Eur. J. Biochem. 177, 569 – 574 (1988)
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Photoaffinity labeling of the allosteric AMP site of biodegradative threonine dehydratase of *Escherichia coli* with 8-azido-AMP

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(Received April 27/July 19, 1988) – EJB 88 0493

The photoreactive AMP analog, 8-azido-AMP, stimulated the activity of biodegradative threonine dehydratase of *Escherichia coli* in a reversible manner and, like AMP, decreased the K_m for threonine. The concentrations required for half-maximal stimulation by AMP and 8-azido-AMP were 40 µM and 1.5 µM, respectively, and the maximum stimulation by 8-azido-AMP was 25% of that seen with AMP. Gel-filtration experiments revealed that 8-azido-AMP stabilized a dimeric form of the enzyme, whereas AMP promoted a tetrameric species. When present together, AMP and 8-azido-AMP showed mutual competition in influencing catalytic activity as well as the conformational state of the protein. Photolabeling of AMP-free dehydratase with 8-azido-[2-^{3}H]AMP resulted in a time and concentration-dependent enzyme inactivation and concomitant incorporation of 8-azido-AMP into protein. At low 8-azido-AMP concentrations, incorporation of about 1 mol 8-azido-AMP/mol dehydratase tetramer was correlated with almost complete inactivation of the enzyme. The presence of AMP in the photolabeling reaction greatly reduced the extent of enzyme inactivation and 8-azido-AMP binding. Ultraviolet irradiation with 20 µM ^{3}H-labeled 8-azido-AMP revealed one tryptic peptide, Thr^{230}-Thr-Gly-Thr-Leu-Ala-Asp-Gly-Cys-Asp-Val-Ser-Arg^{242}, with bound radioactivity. This peptide, labeled at low concentration of 8-azido-AMP, most likely represents the AMP-binding region on the dehydratase molecule.

Since the initial report [1] that the biodegradative threonine dehydratase of *Escherichia coli*, which catalyzes the pyridoxal phosphate-dependent dehydration of threonine and serine to ammonia and corresponding 2-oxo acids, is stimulated by AMP, considerable evidence has been gathered on the effect of this nucleotide on the conformational states, catalytic mechanism, and regulation of enzyme activity by various cellular metabolites. For example, the enzyme can bind 4 mol AMP/mol protein [2], and the AMP-free enzyme exists as a protomer of M_r 35000, whereas the AMP-bound form is a tetramer of M_r 140000 which exhibits a 25-fold decrease in the K_m for threonine [2 – 4]. The stimulatory effect of AMP has been correlated with obligatory oligomerization of the protomeric species [4]. Furthermore, allosteric inhibition of enzyme activity [5 – 7] and catabolite inactivation by covalent protein modification of the dehydratase by intermediary metabolites [7 – 9] are known to be precisely regulated by subtle variations in the concentration of AMP. Nevertheless, the binding site for AMP on the enzyme molecule remains unidentified. Recently, the complete amino acid sequence of the *E. coli* threonine dehydratase has been determined in this laboratory from the nucleotide sequence of the cloned gene [10, 11], and the active-site pyridoxal-phosphate-bound lysine residue involved in the dehydration reaction has been localized on the protein primary structure [11, 12]. This report is concerned with photolabeling of the binding site of 8-azido-AMP, a photoaffinity analog of AMP. The experiments revealed that 8-azido-AMP can mimic the allosteric modifier AMP and binds to a unique site on the dehydratase molecule which most likely represents the binding region of AMP.

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Enzyme. L-Threonine dehydratase (EC 4.2.1.16).

EXPERIMENTAL PROCEDURES

Materials

HPLC-grade acetonitrile and trifluoroacetic acid were obtained from J. T. Baker and Pierce, respectively. 8-Azido-[2-^{3}H]cAMP, ammonium salt (specific activity 13.1 Ci/mmol), was purchased from New England Nuclear, whereas 8-azido-AMP and cAMP phosphodiesterase were from Sigma. Plates for thin-layer chromatography (0.1 mm cellulose MN 300 UV_{259}) were purchased from Brinkman Instruments Co. Trypsin (treated with tosylphenylalanylchloromethane) was supplied by Worthington. All other chemicals were of the highest purity commercially available and have been previously described [7, 9].

