Phenylalanyl Transfer Ribonucleic Acid Synthetase from *Escherichia coli*. Reaction Parameters and Order of Substrate Addition*

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ABSTRACT: Reaction parameters and catalytic indices for Phe-tRNA synthetase catalyzed ATP–PP_i exchange and acylation of $tRNA^{Phe}$ have been defined. The exchange reaction occurs at a significantly greater rate than the formation of Phe-tRNA, and is inhibited by $tRNA^{Phe}$ and periodate-oxidized $tRNA^{Phe}$. Studies of the effects of sodium chloride and ethanol on catalytic indices are in accord with proposals of the involvement of ionic and hydrophobic forces in interactions of the ligase with tRNA and L-phenylalanine, respectively. Inhibitory properties of phenylalaninol, *p*-fluorophenylalaninol, 2-amino-3-phenyl ethyl phosphonate, adenosine, and phenylalaninoladenylate are described. The sequence of substrate binding to Phe-tRNA synthetase from *Escherichia coli* B has been investigated using isotope-exchange studies, initial velocity experiments, and studies with inhibitors. The ATP–PP_i exchange proceeds by a random order of addition of both phenylalanine and ATP. Evidence is presented which suggests that PP_i has a much greater affinity for the ligase–phenylalanyladenylate complex than for the free-enzyme form. Similarly, in the formation of Phe-tRNA, all substrates may bind to the enzyme in a random fashion. The release of PP_i from the enzyme is not required for the binding of $tRNA^{Phe}$ but may occur in a nonobligatory manner, indicating the occurrence of both sequential and Ping-Pong mechanisms.

The importance of the aa-tRNA synthetases to the correct translation of a genetic message is well recognized (Novelli, 1967). This group of enzymes, one for each amino acid, catalyzes the formation of specific aa-tRNA molecules in two discrete steps (Berg *et al.*, 1961)

$$\text{aa} + \text{ATP} \rightleftharpoons \text{aa}\cdot\text{AMP} + PP_i$$

$$\text{aa}\cdot\text{AMP} + \text{tRNA} \rightleftharpoons \text{aa}\cdot\text{tRNA} + \text{AMP}$$

One of the most fundamental questions to be answered is how these proteins are able to distinguish between structurally similar substrates with complete fidelity. The answer requires a knowledge of the differences which exist among the various ligases to which specificity may be attributed, and their manifestations with regard to substrate binding and catalysis. Although at first glance these enzymes would appear to be quite similar it is now apparent that they differ in so many respects that gross analogies must be avoided and each studied in detail. We have undertaken a search for common underlying features of this group of enzymes, as well as basic differences which might permit a better understanding of the molecular basis of substrate binding and recognition.

It is well recognized (*cf.* Novelli, 1967) that reaction parameters for the aa-tRNA synthetases may vary greatly for each activating enzyme, and in many cases for enzymes from different sources which activate the same amino acid. In addition, ATP–PP_i exchange and aminoacylation of tRNA often respond differently toward buffers, pH, ionic strength, organic solvents, and magnesium ion concentration. The relevance of these variations to mechanism and function of the synthetases is not clear at this time, but it is apparent that definition of these parameters for each aa-tRNA synthetase is rudimentary to more exacting investigations.

Relatively little is known about the binding sequences of substrates to the aa-tRNA synthetases, and to what extent these enzymes are similar in this respect. However, various reports (see, for example, Santi and Peña, 1971, Rouget and Chapeville, 1968, Allende *et al.*, 1970, and Mitra and Mehler, 1966) suggest that there may be considerable divergence among the activating enzymes in this basic mechanistic feature. Furthermore, since the binding site for a particular substrate probably depends on whether cosubstrates are present, the order of substrate binding may be highly relevant in defining the nature of the interactions which are responsible for recognition and specificity.

In the present investigation, as part of a broader study of small molecule interactions with the aa-tRNA synthetases, fundamental reaction parameters of highly purified PRS[1] from *Escherichia coli* have been established as well as the sequence of substrate addition in ATP–PP_i exchange and aminoacylation of $tRNA^{Phe}$. These studies also provide the foundation for accompanying (Santi and Danenberg, 1971; Santi *et al.*, 1971) and continuing studies of substrate and inhibitor interactions with PRS.

Materials and Methods

Phe-tRNA synthetase was a 311-fold-purified preparation obtained from *E. coli* B cells (General Biochemicals) by the

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[1] Abbreviations used are: PRS, Phe-tRNA synthetase (EC 6.1.1.4) of *E. coli* B; Phe-tRNA, phenylalanyl-tRNA; $tRNA^{Phe}$, tRNA specific for phenylalanine acceptance; $tRNA^{Phe}_{ox}$, $tRNA^{Phe}$ which has been oxidized with periodate; APEP, D,L-1-amino-2-phenyl ethyl phosphonate.

method of Stulberg (1967); acrylamide gel electrophoresis and titration with [^{14}C]Phe-tRNA (Yarus and Berg, 1967) indicated that this preparation was 85–90% pure. Some experiments utilized a 50-fold purified preparation isolated by a modification of the method of Muench and Berg (1966), which included the addition of an ammonium sulfate fractionation (Stulberg, 1967) prior to DEAE-cellulose chromatography. *E. coli* B tRNA was obtained from Schwarz BioResearch and enriched tenfold in tRNA^{Phe} by BD-cellulose chromatography according to the procedure of Litt (1968). Periodate oxidation of tRNA^{Phe} was performed according to the procedure of Yarus and Berg (1969). L-Phenylalanol and DL-*p*-fluorophenylalanol were prepared by $LiAlH_4$ reduction of the corresponding trimethylsilated amino acids as described in the accompanying paper and L-phenylalaninol adenylate was prepared as described by Sandrin and Boissonnas (1966). [^{32}P]PP_i (10^3–10^4 mCi/mmole) and L-[^{14}C]-phenylalanine (350 mCi/mmole) were purchased from New England Nuclear Corp. APEP was obtained from Calbiochem and crystalline Na_2ATP from Sigma. All other materials were reagent grade.

