Takashi, R. (1979) *Biochemistry 18*, 5164–5169.
Tong, S. W., & Elzinga, M. (1983) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 42*, 2211.
Tonomura, Y., Appel, P., & Morales, M. F. (1966) *Biochemistry 5*, 515–521.
Watt, R. M., & Voss, E. W., Jr. (1977) *Immunochemistry 14*, 533–541.
Weeds, A. G., & Taylor, R. S. (1975) *Nature (London) 257*, 54–56.
White, H. D. (1977) *Biophys. J. 17*, 40a.
White, H. D., & Taylor, E. W. (1976) *Biochemistry 15*, 5818–5826.

Crystals of *Bacillus stearothermophilus* Tryptophanyl-tRNA Synthetase Containing Enzymatically Formed Acyl Transfer Product Tryptophanyl-ATP, an Active Site Marker for the 3′ CCA Terminus of Tryptophanyl-tRNA^{Trp} †

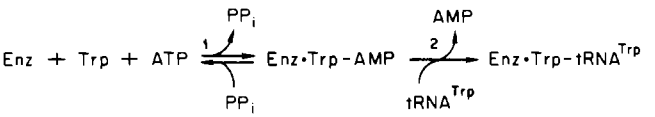
David E. Coleman and Charles W. Carter, Jr.*

ABSTRACT: It has previously been shown that tryptophanyl-tRNA synthetase from *Bacillus stearothermophilus* crystallizes in different forms, depending on the substrates present during crystallization [Carter, C. W., Jr., & Carter, C. W. (1979) *J. Biol. Chem. 254*, 12219–12223]. Radiolabeling experiments show that the tetragonal crystals (type IV), grown in the presence of tryptophan and ATP, contain enzymatically formed 3′(2′)-tryptophanyladenosine 5′-triphosphate (Trp-ATP). Trp-ATP is formed by acyl transfer of the tryptophanyl moiety of an acyladenylate intermediate, Trp-5′-AMP, to a second molecule of ATP bound in the site normally occupied by the 3′ CCA terminus of tRNA^{Trp}. This compound is therefore a chemical marker in type IV crystals for that part of the tRNA binding site on the synthetase. Solution of this crystal structure, now in progress, may therefore provide useful information concerning the mechanism of aminoacylation of tRNA^{Trp} by this enzyme and may help locate its tRNA binding site.

Tryptophanyl-tRNA synthetase catalyzes production of tryptophanyl-tRNA^{Trp} from tryptophan, ATP, and tRNA^{Trp}. Since the enzymes from both *Escherichia coli* (Muench, 1969) and *Bacillus stearothermophilus* (Atkinson et al., 1979) catalyze tryptophan-dependent [^{32}P]pyrophosphate exchange, it is reasonable to assume that aminoacylation normally proceeds via the conventional, two-step reaction (Scheme I). In reaction 1 the α-phosphate of ATP undergoes nucleophilic attack by the carboxyl group of tryptophan to form the adenylate, tryptophanyl-5′-AMP (Trp-AMP).[1] The second reaction (2) involves transfer of the aminoacyl moiety to the 2′- or 3′-hydroxyl group of the ribose moiety on the terminal adenosine of tRNA^{Trp}. We showed previously (Carter & Carter, 1979) that the tryptophanyl-tRNA synthetase from *B. stearothermophilus* can be crystallized in the presence of tryptophan and ATP. The actual ligands associated with this crystal form (type IV) are a matter of considerable interest because of the possibility that the crystals might contain a bound, enzymatically formed ligand.

Two different products can be envisioned for this enzyme in the presence of tryptophan and ATP. Since confusion may arise due to the superficial similarity between the two compounds, it is useful to describe them explicitly. One possibility is that the enzyme makes and binds the acyladenylate intermediate, Trp-5′-AMP (reaction 1 in Scheme I above). The other possibility is that the enzyme also carries out reaction 2 in Scheme I by using a second molecule of ATP as an acyl-group acceptor, synthesizing, and retaining the 3′(2′)-tryptophan ester of adenosine triphosphate (Trp-ATP). This second possibility is raised by previous studies of the tryptophan enzymes from beef pancreas (Weiss et al., 1959) and *E. coli* (Joseph & Muench, 1971b) in which ATP, at high concentrations, was found to serve as an acyl-group acceptor in place of the 3′-terminal adenosine of tRNA^{Trp} in reaction 2 of Scheme I.