Enzyme purification and assay

Biodegradative threonine dehydratase was purified from *E. coli* strain KL227 harboring the plasmid pEC61 with the cloned dehydratase gene [10] as described [7]. The enzyme activity was assayed spectrophotometrically; the standard reaction mixture contained, in a final volume of 1 ml, the following: 100 µmol potassium phosphate buffer pH 8.0, 50 µmol L-threonine, 3 µmol AMP, 10 µmol L-isoleucine (to inhibit the activity of biosynthetic threonine dehydratase if present) and rate-limiting amount of enzyme [7].

Preparation and characterization of 8-azido-[2-^{3}H]AMP

The conversion of 8-azido-[2-^{3}H]cAMP to 8-azido-[2-^{3}H]AMP was carried out in the dark. Aliquots of 8-azido-[2-^{3}H]cAMP (10 µCi, in 100 µl methanol) were dried at 4°C under a stream of nitrogen, immediately dissolved in 100 µl 10 mM Tris/HCl buffer, pH 7.5, and incubated for 5 min at 30°C with cAMP phosphodiesterase (0.1 unit in 10 µl contain-

ing activator and Ca^{2+}). After the incubation period, 0.1 unit fresh enzyme was again added and the incubation continued further for 5 min. Following enzyme treatment, 2 nmol unlabeled authentic 8-azido-AMP was added to the reaction mixtures and 8-azido-[2-^{3}H]AMP formed was separated by thin-layer chromatography using the solvent system 1 M ammonium acetate/95% ethanol (30:75, by vol.) [13]. After a 3-h run, the spots corresponding to 8-azido-AMP (R_F 0.16) were located under short-wavelength ultraviolet light, scraped off and the solid material suspended in 1 ml 0.5 M ammonium bicarbonate solution at 25°C for 30 min to elute 8-azido-[2-^{3}H]AMP. In the above solvent system, the R_F values for authentic azido-cAMP and azido-adenosine, the two potential contaminants of 8-azido-AMP, were R_F 0.5 and R_F 0.69, respectively. After incubation the mixture was centrifuged for 5 min at $5000 \times g$ and the supernatant fluid was lyophilized. In total, 50 µCi 8-azido-[2-^{3}H]cAMP was converted to 8-azido-[2-^{3}H]AMP in 10-µCi aliquots; the purified material was pooled and its purity was confirmed by thin-layer chromatography. The solution was made 1 mM with respect to 8-azido-AMP by adding unlabeled 8-azido-AMP and the exact concentration determined from its molar absorption coefficient at 290 nm of 11800 M^{-1} cm^{-1} [14]. The stock solution of 8-azido-[2-^{3}H]AMP had a specific activity of 21000 cpm/nmol and was stored in small aliquots in the dark under nitrogen at −80°C until further use.

Photolabeling and incorporation of 8-azido-[2-^{3}H]AMP

Photolysis was routinely performed by illuminating enzyme solution in 100 mM potassium phosphate buffer, pH 8.0, in 100−250 µl volume (1 mg/ml protein), held on ice on a small watch glass, with ultraviolet light, using a 'long-wave' lamp (UVL-56) supplied by Ultraviolet Products Inc. Irradiation of enzyme was from a distance of approximately 3 cm for various times indicated in the text. After photolysis the modified enzyme was immediately passed through a Sephadex G-50 column (1 × 5 cm) in the phosphate buffer. The residual enzyme activity was assayed spectrophotometrically and the amount of 8-azido-[2-^{3}H]AMP incorporated into protein was calculated from protein concentration and radioactivity measurements. The individual binding experiments were performed several times and the extent of incorporation of 8-azido-[2-^{3}H]AMP into protein varied ±10%.