ATP–PP_i Exchange Assay. The standard reaction mixture contained 4 mM Na_2ATP, 0.3 mM L-phenylalanine, 25 mM $MgCl_2$, 2 mM [^{32}P]PP_i (*ca.* 2×10^5 cpm), 10 mM 2-mercaptoethanol, 0.05 mg of bovine serum albumin, 100 mM sodium cacodylate (pH 7.0), and a limiting amount of Phe-tRNA synthetase (*ca.* 1.8×10^{-4} unit) in a total volume of 0.5 ml. The reaction was usually initiated by the addition of [^{32}P]PP_i and enzyme was omitted for controls. When one or more of the components was varied, all others were held constant at the concentrations listed above. The mixture was incubated at 37°, and at appropriate times (10 min for single point assays) quenched with 0.7 ml of 15% perchloric acid which contained 0.4 M PP_i. The ATP was adsorbed on Norite, collected on Whatman GF/C glass filters as described by Calendar and Berg (1966), and counted on a Nuclear-Chicago planchet counter.

Aminoacylation of tRNA^{Phe}. The standard reaction mixture contained 5 mM Na_2ATP, 30 μM L-[^{14}C]phenylalanine (*ca.* 2×10^5 cpm), 0.6 μM tRNA^{Phe} (*ca.* 40 A_{260} of crude or 4 A_{260} of purified), 25 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 0.125 mg of bovine serum albumin, 6 mM KCl, 125 mM Tris buffer (pH 7.5), and limiting amounts of enzyme (*ca.* 3.5×10^{-5} unit) in a total volume of 0.5 ml. The reaction was initiated by addition of enzyme, and enzyme was omitted for controls. When one or more substrates were varied, all others were held constant at the concentrations listed above. After appropriate time intervals at 37°, the reactions were quenched and Phe-tRNA was precipitated by the addition of 5 ml of ice-cold 2 N HCl. When less than 10 A_{260} of tRNA was used, 10 A_{260} of carrier tRNA (yeast) was added prior to the addition of acid. [^{14}C]Phe-tRNA was isolated on Whatman GF/C glass filters as described by Muench and Berg (1966), placed under toluene containing 0.4% 2,5-diphenyloxazole and 0.01% 1,4-bis[2-(5-phenyloxazolyl)]benzene, and counted in a Packard 3375 liquid scintillation counter with 70% efficiency.

All kinetic constants were determined by double-reciprocal plots (Lineweaver and Burk, 1934), precautions being exercised throughout to ensure true initial velocity conditions. The notation used is that suggested by Cleland (1963a). One unit of enzyme activity is defined as that amount which will produce 1 μmole of product ([^{32}P]ATP or Phe-tRNA) per minute in a 1-ml assay mixture under the conditions described. An A_{260} unit is defined as the amount of tRNA which, when dissolved in 1 ml of 0.1 M potassium phosphate (pH 7.0), gives an absorbance of 1.0 in a 1-cm light path at 260 mμ. In experiments utilizing unbuffered solutions, pH constancy was ensured by inserting a microelectrode into the assay solution.

Results and Discussion

Reaction Parameters. In the absence of buffer, the rate of PRS-catalyzed ATP–PP_i exchange exhibits an optimum at pH 7.2–7.7, with 50% maximal rate at pH 6.5 and 8.2. Stulberg (1967) has reported that the optimal pH for Phe-tRNA formation lies between pH 7.8 and 8.2 in Tris·HCl buffer. It has recently been shown that buffers may have a dramatic effect upon the catalytic properties of the aa-tRNA synthetases (Loftfield and Eigner, 1968; Ritter *et al.*, 1969; Kull *et al.*, 1969; Rubin *et al.*, 1967). In particular, it is noteworthy that in a number of cases (Loftfield and Eigner, 1968) general bases have been observed to stimulate the rate of formation of amino acid hydroxamate and aa-tRNA. In the presence of 100 mM sodium cacodylate or Tris buffers at pH 7.0 there is at best only a very slight increase (<10%) in PRS-catalyzed ATP–PP_i exhcange rate as compared to an unbuffered control. However, under conditions of constant ionic strength (1.0 M), higher concentrations of Tris inhibit the rate of the exchange reaction, with 50% inhibition occurring at 0.6 M Tris.