Scheme I



We report here that the tryptophan enzyme from *B. stearothermophilus* also produces Trp-ATP in solution and, further, that this compound is the major ligand bound to fresh type IV crystals. The structure and mode of synthesis of this compound imply that it is a product analogue, resembling the 3′ CCA terminus of Trp-tRNA^{Trp}, and that it therefore serves as a marker for the binding site of this end of tRNA^{Trp} on the synthetase. The mode of binding of Trp-ATP to type IV crystals can aid in elucidating the reaction mechanism of

† From the Departments of Biochemistry and Anatomy, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27514. *Received May 23, 1983.* This work was supported by NIH Research Grant GM26203 and by American Cancer Society Institutional Grant IN-15X.

* Address correspondence to this author at the Department of Biochemistry.

[1] Abbreviations: Trp-ATP, 3′(2′)-tryptophanyladenosine 5′-triphosphate; Trp-AMP, tryptophanyl-5′-AMP; EDTA, ethylenediaminetetraacetate sodium salt; TLC, thin-layer chromatography; PEI, poly(ethylenimine); Bicine, *N,N*-bis(2-hydroxyethyl)glycine.

0006-2960/84/0423-0381$01.50/0

$tRNA^{Trp}$ acylation by this enzyme.

Experimental Procedures

Tryptophanyl-tRNA synthetase from *Bacillus stearothermophilus* was purified as described previously (Carter & Green, 1982), and type IV crystals were grown in 2.0 M potassium phosphate, pH 6.5–7.6, with 0.2 mM tryptophan, 5 mM ATP, and 10 mM $MgCl_2$ as described (Carter & Carter, 1979). They are tetragonal, in space group $P4_12_12$ or $P4_32_12$ (a = 60.9 Å; c = 234.4 Å) with a monomer (M_R 37 000) in the asymmetric unit.

The enzyme was assayed as tryptophan-dependent pyrophosphate exchange activity (Joseph & Muench, 1971a). Aminoacylation of $tRNA^{Trp}$ was measured as described (Joseph & Muench, 1971a). [^{14}C]Tryptophan was from Amersham; [γ-^{32}P]ATP and [α-^{32}P]ATP were from New England Nuclear.

Synthesis and isolation of Trp-ATP in solution was carried out as described by Joseph & Muench (1971b) in a 230 μL volume with 2.5 μM enzyme, 9 mM $MgCl_2$, 0.09 mM [^{14}C]tryptophan (5.5×10^4 nCi/mmol), 1.7 mM [γ-^{32}P]ATP (approximately 5.5×10^4 nCi/mmol), 3 mM reduced glutathione, and 0.09 M potassium Bicine, pH 8.8 (20 °C). After a 15-min incubation at 37 °C, the reaction was quenched by adding 10 μL of 0.25 M EDTA and cooled to 0 °C. The reaction mixture was loaded directly onto a Sephadex G-75 column (29 cm × 0.7 cm) and eluted with 20 mM sodium cacodylate, pH 7.0 (20 °C), 10 mM β-mercaptoethanol, 1 mM EDTA, and 0.1 mM phenylmethanesulfonyl fluoride. Fractions (350 μL) were collected and assayed for enzyme activity, and an aliquot (50 μL) was counted for radioactivity. The pooled enzyme-containing fractions (1.6 mL) were made 8.0 M in urea, warmed to 20 °C for 1 min and chilled to 4 °C. Unlabeled ATP was added as a marker, and the solution was made up to 5.0 mL with 0.05 M potassium acetate, pH 4.8 (4 °C), and loaded onto a DE-52 cellulose (Whatman) column (20 cm × 0.7 cm). This column was washed with 24 mL of starting buffer and eluted with a 120-mL linear gradient (0.05–1.0 M potassium acetate, pH 4.8). Fractions (2.17 mL) were collected, and 200 μL of each was counted directly in Brays solution.