Isolation of tryptic peptides

Purified AMP-free enzyme (3 mg, 21 nmol) in 3 ml 100 mM potassium phosphate buffer, pH 8.0, was illuminated with ultraviolet light for 45 min in the presence of 20 µM 8-azido-[2-^{3}H]AMP (specific activity 20000 cpm/nmol) and the solution was dialyzed against the same buffer to remove excess reagents and photolyzed products of 8-azido-AMP. The dialyzed enzyme was lyophilized, dissolved in 100 mM sodium borate buffer, pH 8.8, containing 6 M guanidine hydrochloride and then reduced and alkylated by sodium iodoacetate as described [15]. The lyophilized carboxymethylated protein was dissolved in 3 ml 100 mM ammonium bicarbonate solution, pH 8.6, and digested for 5 h at 37°C with trypsin (2.5% by mass). The peptide mixture was lyophilized, and dissolved in 1 ml deionized water. Analytical peptide maps were obtained by injecting 1.5−2.0 nmol of digest onto a Beckman Ultrasphere-ODS C_{18} HPLC column (4.6 mm × 15 cm) and eluting with a linear gradient of 0−10% acetonitrile in 0.1% trifluoroacetic acid for 10 min followed by 10−40% acetonitrile in 0.1% trifluoroacetic acid for 50 min at a flow rate of 1 ml/min [11]. Fractions (1 ml) were collected and radioactivity was measured by removing an aliquot for scintillation counting. The fractions containing radioactivity were pooled, concentrated and then rechromatographed on HPLC using a linear gradient of 5−40% acetonitrile in 1 mM phosphate buffer, pH 6.2 (made up by mixing 81.5% monobasic sodium phosphate and 18.5% dibasic potassium phosphate). A single pure radioactive peptide was obtained which was desalted using the trifluoroacetic acid/acetonitrile gradient system described earlier. Starting with 21 nmol enzyme, the overall yield of the peptide was approximately 2 nmol.

Amino acid composition of the peptide was determined by reverse-phase HPLC as described by Tarr [16] in the University of Michigan Protein Sequencing Facility. Manual Edman degradation procedure [16] was also carried out to sequence the purified peptide.

Identification of early-eluted radioactivity during HPLC separation

The fractions containing radioactivity eluted at 2−4 min were pooled, lyophilized and then dissolved in 100 µl water. To this solution was added 2 nmol equivalent of unlabeled 8-azido-AMP preilluminated with ultraviolet light for 45 min and the mixture was separated by thin-layer chromatography using butanol/acetic acid/water (5:2:3, by vol.) solvent system. The spot representing the major photolysis product of 8-azido-AMP (R_F 0.08) was visualized under short-wavelength ultraviolet light and the material contained in it was eluted as described earlier. Upon scintillation counting, 70% of the radioactivity applied on the thin-layer plate was found in the supernatant fluid.

Other methods

Protein concentration was determined by the method of Lowry et al. [17]. The molecular size of threonine dehydratase was estimated by gel filtration at 4°C using a calibrated Bio-Gel P200 column (1 × 80 cm) equilibrated with 100 mM phosphate buffer, pH 8.0, with appropriate suplements as indicated. Cross-linking of photolabeled enzyme with dimethylsuberimidate was carried out by the procedure of Davies and Stark [18].

RESULTS

Stimulation of enzyme activity

The results presented in Fig. 1 show that the AMP-free enzyme is stimulated by 8-azido-AMP in the dark in a concentration-dependent manner; the maximum extent of activation was about 25% of that seen with AMP. The concentration of 8-azido-AMP required for half-maximal stimulation was approximately 1.5 µM, a value which is 25-fold lower than that found for AMP (40 µM). With both modifiers, the K_m for L-threonine remained unchanged at about 12 mM. As was the case with AMP [6, 7], stimulation of enzyme activity by 8-azido-AMP was inhibited by pyruvate and glyoxylate. Control experiments indicated that the stimulatory effect of 8-azido-AMP was completely reversible. The following compounds had no effect on enzyme activity: adenosine, cAMP, and the 8-azido derivatives of adenosine,