In earlier studies of PRS from *E. coli* (Conway *et al.*, 1962; Fangman and Neihardt, 1964) a standard concentration of 10 mM magnesium was used for ATP–PP_i exchange studies, but an investigation of the effect of magnesium ion concentration on the exchange rate was not reported. In recent years it has become apparent that optimum magnesium ion concentrations vary widely among the different synthetases (Novelli, 1967). At 4 mM ATP and 2 mM PP_i, the phenylalanine-dependent ATP–PP_i exchange rate increases with increasing magnesium ion concentration until a maximum rate is achieved at *ca.* 20 mM (Figure 1). A further increase of 10 mM magnesium concentration has no effect on the exchange rate, but slight inhibition (*ca.* 10%) is observed at very high levels. After correcting for the amount of magnesium which is complexed to substrates, values of 14–24 and 5.3 mM may be calculated for the optimal range and apparent K_m of free magnesium ion.[2] Although its role is not known at this time, it is apparent that free magnesium ion is required for PRS activity. Magnesium ion dependence of the rate of formation of Phe-tRNA resembles that of ATP–PP_i exchange with the exception that the optimum drops more sharply at high magnesium concentrations (Figure 1). The amount of purified tRNA^{Phe} that was required for these experiments (~0.15 mg or 1 mM nucleotide equivalents) was likely sufficiently small to have an insignificant effect on the free magnesium ion in solution. In addition, an almost superimposable magnesium dependence curve was obtained using crude tRNA (1.5 mM) excepting that the inhibition at high magnesium concentration was more pronounced. These results indicate that the optimum range of free magnesium concentration for the formation of Phe-tRNA under our standard assay conditions is 16–20 mM, after correcting for

[2] It appears to be a general practice to describe optimal magnesium ion concentration for the aa-tRNA synthetases in terms of Mg:ATP ratio or total magnesium added. However, it is noted that these are not accurate designations since the degree of complexing as well as free magnesium concentration will vary with dilution.

TABLE I: Effect of NaCl and Ethanol on Kinetic Parameters.

Additions	K_m (V_{max})[a]		
	L-Phe[b]	ATP[b]	tRNA[c]
None	6.0×10^{-5} (18)	1.0×10^{-3} (18)	5.8×10^{-8} (0.39)
0.2 M NaCl			15×10^{-8} (0.39)
0.4 M NaCl			35×10^{-8} (0.36)
0.5 M NaCl	4.2×10^{-5} (11)	1.2×10^{-3} (13)	
1.0 M NaCl	3.2×10^{-5} (8.0)	1.4×10^{-3} (11)	
5% EtOH	13.5×10^{-5} (12)	1.1×10^{-3} (9.0)	2.4×10^{-8} (0.16)
10% EtOH	21×10^{-5} (5.6)	1.2×10^{-3} (5.3)	1.7×10^{-8} (0.10)

[a] K_m is given in molar concentration units, and V_{max} as μmoles/(min mg) in a 1.0-ml assay. [b] Constants determined by ATP–PP_i exchange assay using 0.55 μg of purified enzyme. [c] Constants determined by Phe-tRNA formation using 0.11 μg of purified enzyme.

the amount of magnesium complexed to ATP. Stulberg (1967) has reported that maximal rate of Phe-tRNA formation is observed at a Mg:ATP ratio of five, which under his reported assay conditions may be calculated to be equivalent to *ca.* 9 mM free magnesium. A similar value has been reported by Rubin *et al.* (1967) to achieve maximal aminoacylation of $tRNA^{Phe}$. The discrepancy of these values to that described above may be attributed to the different pH and conditions used for assay. The magnesium concentrations chosen for use in the standard assays fall on the plateaus of the curves presented in Figure 1 where the exchange rate is optimal and insensitive to small changes in free magnesium ion concentration. In some experiments described below, where ATP or PP_i were varied, free magnesium ion concentration was held at optimal level and an equivalent amount of magnesium was varied with the ligand.

A number of workers have studied the sensitivity of various aa-tRNA synthetases toward salts and organic solvents (see, for example, Loftfield and Eigner, 1967, Kull *et al.*, 1969, Ritter *et al.*, 1969, and Sarin and Zamecnik, 1965) but unfortunately the complexity of the systems does not permit definitive classical interpretation of these effects. Primarily for descriptive and comparative purposes, the effects of sodium chloride and ethanol on PRS-catalyzed ATP–PP_i exchange and Phe-tRNA formation were studied. Figure 2A shows the effect of added NaCl on the rate of PRS-catalyzed aminoacylation of $tRNA^{Phe}$. The standard assay is conducted in a medium of ionic strength estimated to be 0.14 M greater than the contribution of added NaCl, and this value should be considered in calculations of total ionic strength. A slight increase in the rate of aminoacylation is first observed which reaches an optimum at 50 mM added NaCl ($\mu \simeq 0.2$ M). Further additions result in a steady decrease in rate and at 1 M added NaCl the rate is depressed to 20% of its maximum. the ATP–PP_i exchange rate is also decreased as NaCl is added to standard assay mixture ($\mu \simeq 0.17$ M) with 50% inhibition occurring at 650 mM added NaCl (Figure 2A); additions to 1 M NaCl caused no further depression of the exchange rate. The effects of NaCl on Michaelis–Menten parameters are given in Table I and show that with increasing NaCl the K_m for $tRNA^{Phe}$ is increased but V_{max} remains essentially constant. In the ATP–PP_i exchange assay, there is a decrease in V_{max} and in K_m for phenylalanine, while K_m for ATP remains constant or, at best, increases slightly. The effect of ethanol on the rate of ATP–PP_i exchange under our standard assay conditions is shown in Figure 2B. Unlike Val-tRNA synthetase (Loftfield and Eigner, 1967), the exhange reaction is inhibited to approximately the same extent as the formation of Phe-tRNA, with 50% inhibition occurring at 6–7% ethanol. As shown in Table I, the presence of ethanol decreases the V_{max} of both ATP–PP_i exchange and aminoacylation of $tRNA^{Phe}$. The K_m for phenylalanine is increased as ethanol is added while K_m for ATP remains constant or at best is

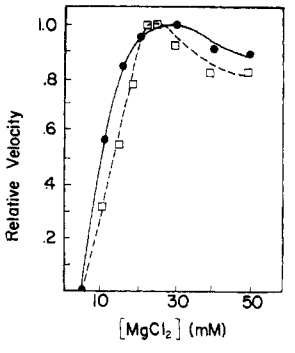


FIGURE 1: Magnesium concentration dependence of the rates of ATP–PP_i exchange (●) and aminoacylation of purified $tRNA^{Phe}$ (□).