Paper electrophoresis (Weiss et al., 1959) was carried out in 20 mM sodium citrate, pH 4.5 (20 °C), by using Whatman 3MM paper in a refrigerated (4 °C) apparatus (Pharmacia). Samples (5 μL) were applied and electrophoresed 2 h 40 min at 11 W, constant power (500 V). Thin-layer electrophoresis in the same buffer was carried out in a similar manner except that samples were applied to dry cellulose TLC plates with fluorescent indicator (Kodak 13254) which were sprayed lightly with buffer and wicked with Whatman 3MM paper. Thin-layer chromatography on poly(ethylenimine)–cellulose plates (PEI–cellulose; Brinkmann Mn30) was carried out as described (Scott et al., 1977).

Crystals were grown with labeled substrates at approximately the specific activities used in solution experiments. Labeled crystals were dried with filter paper and then dissolved in 30 μL of 20 mM sodium cacodylate buffer, pH 7.0, 1 mM EDTA, and 10 mM β-mercaptoethanol. Dissolved crystals were desalted before thin-layer chromatography or electrophoresis by a modification of the centrifuge desalting method described by Christopherson et al. (1980). A 1-mL tuberculin syringe was plugged with glass wool and filled with a slurry of Sephadex G-25 fine, swollen in the buffer used to dissolve the crystals. This apparatus was spun 5 min at 2000 rpm in a Sorval SS34 rotor. A 50-μL sample was applied to the dried gel, and it was spun again. The needle guard itself was used as a collection tube. Desalting by this method was effective enough to permit analysis of single crystal samples by electrophoresis and chromatography.

For the purpose of determining the stoichiometry of binding to labeled crystals, large single crystals were selected, examined, and measured as described by Monteilhet & Blow (1978), with two modifications. First, the dissolved crystals were desalted twice as above. Second, the enzyme content of the crystals was estimated on the basis of crystal volume and verified by means of the pyrophosphate exchange assay.

Results

Both tryptophan and ATP are required for growth of type IV crystals. Moreover, the ATP concentration necessary for type IV crystals (5 mM) is unusual in that it is about 50 times greater than the K_m of the *E. coli* enzyme for ATP (9.0×10^{-5} M at pH 8.8) and causes about 70% inhibition of aminoacylation (Joseph & Muench, 1971a). It is also about 10^3 times greater than the concentration required for formation of tryptophanyladenylate (Trp-5′-AMP) by the *B. stearothermophilus* enzyme (5.1×10^{-6} M; Fersht et al., 1975). These observations suggest that some enzymatic process other than amino acid activation is required for growth of type IV crystals. The substrate requirements for type IV crystals (0.2 mM tryptophan and 5 mM ATP) are remarkably similar to conditions in which synthesis of Trp-ATP is observed for the *E. coli* enzyme (Joseph & Muench, 1971b). We have therefore studied synthesis of this compound by the *B. stearothermophilus* enzyme.

Enzymatic Production of Trp-ATP in Solution. Incubation of the enzyme under conditions appropriate for acylation of tRNA but without tRNA and in the presence of [^{14}C]tryptophan and [γ-^{32}P]ATP generates a complex of enzyme, ^{14}C label, and ^{32}P label which can be isolated as such by Sephadex G-75 gel filtration (Figure 1a). Both labels elute with the enzyme in a ratio of 1–2 mol/mol of enzyme dimer. Extraction of the purified complex in 8.0 M urea, followed by ion-exchange chromatography on DE-52 cellulose, yields one peak containing both labels, as observed by Joseph & Muench (1971b) (Figure 1b). [^{14}C]Tryptophan and [γ-^{32}P]ATP elute close to the position of unlabeled ATP in the ratio of approximately 1:1.

Fractions 5–11 in Figure 1a contain protein-bound ^{14}C label that was not retained by the DE-52 resin. To investigate the possibility that this material was the aminoacyladenylate intermediate, Trp-AMP, an incubation was performed by using ATP labeled in the α-position in the place of [γ-^{32}P]ATP. No ^{32}P label was found with the ^{14}C label in this wash peak, thus eliminating the possibility that Trp-AMP was bound to the protein under these conditions.

Samples prepared with unlabeled ATP and [^{14}C]tryptophan were also analyzed by paper electrophoresis at pH 4.5 as described by (Weiss et al., 1959). Autoradiography revealed a spot running anodically just ahead of AMP in the position of Trp-ATP as established by these authors. A modification of this system was employed in order to obtain less diffuse spots. A cellulose thin-layer plate was used in place of filter paper. With this system Trp-ATP runs in the position of AMP and tryptophan migrates slightly, if at all.