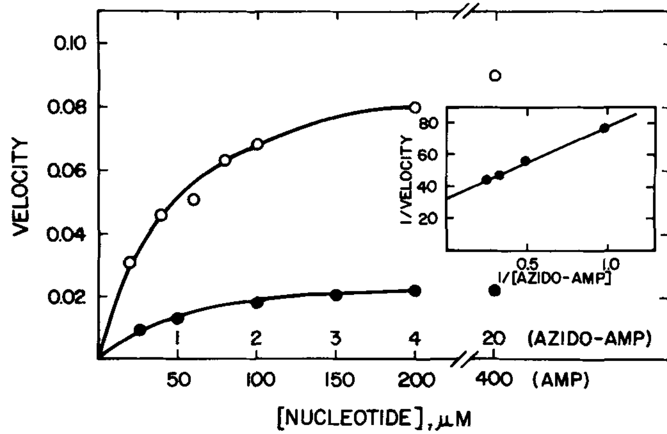

Fig. 1. *AMP and 8-azido-AMP saturation curves.* AMP-free enzyme was prepared by gel-filtration of purified dehydratase through a Sephadex G-50 column (5 × 1 cm) equilibrated with 100 mM phosphate buffer, pH 8.0; 7.5 μg protein was assayed by the spectrophotometric procedure. (○) AMP; (●) 8-azido-AMP. Velocity is expressed as $\Delta A_{310\,nm}$/min. The inset shows a double-reciprocal plot of the data for 8-azido-AMP

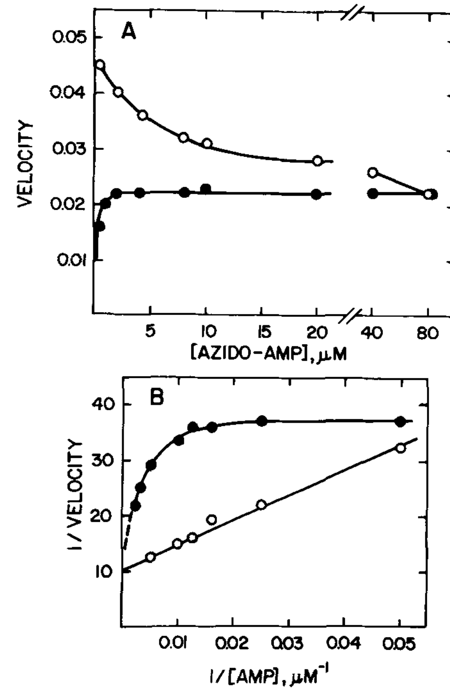

Fig. 2. *Competitive effects of AMP and 8-azido-AMP on dehydratase activity.* AMP-free enzyme (7.5 μg) was assayed spectrophotometrically with increasing concentrations of (A) 8-azido-AMP with (●) no AMP and (○) 40 μM AMP or (B) AMP with (○) no 8-azido-AMP and (●) 100 μM 8-azido-AMP

cAMP, ADP and ATP. These observations suggest that 8-azido-AMP, a structural analog of AMP, mimics the allosteric modifier AMP most likely by interacting at the same site on the protein molecule.

To measure directly the competition between AMP and 8-azido-AMP on threonine dehydratase, enzyme activity was monitored with various combinations of these ligands in the assay mixture. The data in Fig. 2A show that at 40 μM AMP (the concentration required for half-maximal stimulation), increasing concentrations of 8-azido-AMP gradually decreased the AMP-mediated stimulation of enzyme activity, and at 80 μM 8-azido-AMP the AMP effect was completely abolished. In a reciprocal experiment at a fixed, saturating level (100 μM) of 8-azido-AMP, no increase in enzyme activity was seen up to an AMP concentration of about 100 μM, although higher levels of AMP counteracted the effect of 8-azido-AMP (Fig. 2B); an extrapolation of the Lineweaver-Burk plot shows that at infinite AMP concentration identical V_{max} values were seen in the presence or absence of 8-azido-AMP, revealing kinetic competition between these ligands. The order of addition of AMP and 8-azido-AMP to the enzyme had no effect on the final enzyme activity.

