Phenylalanyl Transfer Ribonucleic Acid Synthetase from *Escherichia coli*. Analysis of the Adenosine Triphosphate Binding Site*

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ABSTRACT: The ATP binding site of Phe-tRNA synthetase of *Escherichia coli* B has been studied by the use of competitive inhibitors. The major source of binding energy is derived from the 6-aminopurine moiety of ATP; the remaining interactions may be described as an intricate balance of opposing and reinforcing forces which contribute little to the net free energy of binding but are important for specificity and catalysis. The β- and γ-phosphates of ATP do not appear to interact with the free enzyme or the enzyme–phenylalanine complex, and may be deleted with little effect on binding. In contrast, the α-phosphate is very detrimental to binding, and its omission leads to large increases in the extent of complexation. Binding studies of a number of deoxyadenosines demonstrate that individual contributions to binding from the hydroxyl groups and furanosyl ring oxygen are small. The contributory interactions of the ribose moiety of ATP are best accounted for by hydrophobic microregions of the enzyme binding site. This is illustrated by 5′-deoxyadenosine ($K_i = 1 \times 10^{-6}$ M), which binds 15-fold tighter than adenosine and 1000-fold better than ATP. A number of 9-alkyl- and -hydroxyalkyladenines have been examined as possible congeners of adenosine. These bind as well as ATP, but much poorer than adenosine analogs having a five-membered ring attached to the 9 position of the purine. It is proposed that the role of the ribofuranosyl group of ATP is mainly one of juxtaposing groups in their proper orientations for specificity and catalysis. 2′-dATP serves as a substrate for pyrophosphate exchange and acylation of tRNA almost as effectively as ATP. It appears that differences exist in the ATP binding sites of various activating enzymes, and molecules such as adenine, adenosine, AMP, and ADP, as well as their 2′-deoxy counterparts, may play a role in the control of protein biosynthesis by inhibition of one or more of the aa-tRNA synthetases.

Studies of substrate interactions with the aa-tRNA synthetases have for the most part been concerned with recognition and binding of cognate tRNAs and amino acids. All of the activating enzymes utilize ATP as a common substrate but, with the exception of a few reports, little is known about the nature of its binding site. What are the important points of interaction which lead to complexation? How specific is binding with respect to the sugar and phosphate groups of ATP? Can localized microenvironments of the enzyme binding site be described? Are the ATP binding sites of various activating enzymes similar? These and related questions have been studied with purified Phe-tRNA synthetase from *Escherichia coli* by the use of competitive inhibitors. Similar studies of binding of L-phenylalanine are described in an accompanying report (Santi and Danenburg, 1971).

Materials and Methods

Phe-tRNA synthetase was isolated from *E. coli* B by the method of Stulberg (1967) and ATP–[^{32}P]PP$_i$ exchange assays were performed under the standard conditions previously described (Santi *et al.*, 1971). Inhibition constants (K_i) were determined by Lineweaver–Burk (1934) plots varying MgATP at fixed levels of L-phenylalanine (0.3 mM), PP$_i$ (2.0 mM), and magnesium chloride (19 mM). The magnesium concentration used falls on the broad optimum where the exchange rate is insensitive to small variations which might result from complexation of magnesium ion to the inhibitors studied. When inhibitors known to complex with magnesium ion were tested at high concentration, an equivalent amount of magnesium chloride was generally added to ensure against Mg^{2+} drainage. Unless otherwise specified, where K_i values are given, inhibitors were competitive with respect to ATP.