An incubation with [^{14}C]tryptophan and ATP was analyzed, with standards, on both PEI–cellulose plates and by thin-layer electrophoresis. In both cases the position of a minor spot coincided with that of tryptophan. The position of the major spot on the thin-layer electrophoresis system corresponded to that of Trp-ATP on the paper electrophoresis system (Weiss et al., 1959). On the PEI–cellulose system the position of the

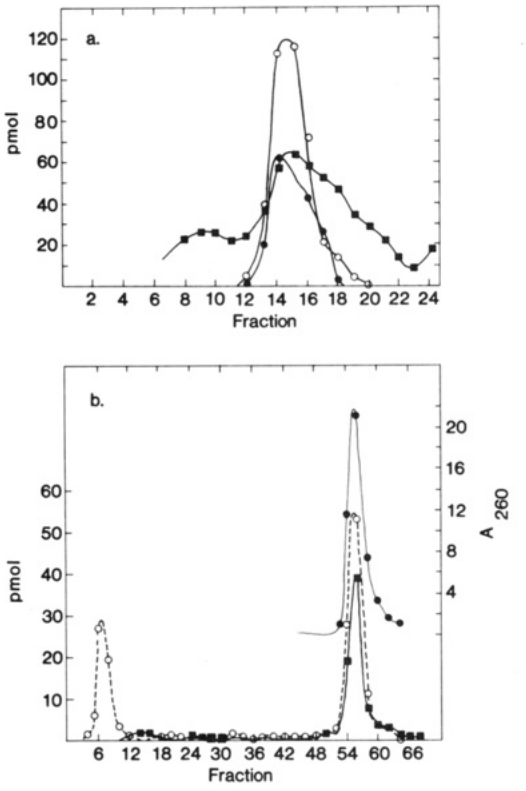


FIGURE 1: (a) Sephadex G-75 gel filtration chromatography of an enzyme–tryptophanyl-ATP complex from a double-labeling experiment. The void volume is contained in fractions 11–16; the included volume begins at fraction 24. Tryptophanyl-tRNA synthetase (●), [^{14}C]tryptophan (○), and [γ-^{32}P]ATP (■) are given picomoles and therefore indicate stoichiometry. (b) DEAE-cellulose (DE-52) chromatography of Trp-ATP obtained by extracting the excluded volume peak from the experiment shown in (a) (fractions 11-16). Absorbance at 260 nm (●) indicates the position of unlabeled ATP. [^{14}C]Tryptophan (○) and [γ-^{32}P]ATP (■) are given in picomoles and indicate stoichiometry.

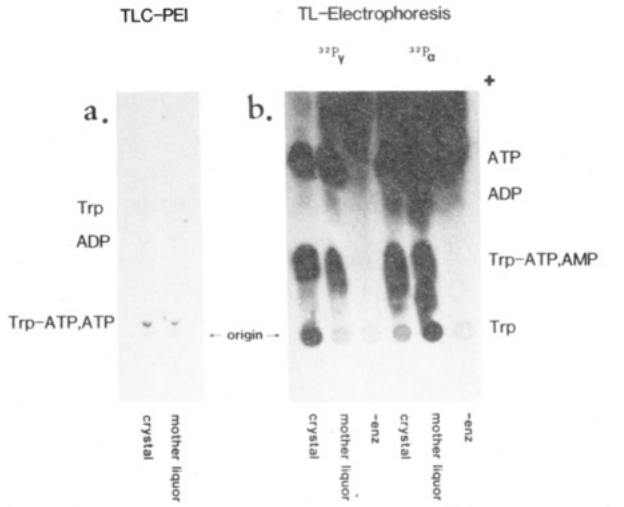


FIGURE 2: Autoradiograms which demonstrate that Trp-ATP is bound to type IV crystals. (a) Products labeled with [^{14}C]tryptophan and chromatographed on poly(ethylenimine) plates (Scott et al., 1977). Labeled products include Trp-ATP, free tryptophan, and a compound with the migration behavior of ADP, probably Trp-ADP. The major band represents Trp-ATP, which appears both in the crystal and in the mother liquor. (b) Products labeled with [γ-^{32}P]ATP (first three lanes from left) and [α-^{32}P]ATP (last three lanes from left) and separated by thin-layer electrophoresis. In this experiment bound adenylate, Trp-AMP, would show up below the round spots where sample was applied because it is positively charged. The overloading serves here to demonstrate that there is no detectable Trp-AMP, which would appear as a difference between the first three and the last three lanes. Third and sixth lanes are minus enzyme controls.