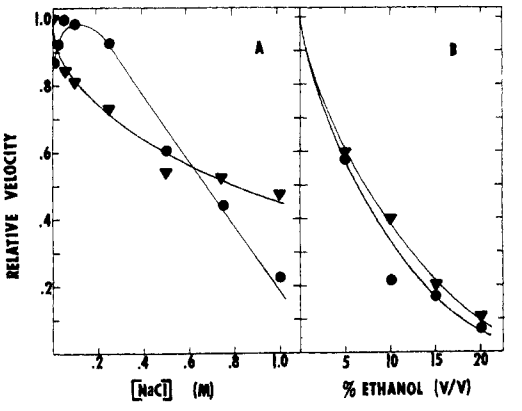


FIGURE 2: The effect of sodium chloride and ethanol on the rate of Phe-tRNA formation (●) and ATP–PP_i exchange (▼).

TABLE II: Catalytic Indices of Purified Phe-tRNA Synthetase.

Substrate	K_m ATP–PP_i Exchange	K_m tRNA Acylation
L-Phenylalanine	0.5×10^{-4} M	5.5×10^{-6} M
ATP	8.0×10^{-4} M	4.6×10^{-4} M
PP_i	4.0×10^{-4} M	
$tRNA^{Phe}$		1.0×10^{-7} M
V_{max} (μmoles)/(min mg)	18	0.45
Catalytic constant (mole of product/ mole of PRS·min)	4200	105

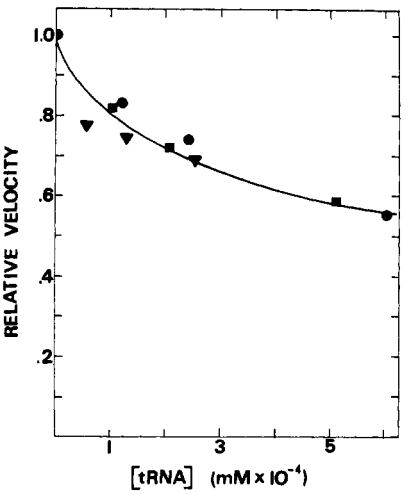


FIGURE 3: ATP–PP_i exchange rate in the presence of crude $tRNA^{Phe}$ (●), 10-fold purified $tRNA^{Phe}$ (■), and $tRNA^{Phe}_{ox}$ (▼).

only slightly increased. In contrast, K_m for $tRNA^{Phe}$ is significantly decreased as ethanol is added to the reaction mixture.

If the simplifying assumptions are made that K_m reflects the dissociation constant of a substrate, and that the effect of added salt or organic solvent is primarily one of altering the medium, some tentative conclusions may be reached which are in accord with current conceptual views of the nature of substrate interactions with PRS. The effects of NaCl and ethanol on K_m of $tRNA^{Phe}$ imply that the affinity of $tRNA^{Phe}$ for PRS is inversely related to the dielectric constant of the medium. A similar result has been reported (Loftfield and Eigner, 1967) for $tRNA^{Val}$ and has been interpreted to support the intuitively apparent importance of ionic bonding in ligase–tRNA interactions. Some verification for this conclusion is also provided by the observation that increasing amounts of NaCl have a highly disruptive effect on a number of tRNA–ligase complexes when isolated by gel filtration (Knowles *et al.*, 1970). It must be emphasized that these effects could be due in part or entirely to conformational changes of tRNA which cannot be distinguished from the effects discussed above.

In contrast to the behavior of tRNA, the binding of phenylalanine parallels the change in dielectric constant; added salt results in a decrease in K_m whereas ethanol causes an increase. Binding studies of analogs of phenylalanine (Santi and Danenberg, 1971) indicate that the free energy of binding of phenylalanine to PRS is in large part due to hydrophobic interactions. With this in mind it is tempting to relegate the observed salt and solvent effects to perturbations of these forces. The apparent increase in the affinity of phenylalanine for the enzyme in the presence of NaCl could very well be due to a "salting out" of phenylalanine from the bulk aqueous phase into a nonpolar site of the enzyme. On the other hand, ethanol, a solvent known to be detrimental to intermolecular hydrophobic interactions, would result in a decrease of the affinity of phenylalanine for the enzyme. Although the exact nature of the intermolecular forces responsible for binding ATP to PRS is not understood at this time, binding studies of analogs (Santi *et al.*, 1971) suggest that ionic interactions of the phosphate groups do not contribute to binding. In this respect it is of interest to note that changing dielectric constants has little effect on K_m for ATP.