The 9-(ω-hydroxyalkyl)adenines, 9-(6-hydroxyhexyl)adenine phosphate, *cis*- and *trans*-9-(4-hydroxymethylcyclohexyl)-adenines, and *cis*-9-(3-hydroxymethylcyclopentyl)adenine were gifts of V. A. Peña of this laboratory. 5′-Deoxyadenosine was donated by Dr. R. Abeles of Brandeis University, 4′-CH$_2$-adenosine[1] was obtained from Dr. Y. W. Shealy of Southern Research Institute, 2′,3′-dideoxyadenosine was a gift from Dr. R. K. Robins, and α,β-methyleneadenosine was obtained from Miles Laboratories, Inc. 9-Methyladenine was prepared as described by Myers and Zeleznick (1963). 9-Ethyl- (Montgomery and Temple, 1957), 9-propyl- (Browne *et al.*, 1968), 9-butyl- (Montgomery and Temple, 1958), and 9-pentyl- (Temple *et al.*, 1962) adenines were prepared by direct alkylation of sodium adenine in *N*,*N*′-dimethylformamide as described by Leonard *et al.* (1965). Adenosine 5′-methylphosphonate was prepared by the method of Myers *et al.* (1965). All other materials were obtained commercially and were the highest purity available.

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[1] Abbreviations used are: PRS, Phe-tRNA synthetase (EC 6.1.1.4) of *Escherichia coli* B; Phe-tRNA, phenylalanyl-tRNA; tRNA^{Phe}, tRNA specific for phenylalanine acceptance; Phe-AMP, phenylalanyl-adenylate; 4′-CH$_2$adenosine, 9-[β-D,L-2α,3α-dihydroxy-4-β-(hydroxymethyl)cyclopentyl]adenine.

TABLE I: Binding of 6-Aminopurine Nucleotides and Nucleotide Analogs to Phe-tRNA Synthetase.[a]

Compound	K_i (mM)
ATP	1.0[b]
ADP	2.4
AMP	3.7
dATP	1.6 (K_m)[c]
dAMP	6.2
α,β-Methyleneadenosine triphosphate	Noncompetitive
Adenosine 5′-methylphosphonate	Noncompetitive
9-(6-Hydroxyhexyl)adenine phosphate	3.0

[a] Inhibitions were noncompetitive with respect to L-phenylalanine. [b] Determined kinetically (Santi *et al.*, 1971). [c] Determined under standard assay conditions.

TABLE III: Inhibition of ATP–PP_i Exchange by 9-Alkyl- and 9-Hydroxyalkyladenines.

Compound	K_i (mM)
9-(3-Hydroxypropyl)adenine	0.59
9-(4-Hydroxybutyl)adenine	2.6
9-(5-Hydroxypentyl)adenine	0.49
9-(6-Hydroxyhexyl)adenine	0.24
Adenine	0.5
9-Methyladenine	1.4
9-Ethyladenine	0.85
9-Propyladenine	0.60
9-Butyladenine	0.43
9-Pentyladenine	0.15
9-Isopentyladenine	0.19
cis-9-(4-Hydroxymethylcyclohexyl)adenine	0.25
trans-9-(4-Hydroxymethylcyclohexyl)adenine	0.72

Results

In Table I are given binding constants or K_m values for a number of 6-aminopurine nucleotides and analogs when tested as inhibitors or substrates of PRS. In agreement with the kinetics expected for the random order of substrate binding to PRS (Santi *et al.*, 1971) the inhibitors, except for α,β-methyleneadenosine triphosphate and adenosine 5′-methylphosphonate, were competitive with respect to ATP and noncompetitive with respect to L-phenylalanine. The inhibition constants for the noncompetitive inhibitors were not determined since it is believed (see Discussion) that these analogs bind to two distinct sites of PRS. The dissociation constant listed for the PRS·ATP complex is that determined kinetically (Santi *et al.*, 1971) and, within experimental error, is identical with the K_m for ATP. In the absence of ATP, PRS catalyzes a L-phenylalanine-dependent incorporation of PP_i into dATP, presumably through a Phe-dAMP intermediate. The K_m for dATP is similar to ATP but V_{max} is depressed threefold with the alternate substrate. dATP is also an effective substrate for Phe-tRNA formation, giving a K_m value of 9×10^{-4} M and a 20% depression in V_{max} as compared to ATP. It is noted that the conditions used for these experiments were those derived for ATP and were not optimized for dATP.