Changes in the enzyme structure

Previous reports [3, 4] indicated that AMP converts the monomeric form of threonine dehydratase to its tetramer and that enzyme oligomerization is necessary for the AMP-dependent stimulation of enzyme activity. Gel-filtration of dehydratase through Bio-Gel P200 in the absence and presence of 3 mM AMP, as expected, showed molecular forms of M_r 35000 and M_r 140000, respectively, corresponding to monomer and tetramer (Fig. 3, a and b). On the other hand, the elution profile of the enzyme in the dark in buffer with 100 μM 8-azido-AMP (Fig. 3, c) indicated a dimeric form of the enzyme with M_r = 70000. Interestingly, when the gel-filtration buffer contained 100 μM 8-azido-AMP and 3 mM AMP, the enzyme was also eluted at a position corresponding to that of a dimer (Fig. 3, d). Thus, AMP and 8-azido-AMP, individually, stabilize different conformational states of the

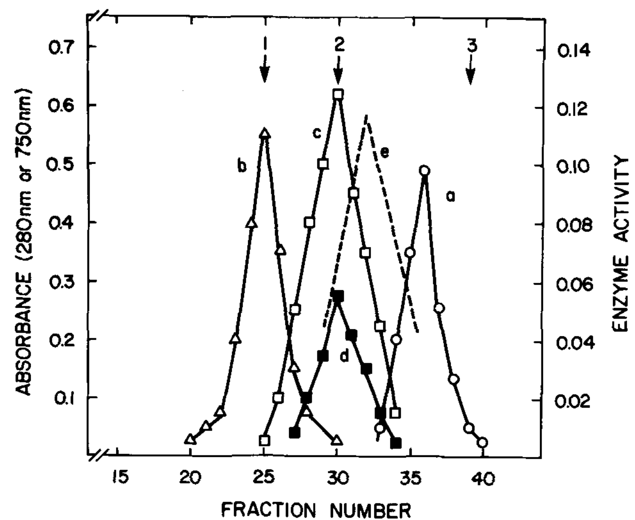

Fig. 3. *Gel-filtration profiles of threonine dehydratase in the presence or absence of AMP and 8-azido-AMP.* Aliquots of AMP-free enzyme (1 mg) were separately passaged through a calibrated Bio-Gel P200 column (80 × 1 cm) at 4°C, in the light or in the dark, equilibrated with 100 mM phosphate buffer, pH 8.0, supplemented with various ligands as indicated. The flow rate was 6 ml/h and 1-ml fractions were collected. The elution profiles were determined by assaying for dehydratase activity, monitoring absorbance at 280 nm or by estimating protein concentration by the Lowry procedure as designated. Curve a, enzyme in buffer only, activity assay; curve b, enzyme with 3 mM AMP, activity assay; curve c, enzyme with 100 μM 8-azido-AMP eluted in the dark, A_{280} nm; curve d, enzyme with 100 μM 8-azido-AMP plus 3 mM AMP eluted in the dark, activity assay; curve e, photolabeled enzyme (ultraviolet irradiated with 100 μM 8-azido-AMP for 45 min) with 3 mM AMP, protein assay. Numbered arrows indicate the position of the reference markers: 1, yeast alcohol dehydrogenase (M_r = 142000); 2, bovine serum albumin (M_r = 68000); 3, trypsinogen (M_r = 24000)