major ^{14}C-containing spot coincided with that of ATP. Identification of this spot as Trp-ATP therefore rests on consistent correspondences between its behavior on the DE-52 cellulose, thin-layer, and paper electrophoresis systems.

Characterization of Trp-ATP in Crystals. Labeled crystals were analyzed by thin-layer electrophoresis and thin-layer chromatography on PEI–cellulose plates (Figure 2).

Type IV crystals grown in 2.0 M potassium phosphate with ATP and [^{14}C]tryptophan were isolated, dried with filter paper, and quickly dissolved. The high phosphate concentration in the mother liquor severely disrupts the PEI–cellulose thin-layer system. Thus, the dissolved crystal solution was first desalted on a microdesalting column as described under Experimental Procedures. A 20-μL sample of mother liquor was similarly prepared. In 2.0 M potassium phosphate the liquid phase contains about one-third and the crystalline phase contains two-thirds of the total protein. Mother liquor samples in Figure 2 therefore represent protein-bound ligands. The samples were applied to a prewashed PEI–cellulose plate, and the plate was developed in 1.0 M formic acid/0.5 M lithium chloride (Scott et al., 1977). After drying, the plate was autoradiographed for 3 days at −70 °C with an intensification screen. Autoradiography revealed that the major spot is in the position of Trp-ATP for both crystal and mother liquor (Figure 2a).

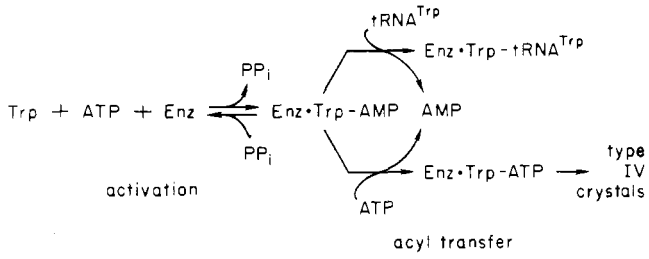
For both crystal and mother liquor two, weak, additional spots are seen in Figure 2a running in the positions of tryptophan and ADP. These spots are most likely free tryptophan and Trp-ADP, a hydrolysis product of Trp-ATP. The relative amount of the latter compound is variable and apparently increases with the age of the crystal. A minus enzyme desalting control contains no visible ^{14}C label.

To test for the presence of Trp-AMP, crystals were grown with tryptophan and [α-^{32}P]ATP. Crystals were isolated and desalted as above and analyzed by thin-layer electrophoresis because this system separates the three ^{32}P-containing compounds of interest. Autoradiography indicates no material running as Trp-AMP, which should have migrated slightly toward the cathode (right half of Figure 2). Nor is there any significant difference between incubations with α-^{32}P- and γ-^{32}P-labeled ATP. Type IV crystals therefore do not contain the aminoacyladenylate, Trp-AMP.

We can address the question of bound products in type IV crystals as follows. Experiments with [^{14}C]tryptophan show that the major form of bound tryptophan is Trp-ATP. Experiments which follow labeled nucleotide by thin-layer electrophoresis (Figure 2b) are more difficult to interpret unambiguously. The presence of ATP in the minus enzyme control for the α-^{32}P-labeled experiment (first lane in Figure 2b) shows that the desalting process is less effective for experiments with labeled nucleotides (Figure 2b). This is because Sephadex G-25 partially excludes ATP (data not shown). Despite this uncertainty it appears from Figure 2 that a substantial fraction of the bound nucleotide is Trp-ATP, although other bound nucleotides may also be present.