In order to evaluate the mode of binding of the ribose moiety of the substrate, it was desirable to have analogs of ATP in which the functional groups of the ribose were systematically omitted or modified. Because of the general difficulties in preparing triphosphate esters and the complication that such analogs might demonstrate substrate properties, we elected to approach this problem by an examination of analogs which did not possess the triphosphate group. It has previously been shown (Santi *et al.*, 1971) that adenosine is bound to PRS *ca.* 67-fold more tightly than the substrate, and gives inhibition patterns which indicate that it is complexed to the ATP binding site. Table II contains inhibition constants of a series of deoxyadenosines, all of which were competitive with respect to ATP. In conjunction with the above, and in order to determine the effect of changing the overall size and stereochemistry of the 9-substituent while retaining a hydroxyl group to assist in binding to PRS, the inhibitory properties of a number of 9-hydroxyalkyladenines were examined. These results are given in Table III, along with the binding constants obtained for adenine and a series of 9-alkyladenines. The latter provide information as to the overall contribution to binding of the purine ring and the terminal hydroxyl functions of the hydroxyalkyladenines.

TABLE II: Inhibition of ATP–PP_i Exchange by Adenosine and Deoxyadenosines.

Compound	K_i (mM)
Adenosine	0.015
2′-Deoxyadenosine	0.0073
3′-Deoxyadenosine	0.13
4′-CH_2adenosine	0.12
5′-Deoxyadenosine	0.001
cis-9-(3-Hydroxymethylcyclopentyl)adenine	0.05
2′,3′-Dideoxyadenosine	0.02

Discussion

The ATP–PP_i exchange reaction catalyzed by the aa-tRNA synthetases requires binding of both ATP and PP_i as their magnesium salts. *A priori*, it would appear reasonable to believe that the same binding site is involved for PP_i and the β,γ-phosphates of ATP. This is supported in some cases by observations that PP_i competes with ATP for its binding site on the tyrosine- (Santi and Peña, 1971) and isoleucine- (Cole and Schimmel, 1970a) activating enzymes. In contrast, we observed (Santi *et al.*, 1971) that PP_i does not compete with ATP for the ATP binding site of PRS, but still has a relatively high affinity for the Phe-AMP–enzyme complex. This was interpreted as evidence that the PP_i binding site of PRS was masked and inaccessible unless the aminoacyladenylate were bound, and suggested the possibility of a conformational change in the activation process. The corollary to this is that the β,γ-phosphates of ATP do not contribute significantly to the free energy of binding to PRS. In support of the above, it has been shown (Table I) that the mono- and diphosphate esters of adenosine have approximately the same affinity for PRS as does ATP. Similarly, if K_m is taken as a measure of dissociation constant, omission of the terminal pyrophosphate moiety of dATP to give dAMP results in only a 2.6-fold loss in binding. The small variation in binding of these analogs

is even more dramatic in view of the different structural features imposed by magnesium complexation. It is tempting to speculate that the high affinity of PP_i for the PRS·PheAMP complex and the lack of binding of the β,γ-phosphates of ATP provide a driving force for aminoacyladenylate formation.

Substitution of the oxygen atom which separates the α- and β-phosphates of ATP to give α,β-methyleneadenosine triphosphate results in noncompetitive inhibition with respect to ATP; the effect is also obtained upon substitution of methyl for one of the phosphate oxygens of AMP, giving adenosine 5′-methylphosphonate. These results are of interest for two reasons. First, they suggest the presence of an alternate binding site for adenosine nucleotides, a conclusion which has been verified by other studies (D. V. Santi, unpublished results). Second, they may indicate the occurrence of a specific interaction between the α,β oxygen of ATP and PRS which is required for productive binding. With regard to the latter, it is noted that such an interaction could be important for specificity and yet not contribute to the net observed binding if it were negated by detrimental interactions at some other site(s). Relevant to this are the observations that the affinity of PP_i for phenylalanyl- (Santi *et al.*, 1971), tyrosyl- (Santi and Peña, 1971), and isoleucyl- (Cole and Schimmel, 1970a) tRNA synthetases is significantly greater than that of ATP. In the minimal mechanism which describes ATP–PP_i exchange, the α,β-oxygen atom of ATP departs as PP_i with a negative charge in the formation of aminoacyladenylate; likewise, in the reverse reaction nucleophilic attack on the latter by pyrophosphate to give ATP must, by microscopic reversibility, involve the same species. It has recently been shown (Cole and Schimmel, 1970b) for the isoleucine-activating enzyme, and is also probably true for others, that the principal substrate forms of ATP and PP_i are $MgATP^{2-}$ and $MgP_2O_7^{2-}$. Since the only difference between PP_i and its counterpart in the ATP molecule is the additional negative charge possessed by the former, it must, in some fashion, be responsible for the high affinity of PP_i for these enzymes. It is possible that a functional group of the enzyme could complex with the α,β oxygen of ATP, perhaps by a specific hydrogen bond or electrostatic interaction which is also responsible for the high affinity of PP_i. From a teleological standpoint, interactions of this type would be advantageous for catalysis since the developing negative charge on PP_i would be partially neutralized in the transition state leading to the aminoacyladenylate. The previous suggestion that a change in the PP_i binding site of PRS occurs with Phe-AMP formation may be accommodated by this model if the complexing group of the enzyme is one involved in a conformational change.

