylate. Therefore, the effect of AMP on the accumulation of this intermediate was tested. Figure 1 shows that in the presence of 5.0 mM AMP and 0.2 M L-threonine, a sizable absorbance peak at 455 mμ is formed. In the absence of AMP, however, only a much smaller peak at 455 mμ appeared.

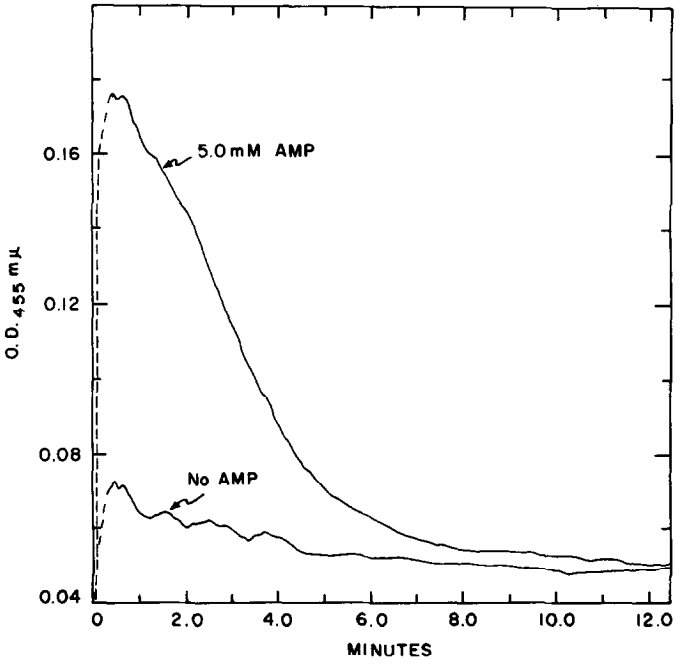

Figure 1 - The effect of 5 mM AMP on absorbance at 455 mμ. Microcuvettes contained purified dehydrase (1.44 mg protein/ml) which had the AMP rigorously removed as described under Experimental Procedure; dithiothreitol, 5 mM; potassium phosphate buffer (pH 8), 0.1 M; L-threonine, 0.2 M. The final volume was 0.25 ml. Rates were recorded on a Gilford Model 2000 spectrophotometer at 28°.

This observation suggests that AMP enhances a step in the dehydration mechanism involved in accumulation of the α,β-elimination product; viz., non-covalent threonine binding, transaldimination with threonine, or dehydration of the threonine-PLP aldimine.

Nakazawa et al. (9) have shown that L-threonine dehydrase exhibits positive circular dichroism (CD) with a maximum near 415 mμ; this CD disappears when L-threonine is added and returns when threonine is exhausted. We attribute this loss of CD to transaldimination between the internal lysyl-PLP aldimine and dehydrase-bound L-threonine. This is suggested from the observation that the competitive inhibitors D-threonine, DL-allothreonine, and L-α-amino-n-butyrate are able to evoke a similar loss in enzyme CD at 415 mμ, whereas, DL-β-hydroxy-n-butyrate, a competitive inhibitor which lacks the α-amino group necessary for aldimine formation causes no change in CD. Furthermore, α-ketobutyrate formation parallels the rate of reappearance of CD at 415 mμ as L-threonine becomes exhausted. Figure 2 shows the effect of AMP and L-threonine on the CD of the dehydrase at 415 mμ. The presence of 5.0 mM AMP causes a marked enhancement in the substrate-induced loss of CD. In the absence of AMP and with ten times more L-threonine, the loss of CD is still less than 50% that observed in the presence of AMP. This finding is consistent with the spectral observations reported above and suggests that the effect of AMP is exerted at one or more of the first three partial catalytic steps in the dehydration mechanism.

Inhibition of the dehydrase by DL-β-hydroxy-n-butyrate and L-α-amino-n-butyrate follows strictly competitive kinetics. Since β-hydroxybutyrate is unable to form an azomethine, it is possible to test the effect of AMP on non-covalent enzyme-substrate interactions which occur prior to trans-aldimination. This assumes that β-hydroxybutyrate forms the same non-covalent intermediate as does L-threonine. Table I shows that AMP causes a 9-fold decrease in the K_i value for DL-β-hydroxy-n-butyrate. Further, AMP causes a > 30-fold decrease in the K_i for L-α-amino-n-butyrate, a

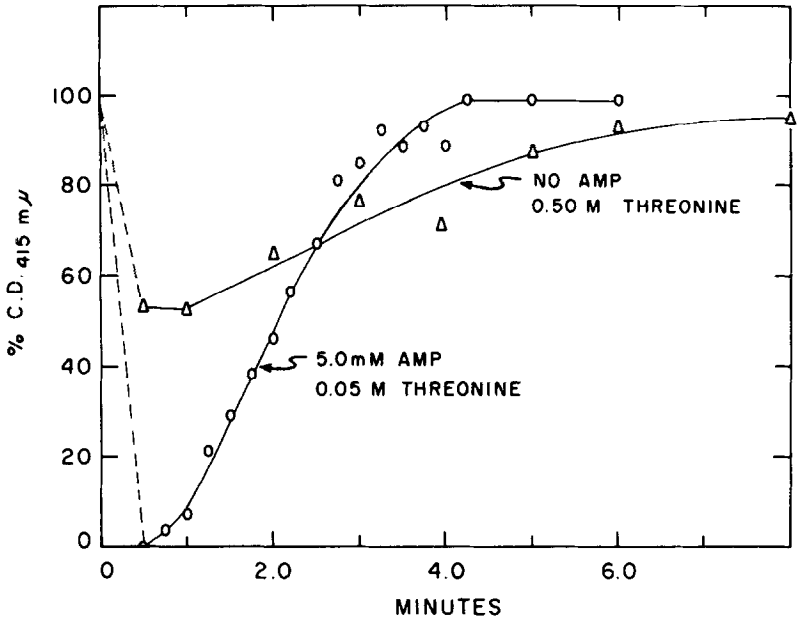

Figure 2 - The effect of 5 mM AMP on the disappearance of CD at 415 mμ. The reaction cuvette contained AMP-free dehydrase [8 mg per ml (circles), 5 mg per ml (triangles)]; dithiothreitol, 5 mM; potassium phosphate buffer (pH 8), 0.1 M; L-threonine as indicated; final volume was 0.6 ml. CD was measured on a Jasco ORD/UV-5 spectropolarimeter and is expressed as percent of the original values to compensate for differences in protein concentrations.

TABLE I

Effect of AMP on the competitive inhibition of L-threonine dehydrase

	K_i	
Inhibitor	-AMP	+AMP
	mM	
DL-β-Hydroxy-n-butyrate	230	26
L- α-Amino-n-butyrate	>500	15

competitive inhibitor which is unable to undergo dehydration. These AMP-elicited increases in the affinity for competitive inhibitors are similar

to the effect of AMP on the K_m for L-threonine and suggest that the allosteric effect of AMP is exerted on non-covalent enzyme-substrate binding.

It is not possible at this time to establish whether the partial reactions of transaldimination and dehydration are also directly affected by AMP. However, an increase in non-covalent binding alone would be sufficient to enhance the extent of transaldimination and, therefore, the effective rate of dehydration and to cause the changes in absorbancy and CD observed. Further, an increase in non-covalent binding or in the rate constant for one of these partial reactions would be a sufficient condition to cause the observed 30-fold change in K_m for L-threonine elicited by AMP.

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