Kinetic constants obtained for PRS-catalyzed ATP–PP_i exchange and Phe-tRNA formation obtained after permutational variation of reactant concentrations are given in Table II. It will be noted that under the standard assay conditions employed in this study, ATP–PP_i exchange is some 40-fold more rapid than acylation of $tRNA^{Phe}$. This difference is of the same order as observed for the Leu- and Tyr-tRNA synthetase (Calendar and Berg, 1966; Berg *et al.*, 1961) from *E. coli*. Using the reported molecular weight of 181,000 (Kosakowski and Bock, 1970; Stulberg, 1967) and homogeneity of 85% for our enzyme preparation, catalytic constants of 4200 and 105 moles per min per mole of PRS may be calculated for ATP–PP_i exchange and Phe-tRNA formation, respectively. Kosakowski and Bock (1970) have recently reported that PRS from *E. coli* consists of four identical subunits of mol wt 43,000 which are catalytically active only as the tetrameric native protein. Utilizing the method described by Yarus and Berg (1967), we have obtained preliminary results which demonstrate that native PRS may bind only one molecule of Phe-tRNA (D. V. Santi and J. Kasperek, unpublished results). Thus, although we cannot exclude the possibility that more than one catalytic site exists on native PRS, it would appear that only one may function to acylate $tRNA^{Phe}$ at any one time and it may be concluded that the aforementioned catalytic constant for formation of Phe-tRNA represents a true turnover number for PRS. Although the same conclusion may not be reached at this time with regard to ATP–PP_i exchange, the linearity of slope and intercept replots of all exchange studies described later in this report suggests that (a) only one site is functional, or (b) if more than one catalytic site is operative, binding of substrates or inhibitors at one site does not significantly effect parameters at the other(s).

As will be described later, PRS may bind to tRNA in the presence or absence of other substrates. Since it is possible that *in vivo* the enzyme is nearly always bound to tRNA (Novelli, 1967), it was of interest to compare the rates of ATP–PP_i exchange catalyzed by PRS and the PRS·$tRNA^{Phe}$ complex. When $tRNA^{Phe}$ is added to the ATP–PP_i exchange assay, the initial velocity of exchange is markedly decreased (Figure 3) with *ca.* 45% inhibition occurring at 5×10^{-7} M $tRNA^{Phe}$. In these experiments, the amount of PRS was lowered to that described for the aminoacylation of tRNA and [^{32}P]PP_i of high specific activity (*ca.* 2×10^6 cpm/μmole) was used to allow detection of low levels of exchange. Parallel experiments with [^{14}C]phenylalanine were performed which demonstrated that under these conditions there was no accum-

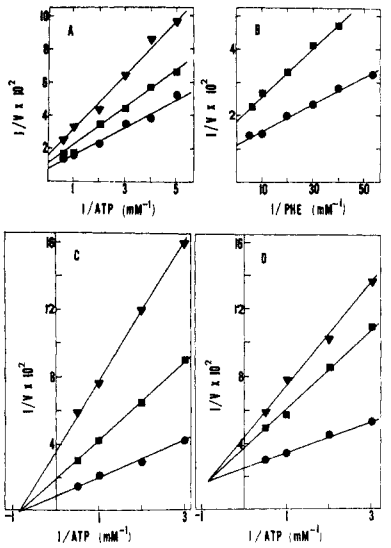


FIGURE 4: Double-reciprocal plots of ATP–PP_i exchange with varying levels of substrates. The free Mg^{2+} concentration was kept at 14 mM and PP_i at 2.0 mM unless otherwise indicated. (A) 0.2 mM (●), 0.1 mM (■), and 0.05 mM (▼) L-phenylalanine; (B) 4 mM (●) and 2 mM (■) ATP; (C) 7.5 mM (●), 0.075 mM (■), and 0.0375 mM (▼) L-phenylalanine; (D) 15 mM (●), 0.075 mM (■), and 0.05 mM (▼) L-phenylalanine with 0.2 mM PP_i. Slope and intercept replots were linear.

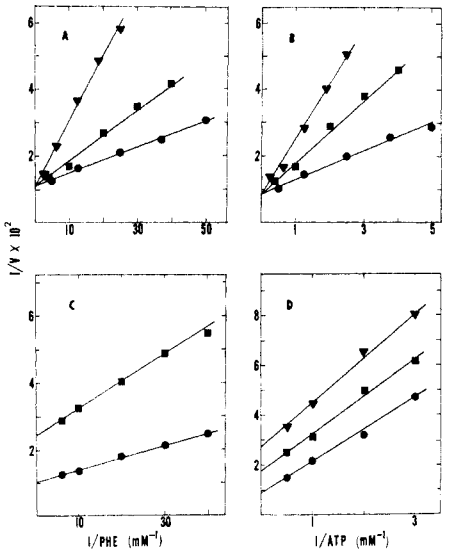


FIGURE 5: Inhibition of ATP–PP_i exchange by L-phenylalaninol and adenosine. (A) Varying phenylalanine in the absence of phenylalaninol (●), and at 0.0045 mM (■) and 0.018 mM (▼); (B) varying ATP in the absence of adenosine (●), and at 0.018 mM (■) and 0.045 mM (▼); (C) varying phenylalanine in the absence of adenosine (●) and at 0.1 mM (■); (D) varying ATP in the absence of phenylalaninol (●), and at 0.05 mM (■) and 0.1 mM (▼). Replots of slopes and/or intercepts *vs.* inhibitor concentration were linear.

ulation of Phe-tRNA. To further preclude the possibility that inhibition of ATP–PP_i exchange was in some way associated with the participation of Phe-tRNA as a reactant, $tRNA^{Phe}$ was oxidized with periodate and its effect on the exchange reaction examined; also shown in Figure 3, $tRNA^{Phe}_{ox}$ which is completely devoid of acceptor activity produces an inhibitory effect on ATP–PP_i exchange similar to that observed with $tRNA^{Phe}$. Furthermore, this effect was shown to be specific for $tRNA^{Phe}$ since inhibition by mixture of tRNA is a function of their content of $tRNA^{Phe}$. Further studies on the inhibitory effects of $tRNA^{Phe}$ on the activation of phenylalanine are in progress.