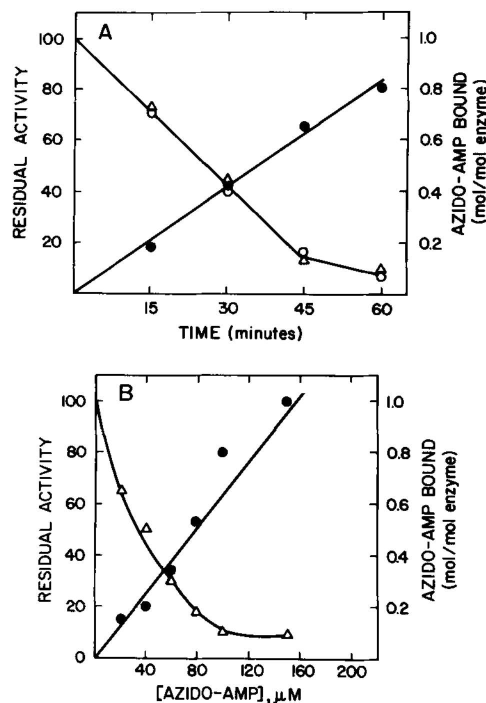

Fig. 4. *Dehydratase inactivation and photoincorporation of 8-azido-AMP.* Aliquots of AMP-free enzyme were illuminated with ultraviolet light in the presence of various concentration of 8-azido-[2-^{3}H]AMP and for the lengths of time indicated; the photolabeled enzyme was reisolated as described in the Experimental Procedures. The residual enzyme activity was determined spectrophotometrically with AMP or 8-azido-AMP and the data are expressed as a percentage of the zero-time control. The amount of 8-azido-[2-^{3}H]AMP incorporated into protein was calculated from protein concentration and radioactivity measurement. (A) Protein, 1 mg/ml; 8-azido-[2-^{3}H]AMP (specific activity 8800 cpm/nmol), 100 μM. (○) and (△), residual activity with 3 mM AMP and 100 μM 8-azido-AMP, respectively; (●) mol 8-azido-AMP bound/mol enzyme. (B) Protein, 0.6 mg/ml; incubation time, 45 min; 8-azido-[2-^{3}H]AMP specific activity, 4500 cpm/nmol; (△) residual activity; (●) mol 8-azido-AMP bound/mol enzyme

protein; 8-azido-AMP appears to play a dominant part in influencing the oligomeric state of the protein.

Photolabeling of the enzyme

Numerous studies have been reported on the use of azidopurine nucleotides as photoaffinity probes to identify nucleotide binding sites on proteins. For example, 8-azido-AMP, 2-azido-AMP, and 8-azido-ATP have been employed to map the AMP- and ATP-binding sites on the pig kidney fructose 1,6-bisphosphatase and the RecA protein of *E. coli*, respectively [14, 19, 20]. Illumination with ultraviolet light of AMP-free threonine dehydratase in buffer containing 100 μM 8-azido-[2-^{3}H]AMP led to a time-dependent inactivation of the enzyme as judged by the loss of stimulation by AMP or 8-azido-AMP, and concomitant incorporation of ^{3}H-labeled 8-azido-AMP into protein (Fig. 4A). A linear relationship was found between the extent of enzyme inactivation and the amount of 8-azido-[2-^{3}H]AMP bound; at greater than 90% enzyme inactivation, 0.8 mol 8-azido-[2-^{3}H]AMP was bound/mol average tetramer.

Fig. 4B shows the results of the photolabeling reaction when enzyme was illuminated with various concentrations of 8-azido-[2-^{3}H]AMP. With increasing ligand concentrations, incorporation of 8-azido-AMP was accompanied by loss of enzyme activity, and, as seen above, approximately 0.7 mol 8-azido-AMP was bound/mol average tetramer with 90% inactivation of the enzyme.

A series of control experiments (data not shown) revealed no significant incorporation of 8-azido-AMP in the dark, or with preilluminated 8-azido-AMP in the light or in the dark. Preillumination of enzyme prior to incubation with 8-azido-AMP incorporated 8-azido-AMP in the light but not in the dark. The presence of AMP during the photolabeling reaction decreased the incorporation of 8-azido-AMP by more than 85%. Further, enzyme inactivated partially (about 30%) by photolabeling with 20 μM 8-azido-[2-^{3}H]AMP (cf. Fig. 4B) and passed through a Sephadex G-50 column to remove unreacted reagents, showed identical K_m values for L-threonine and AMP to that found with the native enzyme, indicating that the unlabeled enzyme was fully functional.

The results presented in Fig. 3 showed that noncovalent interaction of 8-azido-AMP with threonine dehydratase in the absence of AMP promoted the formation of a dimeric species of M_r = 70000. Gel-filtration of photolabeled enzyme in buffer with AMP exhibited an altered elution profile corresponding to a molecular species of M_r = 56000 (Fig. 3, e); the same elution profile was observed when photolabeled enzyme was eluted in phosphate buffer in the absence of AMP. However, cross-linking of the photolabeled enzyme with dimethylsuberimidate followed by SDS/PAGE (data not shown) indicated that this form of the enzyme also exists as a dimer of two dehydratase subunits but it represents a different conformational state than that exhibited by the dehydratase dimer with noncovalently bound 8-azido-AMP.