Stoichiometries can be determined from the results of solution experiments using the data in Figure 1a. Both ATP and tryptophan are bound to the soluble enzyme in amounts greater than one per dimer enzyme molecule (1.1 ATP; 2.0 tryptophan). The soluble enzyme binds a considerable amount of free tryptophan (50% of the bound amino acid; fractions 5–11 in Figure 1b) in addition to Trp-ATP. Scintillation counting and enzymatic assays of dissolved crystals indicate that crystals labeled with [^{14}C]tryptophan contain two labeled equivalents per dimer. Densitometry of the left-hand auto-

Scheme II



radiograph in Figure 2a indicates that about 90% of the ^{14}C label obtained from crystals is present as Trp-ATP, in contrast to the situation observed in solution.

Crystal density measurements (Low & Richards, 1952) are inconsistent with occupation of the crystallographic asymmetric unit by a dimer. Thus, we believe that 2-fold molecular symmetry is observed in the dimer of the crystalline complex. That is, the asymmetric unit to type IV crystals is a monomer, M_r 37 000, which is almost fully occupied by Trp-ATP. Chances are good, therefore, that we will be able to locate that ligand in electron density maps of the structure.

In order to tighten the connection between Trp-ATP synthesis and acyl transfer, on the one hand, and type IV crystal growth on the other hand, we have observed the effect of including 2.0 mM pyrophosphate on all three of these processes. Pyrophosphate completely inhibits acylation of $tRNA^{Trp}$ and inhibits Trp-ATP synthesis by 78%, relative to the amount of product formed in the absence of pyrophosphate. Growth of type IV crystals is completely eliminated by pyrophosphate. Crystals which do grow resemble a different crystal form (type II; Carter & Carter, 1979) grown in the presence of tryptophan alone. These results establish a strong correlation between type IV crystals and the enzymology of acyl transfer. Evidently, the catalysis of pyrophosphate exchange by the synthetase precludes acyl transfer to tRNA, Trp-ATP synthesis, and type IV crystal growth.

Discussion

The conclusion that Trp-ATP is an acyl transfer product was originally based on chemical synthesis of 3′(2′)-tryptophanyl-ATP as a standard for thin-layer chromatography and on analysis of the chemical reactivity of the enzymatic product (Weiss et al., 1959). It also provides the most sensible explanation for the retention of the γ-phosphate in the tryptophanyl derivative. Dependence of type IV crystal growth on a high ATP concentration suggests that an enzymatic process other than formation of the aminoacyladenylate is required for growth of type IV crystals and that this process is probably synthesis of Trp-ATP. The fact that these processes and acylation of tRNA are all sensitive to inhibition by pyrophosphate indicates that they all require and consume the adenylate intermediate. Synthesis of Trp-ATP therefore probably occurs subsequently to amino acid activation. We therefore can revise Scheme I given in the introduction as follows. Trp-ATP is synthesized by acyl transfer to a second ATP molecule in a process analogous to acylation of tRNA (Scheme II). The stoichiometric amount of bound Trp-ATP in type IV crystals suggests the precursor–product relationship shown in Scheme II. If the K_m for binding the second molecule of ATP at the tRNA binding site is very much higher than that for binding the first at its normal site, this would explain the high ATP concentration required for type IV crystal growth. Finally, Scheme II accounts for the inhibitory effect of pyrophosphate on all three processes listed above. Pyrophosphate exchange competes with these processes by reversing

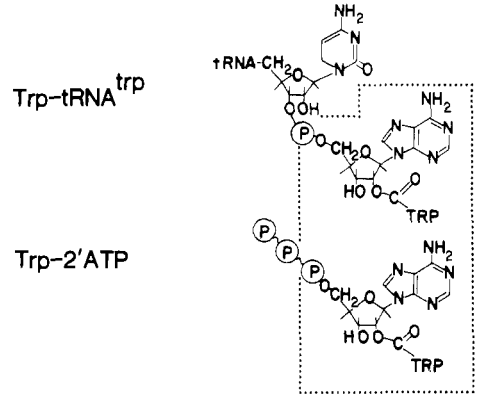


FIGURE 3: Acyl-transfer products formed by *B. stearothermophilus* tryptophanyl-tRNA synthetase. Comparison of Trp-ATP with the 3′ terminus of tryptophanyl-$tRNA^{Trp}$. Tryptophanyl moiety is shown on the 2′-hydroxyl group in both cases, but it is unknown which position (2′ or 3′) is esterified in the enzyme–product complexes.

the synthesis of the Trp-AMP intermediate.