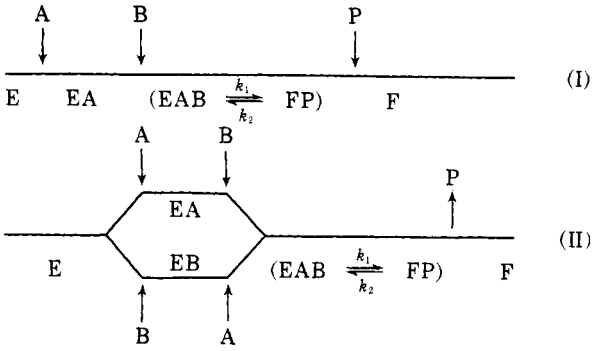
Order of Substrate Addition. Reports have recently been published in which binding mechanisms for the leucyl- (Rouget and Chapeville, 1968) and Tyr- (Santi and Peña, 1971) tRNA synthetases of *E. coli* and Thr-tRNA synthetase from rat liver (Allende *et al.*, 1970) have been proposed. Rouget and Chapeville have examined the effects of a number of inhibitors in the ATP–PP_i exchange and concluded that the addition of substrates was ordered, with ATP being the initially bound substrate. Utilizing initial velocity studies, Allende *et al.* have obtained evidence that the formation of Thr-tRNA proceeds by a Ping-Pong (bi-uni-uni-bi) mechanism in which ATP binds to the enzyme initially followed by threonine; after formation of the aminoacyladenylate and obligatory release of PP_i, $tRNA^{Thr}$ adds to the enzyme–threonyladenylate complex and is acylated. In contrast, kinetic studies of the tyrosine activating enzyme support a random order of substrate addition.

A number of works have also been reported which, although the objective was not to establish the order of substrate binding, permit suggestions of partial mechanisms; although further studies are necessary before stringent conclusions may be drawn, divergent mechanisms are apparent. Boyko and Fraser (1964) have published data which indicate that binding of ATP to Gly-tRNA synthetase from rat liver is not obligatory for the addition of glycine. The numerous synthetase·tRNA complexes that have been isolated by gel filtration (Knowles *et al.*, 1970; Lagerkvist and Waldenstrom, 1965: Lagerkvist and Rymo 1969) and on nitrocellulose membranes (Yarus and Berg, 1967, 1969) indicate that in these cases, cognate tRNA may bind to the free-enzyme form and, unless these represent dead-end complexes, the mechanisms are probably not Ping-Pong. Also pertinent are the observations that certain aa-tRNA synthetases do not catalyze ATP–PP_i exchange unless the appropriate tRNA is present (Mitra and Mehler, 1966; Ravel *et al.*, 1965). It is possible that for the latter synthetases the binding of tRNA may be obligatory for binding of one or both substrates; alternatively, binding of ATP and amino acid may be independent of tRNA, which might serve as an allosteric activator for aminoacyladenylate formation. In the case of the Arg-tRNA synthetase of *E. coli* the former is most likely the case since tRNA is required for PP_i incorporation into ATP even when synthetic arginyladenylate is utilized as substrate (Mehler and Mitra, 1967).

ATP–PP_i exchange. Since PRS-catalyzed ATP–PP_i exchange does not occur in the absence of L-phenylalanine, it would appear reasonable to surmise at the outset that the release of pyrophosphate occurs after the binding of both substrates. Direct evidence for this was obtained by variation of ATP and L-phenylalanine at different fixed levels of the other substrate. Double-reciprocal plots (Figure 4A,B) of both experiments yielded lines which intersected at the left of the $1/v$ axis (L-phenylalanine and ATP have Michaelis constants of 0.05 and 0.8 mM, respectively); since parallel lines would have been obtained if the reaction proceeded by a Ping–Pong mechanism (Cleland, 1963b), it may be concluded that both substrates add to the enzyme before a product is released.

It appears to be the general case for the aa-tRNA synthetases that the aminoacyladenylate intermediate is very tightly

bound to the enzyme and its dissociation is not relevant throughout the course of the reaction. As pointed out by Cole and Schimmel (1970), this presents an unusual situation in that the "product," PP_i, is formed in stoichiometric amounts with a modified enzyme form and the equilibrium condition is reached after a single turnover. For a two-substrate sequential reaction of this type, the binding of substrates may proceed by ordered (mechanism I) or random (mechanism II) pathways, where P is pyrophosphate and F is the enzyme-bound aminoacyladenylate, or equivalent species; in mechanism I, A is the first substrate to add and B the second.



Kinetic expressions corresponding to the above mechanisms have been reported (Cedar and Schwarz, 1969) and are reproduced[3] using the notations suggested by Cleland (1963a) in inverted forms in eq 1–3

$$\frac{1}{v} = \frac{1}{V}\left(1 + \frac{K_a B}{K_{ia}K_b}\right)\left(1 + \frac{K_{ib}}{B} + \frac{K_{ip}}{P} + \frac{K_{ia}K_{ib}}{AB}\right) \quad (1)$$

$$\frac{1}{v} = \frac{1}{V}\left(1 + \frac{K_{ib}}{B} + \frac{K_{ip}}{P} + \frac{K_{ia}K_{ib}}{AB}\right) \quad (2)$$

$$\frac{1}{v} = \frac{1}{V_x}\left(1 + \frac{K_{xb}}{B} + \frac{K_{xp}}{P} + \frac{K_{ia}K_{xb}}{AB} + \frac{K_{xa}}{A}\right) \quad (3)$$

where $K_{xb} = k_2K_b/(k_1 + k_2)$, $K_{xa} = k_2K_a/(k_1 + k_2)$, $K_{xp} = k_1K_{ip}/(k_1 + k_2)$, $V_x = k_1k_2E_t/(k_1 + k_2)$, and $V = V_1K_{ib}/K_b$. Analogous equations have been derived by Cole and Schimmel (1970) which differ from the above in the definitions of constants and notation used. The rate of the exchange reaction for ordered mechanism I where P exchanges with A is given in eq 1. Equation 2 corresponds to the same mechanism, only where P exchanges with the obligatory second substrate B. The random mechanism II is described by eq 3.