Identification of the 8-azido-AMP binding site

Threonine dehydratase was photolabeled with 20 μM 8-azido-[2-^{3}H]AMP yielding approximately 0.2 mol bound 8-azido-AMP/mol tetramer with 30% loss of enzyme activity (see Fig. 4B). Following illumination, the enzyme was reduced and alkylated, and the carboxymethylated protein sample was subjected to trypsin digestion. When the tryptic peptides were separated on HPLC, more than 80% of radioactivity (7300 cpm) was eluted at 35–37 min, with a small amount (<5%) emerging at 2–4 min (Fig. 5A). The fractions containing high radioactivity were pooled, lyophilized and rechromatographed on HPLC using a linear phosphate gradient, yielding one pure radioactive tryptic peptide (Fig. 5B). The radioactivity eluted between 2–4 min was identified as free 8-azido-[2-^{3}H]AMP derivative as described in Experimental Procedures, indicating that a small amount of labile enzyme-ligand adduct(s) was formed during photolabeling with 8-azido-AMP.

The amino acid composition of the purified peptide showed the following residues (mol/mol peptide): $Ala_{1.1}$, $Arg_{1.0}$, $Asx_{1.4}$, $CmCys_{1.1}$, $Gly_{1.8}$, $Leu_{1.2}$, $Ser_{0.8}$, $Thr_{2.3}$, $Val_{1.3}$. The amino acid sequence of the first 10 residues of the peptide by the manual Edman degradation procedure was found to be Thr-Thr-Gly-Thr-Leu-Ala-Asp-Gly-Cys-Asp. From the amino acid sequence of the dehydratase reported earlier [11] the peptide corresponded to amino acid residues Thr^{230}-Thr-Gly-Thr-Leu-Ala-Asp-Gly-Cys-Asp-Val-Ser-Arg^{242} on the protein primary structure. These results indicate that photoaffinity labeling of threonine dehydratase with 8-azido-AMP, an analog of AMP, led to covalent binding of