Much effort has been devoted to locating the tRNA binding sites in crystalline tyrosyl- and methionyl-tRNA synthetases (Irwin et al., 1976; Monteilhet & Blow, 1978, Rubin & Blow, 1980; Zelwar et al., 1976, 1982). Despite such efforts it has not been possible to locate the binding site either by inspection and model building or by soaking crystals in various analogues of the tRNA, including the oligonucleotide, CCA (Monteilhet & Blow, 1978; Rubin & Blow, 1980). Trp-ATP resembles the 3′-terminal fragment, 3′(2′)-tryptophanyladenosine, of the normal product Trp-$tRNA^{Trp}$. The corresponding parts are enclosed by the dotted line in Figure 3. Its position in type IV crystals therefore should help locate the tRNA binding site on the enzyme. We have used the ability of the tryptophanyl enzyme to acylate ATP as a means of labeling a region of the enzyme not previously characterized for other synthetases.

Yeast phenylalanyl-tRNA synthetase has been shown to transfer the phenylalanyl moiety to adenosine and CpCpA acceptors, but only in the presence of modified $tRNA^{Phe}$ lacking terminal residues (Renaud et al., 1981). It has also been shown that binding of $tRNA^{Phe}$ to phenylalanyl synthetase causes the enzyme to undergo a conformational change (Krauss et al., 1976; Ehrlich et al., 1980). These observations have been interpreted to mean that a tRNA-induced conformational change is necessary in order for acyl transfer to proceed. The requirement for a macromolecular triggering ligand by the phenylalanyl enzyme is in contrast to our results which do not show a requirement for modified $tRNA^{Trp}$.

It is interesting that the terminal adenosine plays an important role in the $tRNA^{Phe}$-dependent conformational change in the phenylalanyl enzyme (Von der Haar & Gaertner, 1975; Krauss et al., 1977). Von der Haar & Gaertner (1975) have proposed that the 3′-terminal adenosine plays a role analogous to an allosteric effector. In light of this observation, it is possible that the tryptophan enzyme must also change conformation prior to acyl transfer but that ATP can act by itself to effect this change. The polymorphism of the tryptophanyl enzyme crystals (Carter & Carter, 1979) may allow us to pursue this question in detail, once more is known about the structures of different crystal forms.

The ability of tryptophanyl-tRNA synthetase to transfer tryptophan to acceptors other than cognate tRNA may be important in another context entirely. It has been reported that the seryl- and leucyl-tRNA synthetases transfer their aminoacyl moieties to the hydroxyamino group of 4-(hydroxyamino)quinoline 1-oxide (Tada & Tada, 1975). This

process is thought to provide the ultimate pathway for activating 4-nitroquinoline 1-oxide into a carcinogen.

Acknowledgments

We are grateful to A. H. Lockwood for suggesting the use of cellulose thin-layer plates for electrophoresis.

Registry No. Trp-ATP, 88015-39-8; tryptophanyl-tRNA synthetase, 9023-44-3.

References

Atkinson, T., Banks, G. T., Bruton, C. J., Comer, M. J., Jakes, R., Kalmagharan, T., Whitakee, A. R., & Winter, G. P. (1979) *J. Appl. Biochem. 1*, 247–258.

Carter, C. W., Jr., & Carter, C. W. (1979) *J. Biol. Chem. 254*, 12219–12223.

Carter, C. W., Jr., & Green, D. C. (1982) *Anal. Biochem. 124*, 327–332.

Christopherson, R. I., Jones, M. E., & Finch, L. R. (1979) *Anal. Biochem. 100*, 184–187.

Ehrlich, R., Lefevre, J.-F., & Remy, P. (1980) *Eur. J. Biochem. 103*, 145–153.

Fersht, A. R., Ashford, J. S., Bruton, C. J., Jakes, R., Koch, G. L. E., & Hartley, B. S. (1975) *Biochemistry 14*, 1–4.

Irwin, M. J., Nyborg, J., Reid, B. R., & Blow, D. M. (1976) *J. Mol. Biol. 105*, 577–586.