In an ordered mechanism in which addition of ATP is the obligatory first step (eq 1), increasing concentration of phenylalanine should lead to uncompetitive substrate inhibition since high concentrations of phenylalanine, by combining with E·ATP, would inhibit the release of ATP. On the other hand, for an ordered mechanism in which phenylalanine adds first, a pattern with lines intersecting on the $1/v$ axis is predicted (eq 2) when ATP is the variable substrate. Figure 4 shows that the latter possibility could be eliminated at the outset. If substrates add to the enzyme in a random mechanism, a pattern would be obtained in which all lines intersected to the left of the vertical axis, notwithstanding the concentration of phenylalanine. Initial rates of ATP–PP_i exchange determined at varying concentrations of ATP (0.33–2 mM) and phenylalanine (0.0375–7.5 mM) support a random mechanism as shown in Figure 4C where it is apparent that substrate inhibition is not observed at very high phenylalanine concentrations and all lines converge well to the left of the ordinate.

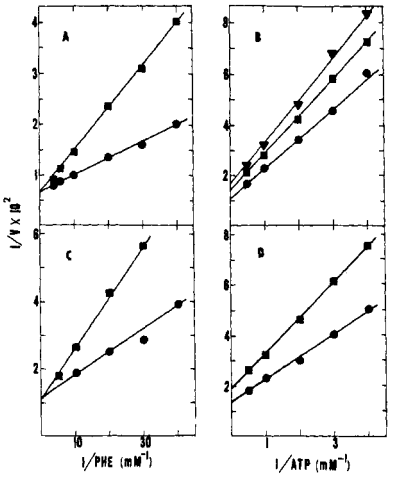


FIGURE 6: Inhibition of ATP–PP_i exchange by D,L-*p*-fluorophenylalaninol and D,L-APEP. (A) Varying phenylalanine in the absence of inhibitor (●) and at 0.6 mM (■) *p*-fluorophenylalanine; (B) varying ATP in the absence of inhibitor (●), and at 1.0 mM (■) and 1.5 mM (▼) *p*-fluorophenylalanine; (C) Varying phenylalanine in the absence of inhibitor (●) and at 0.05 mM (■) APEP; (D) varying ATP in the absence of inhibitor (●) and at 0.2 mM (■) APEP.

Confirming evidence for the random order of addition of substrates in the PRS-catalyzed ATP–PP_i exchange was obtained by the use of competitive inhibitors (Cleland, 1963b, 1970). In the case of the ordered mechanism, a competitive inhibitor for the substrate which adds to the enzyme first (A, mechanism I) will also demonstrate competitive[4] inhibition with respect to the second substrate (B) under equilibrium conditions. A competitive inhibitor for the second substrate will produce uncompetitive inhibition when the initially bound substrate is varied. On the other hand, for the random pathway (mechanism II), a competitive inhibitor for either substrate will demonstrate noncompetitive inhibition with respect to the other.

Adenosine and L-phenylalaninol were found to be potent linear competitive inhibitors of ATP and L-phenylalanine, respectively (Figure 5A,B); under standard ATP–PP_i exchange reaction conditions K_i values of 6.1×10^{-6} M for L-phenylalaninol and 1.5×10^{-5} M for adenosine were obtained. With adenosine as the inhibitor and L-phenylalanine as the variable substrate (Figure 5C), noncompetitive kinetics were observed. Upon examination of the inhibition by phenylalaninol with ATP as the variable substrate (Figure 5D), Lineweaver–Burk plots gave lines that were "nearly" parallel, suggesting uncompetitive inhibition. However, upon close examination, and after a number of repetitions, it was apparent that the slopes of the lines were *not* identical and converged far to the left of the $1/v$ axis. In view of the deceptive nature of the plots obtained with phenylalaninol, other competitive inhi-

[3] Equation 1 differs from that originally reported for asparagine synthetase because of a typesetting error in the latter.

[4] It is noted that the inhibition patterns obtained under equilibrium conditions differ from those obtained at initial velocity.

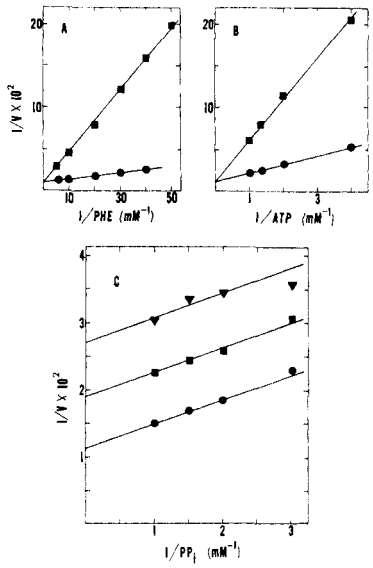


FIGURE 7: Inhibition of ATP–PP_i exchange by L-phenylalaninol adenylate. (A) Varying phenylalanine in the absence of inhibitor (●) and at 0.01 mM (■); (B) varying ATP in the absence of inhibitor (●) and at 0.01 mM (■); (C) varying PP_i in the absence of inhibitor (●) and at 0.0125 mM (■) and 0.025 mM (▼).