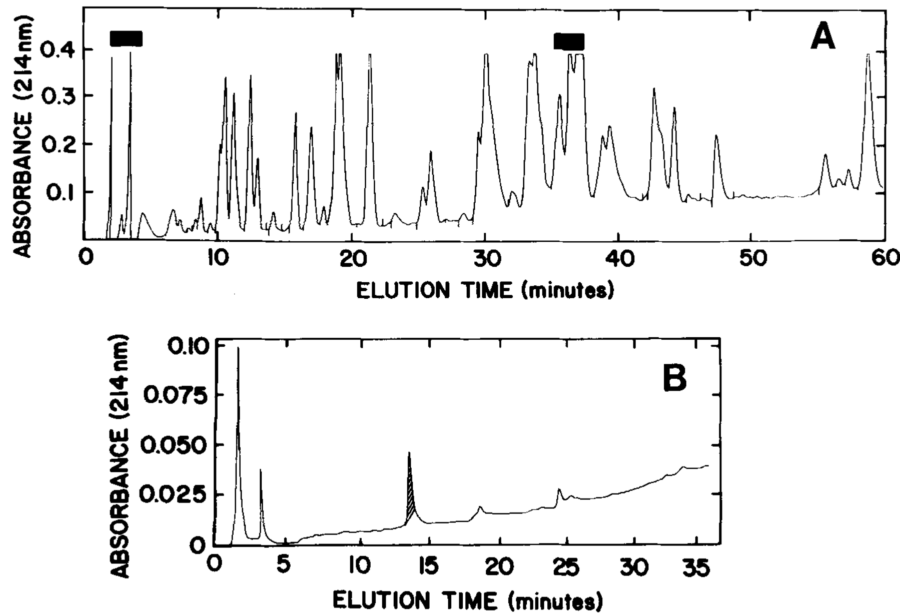

Fig. 5. *Separation of tryptic peptides by HPLC of dehydratase photolabeled with 8-azido-[2-³H]AMP.* (A) AMP-free enzyme was photolabeled with 20 μM 8-azido-[2-³H]AMP. For detailed protocol, see Experimental Procedures. The solid boxes indicate fractions containing radioactivity. (B) Fractions eluted at 35–37 min containing radioactivity (see A) were rechromatographed employing the phosphate/acetonitrile gradient described in Experimental Procedures. The shaded peak identifies radioactive peptide

the modifier to a unique peptide which most likely represents the binding region for AMP on the protein molecule.

DISCUSSION

Experiments summarized above demonstrate that 8-azido-AMP mimics the allosteric modifier AMP in stimulating threonine dehydratase activity by lowering the K_m for threonine, changing the conformational state of the protein, and modulating the extent of inhibition by pyruvate and glyoxylate. Furthermore, when present together, AMP and 8-azido-AMP compete with each other in the following ways: (a) 8-azido-AMP abolishes the stimulation of enzyme activity by low concentration of AMP; (b) the V_{max} value of the dehydratase at infinite AMP concentration is unaffected by the presence of 8-azido-AMP; (c) addition of AMP during photolabeling reaction greatly reduces the incorporation of 8-azido-AMP into protein. These observations are consistent with the notion that the two ligands most likely occupy the same binding site on the enzyme molecule. Thus, the tryptic peptide, $Thr^{230}-Arg^{242}$, isolated following photoaffinity labeling with low concentration (20 μM) of 8-azido-AMP may represent the binding region for AMP on the dehydratase protein.

The results depicted in Figs 1 and 3 show that 8-azido-AMP, despite its high affinity for the enzyme, failed to stimulate enzyme activity to the same extent as that seen with AMP, and stabilized a dimeric form of the enzyme in contrast to AMP which promoted the formation of a tetrameric species. These differences presumably reflect the conformational forms of these nucleotides in solution [21, 22] and the presence of the bulky, ionizable azido group on the AMP analog which may play a significant part in the protein-ligand interaction. It is conceivable that the azido substituent of 8-azido-AMP may in some way facilitate its binding to the enzyme and, when bound, prevents the formation of dehydratase tetramer by altering the conformation of the enzyme subunits. It may be recalled that the conversion to tetramer of the monomeric species by AMP is necessary for the AMP-mediated stimulation of enzyme activity [3, 4].

An interesting aspect of the data reported here is that while noncovalent interaction of threonine dehydratase with 8-azido-AMP stimulated enzyme activity, covalent binding of 1 mol 8-azido-AMP led to enzyme inactivation. This is reminiscent of the earlier reports [8, 23] that catabolite inactivation of dehydratase by pyruvate involves covalent binding of 1 mol of the ligand followed by dissociation of the tetramer to yield inactive dimer. Because covalent modification of the dehydratase by 8-azido-AMP also stabilizes a dimeric form of the protein, it is plausible that a common mechanism underlies the basis for enzyme inactivation by pyruvate and 8-azido-AMP.

During these studies it became apparent (our unpublished data) that photolabeling of dehydratase at high concentrations (>300 μM) of 8-azido-AMP resulted in additional labeling of the protein, unaffected by the presence of AMP. An analysis of the tryptic peptides showed a new labeled peptide (in addition to the one labeled at 20 μM 8-azido-AMP) corresponding to residues $Val^{279}-Lys^{296}$ (Val-Val-Thr-Glu-Gly-Ala-Gly-Ala-Leu-Ala-Cys-Ala-Ala-Leu-Leu-Ser-Gly-Lys) on the protein primary structure. These findings are contrary to the general expectation [24] of random nonspecific incorporation of labeled azido-purine analogs into proteins at high ligand concentrations. In view of this, the significance of selective covalent attachment of 8-azido-AMP at the 'second-site' peptide remains to be uncovered.

This work was supported by grant GM21436 from the United States National Institutes of Health.

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