Joseph, D. R., & Muench, K. H. (1971a) *J. Biol. Chem. 246*, 7602–7609.

Joseph, D. R., & Muench, K. H. (1971b) *J. Biol. Chem. 246*, 7610–7615.

Krauss, G., Riesner, D., & Maass, G. (1976) *Eur. J. Biochem. 68*, 81–93.

Krauss, G., Riesner, D., & Maass, G. (1977) *Nucleic Acids Res. 4*, 2253–2262.

Low, B., & Richards, F. M. (1952) *J. Am. Chem. Soc. 74*, 1660–1666.

Monteilhet, C., & Blow, D. M. (1978) *J. Mol. Biol. 122*, 407–417.

Muench, K. H. (1969) *Biochemistry 8*, 4872–4888.

Renaud, M., Bacha, H., Remy, P., & Ebel, J. P. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 1606–1608.

Rubin, J., & Blow, D. M. (1980) *J. Mol. Biol. 145*, 489–500.

Scott, J. F., Eisenberg, S., Bertsch, L. L., & Kornberg, A. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 193–197.

Tada, M., & Tada, M. (1975) *Nature (London) 255*, 510–512.

Von der Haar, F., & Gaertner, E. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 1378–1382.

Weiss, S. B., Zachau, H. G., & Lipmann, F. (1959) *Arch. Biochem. Biophys. 83*, 101–114.

Zelwar, C., Risler, J. L., & Monteilhet, C. (1976) *J. Mol. Biol. 102*, 93–106.

Zelwar, C., Risler, J. L., & Brunie, S. (1982) *J. Mol. Biol. 155*, 63–81.

Inhibitory Effect of Duramycin on Partial Reactions Catalyzed by (Na^+,K^+)-Adenosinetriphosphatase from Dog Kidney†

Shun Nakamura and Efraim Racker*

ABSTRACT: The polypeptide antibiotic duramycin inhibited the (Na^+,K^+)-adenosinetriphosphatase purified from dog kidney. An analysis of its mode of action revealed that the formation of phosphoenzyme from P_i but not from ATP was inhibited. The rate of dephosphorylation of the phosphoenzyme formed from ATP was markedly reduced. In contrast to quercetin, duramycin did not inhibit K^+-dependent *p*-nitrophenylphosphatase activity. The effect of duramycin was completely reversed by phospholipids.

Duramycin, an antibiotic isolated from the culture broth of *Streptomyces cinnamoneus* forma *Azacoluta*, inhibits the growth of some Gram-positive rods, yeasts, and fungi (Shotwell et al., 1958). It is an unusual polypeptide containing rare amino acids such as lanthionine, methyllanthionine, and lysinoarginine (Gross & Brown, 1976).

It was observed recently that duramycin inhibits the chloride-dependent ATP-driven proton pump of clathrin-coated vesicles at concentrations that have little or no effect on the mitochondrial proton pump [Stone et al. (1983) and unpublished results]. Duramycin inhibits also the ouabain-sensitive and -insensitive hydrolysis of ATP by plasma membranes of Ehrlich ascites tumor cells but has no effect on the soluble mitochondrial ATPase (Racker et al., 1984).

In view of these observations, we undertook a study of the mode of action of duramycin on a well-characterized (Na^+,K^+)-ATPase preparation from dog kidney (Jørgensen, 1974). This enzyme is known to catalyze a series of reactions involving two intermediates, E_1–P and E_2–P, during the hydrolysis of ATP to ADP and P_i. In this paper, we describe the effect of duramycin on the partial reactions catalyzed by the purified enzyme. We also report the reversal of duramycin inhibition by addition of phospholipids.

Experimental Procedures

Materials. Radioactive orthophosphate ([^{32}P]P_i), obtained from ICN Pharmaceuticals, Inc., was incubated at 100 °C in 3 N HCl for 3 h and stored at −20 °C. Prior to use, it was

† From the Section of Biochemistry, Molecular and Cell Biology, Division of Biological Sciences, Cornell University, Ithaca, New York 14853. *Received August 3, 1983.* This investigation was supported by Grant BC-156 from the American Cancer Society and by U.S. Public Health Service Grant CA-08964 from the National Cancer Institute, DHHS.

0006-2960/84/0423-0385$01.50/0