It is interesting that adenine is bound to PRS more tightly than ATP. In view of the detrimental interaction of the α-phosphate group, this result might initially be interpreted to indicate that the ribose moiety of ATP has little direct interaction with the enzyme. However, this cannot be the case since the K_i for adenine greatly exceeds those of its nucleoside analogs. Clearly, complexation of a large polyfunctional molecule to an enzyme involves an intricate balance of opposing and reinforcing forces. Individual interactions need not contribute to the net free energy of binding but probably play a determinant role in specificity and recognition. Furthermore, a particular structural modification could perturb interactions between the enzyme and functions removed from the modified site. Thus, although it is apparent that the 6-aminopurine is necessary for complexation, the extent of its contribution to binding may be dependent upon the nature of the 9-substituent. For example, if the unfavorable interactions which exist between the α-phosphate of adenosine nucleotides and PRS perturb the binding of the adenine and/or ribose moieties, deletion of the phosphate group would relieve this stress and permit more favorable interactions to occur at these other sites. In this manner, the large increases obtained with analogs not possessing a phosphate may in part reflect the optimization of binding of removed sites.

In an attempt to ascertain the role of the oxygen functions of the ribose moiety in complexation of ATP to PRS, inhibitory properties of a number of deoxyadenosines (Table II) were examined. Although precise analyses of the results are complicated by unpredictable changes in conformation and solvation which likely accompany minor structural modifications, structural requirements for binding have been obtained and limited interpretation of the forces involved in complexation may be made. It is apparent that the 2′-hydroxyl of the ribofuranosyl group is not necessary for complexation to PRS since the K_i values of 2′-deoxyadenosine and dAMP are within a factor of 2 of the corresponding ribofuranosyl analogs; also as will be discussed later, dATP is an effective substrate for pyrophosphate exchange and $tRNA^{Phe}$ esterification. The observation that the K_i of 3′-deoxyadenosine is tenfold greater than that of adenosine initially suggested that the 3′-hydroxyl might be an important binding point. However, this possibility was rejected upon finding that 2′,3′-dideoxyadenosine is bound as well as adenosine. These data are currently interpreted as evidence that the 2′-hydroxyl group is involved in an unfavorable interaction which is in some way neutralized by the presence of the adjacent 3′-hydroxyl group, perhaps as a result of intramolecular hydrogen-bond formation. The omission of the 2′,3′-*cis*-glycol system of adenosine converts this polar region into one that is hydrophobic. The fact that this change does not affect binding suggests that either this region is not a contact point, or involves specific solvation effects which are too complex for definition at this time. If the former is correct, the noncontact area connot be very large since the bulky 2′,3′-isopropylidene group is severely detrimental to binding. Substitution of the oxygen of the furanoside ring of adenosine by CH_2 results in a ninefold loss in binding. On the other hand, the K_i of *cis*-9-(3-hydroxymethylcyclopentyl)adenine, a carbocyclic adenosine analog which also lacks the 2′- and 3′-hydroxyl groups, is only three times greater than that of adenosine, suggesting that in this case a ring oxygen is not a critical binding point and supporting the previous evidence that the 2′- and 3′-hydroxyl groups are not necessary for binding. The tight binding of this compound is not a result of nonspecific interactions since the *cis*- and *trans*-9-(4-hydroxymethylcyclohexyl)adenines bind considerably poorer. It is noted that the above compounds were tested as enantiomeric mixtures and the K_i values could be high by as much as a factor of 2.

The inhibitors discussed thus far possess substituents on the 9-position of the adenine which are similar in basic dimensions and stereochemistry to the ribofuranosyl group of the substrate, ATP. In order to ascertain whether a rigid steric requirement for a five-membered ring exists in the corresponding binding region of PRS, the inhibitory power of a number of 9-alkyl- and 9-hydroxyalkyladenines was examined. Although the numerous conformations which may be assumed by the 9-substituents of these analogs make them of limited usefulness in probing the topography of the binding site, their flexibility could permit optimization of interactions with the suspected hydrophobic microregions of the enzyme. Furthermore, the hydroxyl group, where present, could simulate inter-

actions of the hydroxyls of adenosine. With the exception of the 1-methyl- and 4-hydroxybutyladenines, all analogs have fairly similar affinities for PRS and bind tighter than the substrate, ATP. The K_i for 9-methyladenine is threefold larger than that of adenine and, if bound to the same site as the purine moiety of the substrate, indicates a detrimental steric interaction between PRS and the C_1' position of ATP or related analogs. As the alkyl chain is lengthened from methyl to *n*-pentyl, 1.37 kcal/mole in binding energy is obtained which is best explained as a result of hydrophobic interactions. The incremental increase in binding per methylene group corresponds to 0.2–0.3 kcal/mole except in going from *n*-butyl to *n*-pentyl, where 0.64 kcal/mole in binding energy is obtained. The hydroxyalkyladenines show no indication of additional contribution to binding by the terminal hydroxyl group, and demonstrate inhibitory powers equal to or poorer than the 9-alkyladenines. As with the latter series, extension of the carbon chain separating the hydroxyl and purine moieties generally enhances binding; an exception is observed with 4-hydroxybutyladenine which binds significantly poorer than other analogs in this series. Clearly, there is no assurance that the 9-alkyl and hydroxyalkyl chains interact with PRS in the ribosyl binding region. However, it is interesting that conversion of 9-(6-hydroxyhexyl)adenine into its monophosphate ester results in a 12-fold increase in K_i. Although this decrease in binding may reflect a nonspecific repulsion of the polar phosphate from a hydrophobic region of the enzyme, it is the same effect observed upon phosphorylation of adenosine and 2′-deoxyadenosine.

A number of observations indicate that the nature of the ATP binding site may differ among the various synthetases, and the case examined here may be unique in many respects. Other studies in progress in this laboratory demonstrate that, in contrast to PRS, phosphate moieties of ATP are necessary for complexation to the Tyr-tRNA synthetase and contribute to binding to the isoleucine- and valine-activating enzymes. Mitra and Mehler (1969) have observed that the tyrosine- and lysine-activating enzymes from *E. coli* may not effectively utilize dATP in the PP_i exchange assay although it serves as a substrate for the esterification of cognate tRNA molecules. For the latter reaction, these workers observed much larger K_m values for the deoxyribonucleotide but similar maximum velocities and concluded that dATP was not a relevant *in vivo* energy source for aa-tRNA synthesis. As shown in this report, PRS may utilize dATP almost as effectively as ATP for both pyrophosphate exchange and formation of Phe-tRNA; this suggests that dATP may be involved in the *in vivo* synthesis of the Phe-tRNA. Furthermore, the competition for the ATP binding site by such naturally occurring molecules as AMP, adenosine, and adenine suggest that these may play roles in the control of protein biosynthesis. The relevance of the aforementioned possibility that PRS may be more susceptible than other activating enzymes with regard to this inhibition is not known at this time.

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