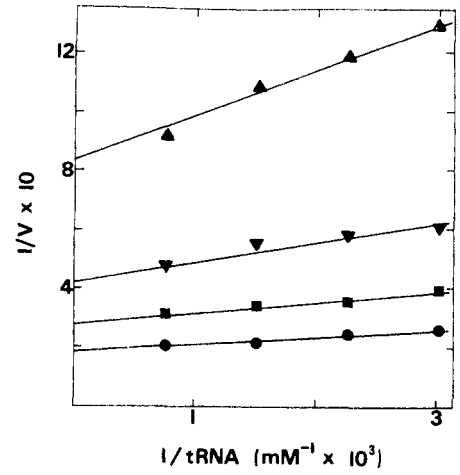


FIGURE 8: Double-reciprocal plots of Phe-tRNA varying $tRNA^{Phe}$ with fixed levels of ATP and phenylalanine at constant ratio. The concentrations of L-phenylalanine–adenosine triphosphate used were 0.012–5.0 mM (●), 0.006–2.5 mM (■), 0.004–1.67mM (▼), and 0.003–1.25 mM (▲). Slope and intercept replots as a function of reciprocal concentration of changing fixed substrates were parabolic.

bitors were sought which might better define the nature of inhibition. As reported by Conway *et al.* (1962), *p*-fluorophenylalanine is a substrate for PRS-catalyzed ATP–PP_i exchange ($K_m = 5.6 \times 10^{-4}$ M), and Figure 6A shows that D,L-*p*-fluorophenylalaninol is a competitive inhibitor with respect to phenylalanine ($K_i = 2 \times 10^{-4}$ M). It is readily apparent from Figure 6B that the inhibition by *p*-fluorophenylalanine with respect to ATP is noncompetitive. Similarly, APEP, a good competitive inhibitor with respect to phenylalanine ($K_i = 4.0 \times 10^{-5}$ M), is noncompetitive with respect to ATP (Figure 6C,D). These data are only consistent with a random order of binding (mechanism II) of substrates to Phe-tRNA synthetase.

Cassio *et al.* (1967) and Rouget and Chapeville (1968) have observed that aminoalkyladenylates, structural analogs of the intermediate aminoacyladenylates, are potent and specific inhibitors of the aa-tRNA synthetases. We have also prepared phenylalaninol adenosine monophosphate and have found it to be competitive with both L-phenylalanine ($K_i = 1.1 \times 10^{-6}$ M) and ATP ($K_i = 1.1 \times 10^{-6}$ M), and uncompetitive with respect to PP_i in the ATP–PP_i exchange assay (Figure 7). This pattern is in accord with the random mechanism in which the inhibitor blocks the adsorbing sites of both substrates.

For the random mechanism II, the abscissa value which defines the point of convergence in Figure 4C corresponds to the negative reciprocal of the apparent dissociation constant (K_{ia}) of ATP. At this point, the velocity of ATP–PP_i exchange is independent of concentration of phenylalanine (B) and subtraction of simultaneous equations with varying concentrations of phenyalanine in eq 5 results in the expression

$$\frac{K_{xb}}{B - B_n} + \frac{K_{ia}K_{xb}}{A(B - B)_n} = 0$$

or

$$\frac{1}{A} = -\frac{1}{K_{ia}}$$

If PP_i competes with ATP for its binding sites an inhibition term must be introduced giving

$$\frac{1}{A} = -\frac{1}{K_{ia}}\left(1 + \frac{P}{K_i}\right)$$

where K_i is the dead-end inhibition constant for PP_i. If this term is significant the apparent K_{ia} will increase with increasing PP_i; if competition for the ATP site by PP_i is not significant K_{ia} will be independent of PP_i concentration and represent a true dissociation constant for ATP. As shown in Figure 4C,D, the values obtained for the apparent K_{ia} for ATP remain constant at 1.1 mM over a 10-fold range in PP_i concentration. Unlike the analogous Ile- (Cole and Schimmel, 1970) and Tyr-tRNA synthetases (Santi and Peña, 1971), the above data suggest that PP_i does not significantly compete for the ATP binding site on the free enzyme. We estimate that the dead-end inhibition constant for PP_i combination with the free enzyme is at least an order of magnitude higher than the concentration used in the standard exchange assay. This is in accord with binding studies of ATP analogs (Santi *et al.*, 1971) which demonstrate that the β,γ-pyrophosphate moiety does not contribute significantly to the free energy of binding of ATP to PRS, and that the α-phosphate is detrimental. Since PP_i has a much greater affinity for the E·Phe-AMP complex than for the free enzyme, it is reasonable to believe that its binding site is altered during the activation process. This could be a result of a change in protein conformation or solvation of the active site which accompanies aminoacyladenylate formation and results in a more favorable environment for binding of PP_i.

FORMATION OF Phe-tRNA. Although a similar binding sequence is expected for ATP and phenylalanine in the overall sequence leading to Phe-tRNA as in the ATP–PP_i exchange, alternative mechanisms are conceivable. For this reason, and in order to ascertain the point of addition of $tRNA^{Phe}$, we chose the approach suggested by Fromm (1967) which utilizes competitive inhibitors of each of the three substrates. After it has been determined whether the reaction is Ping-Pong or sequential by initial velocity experiments, analysis of inhibition

TABLE III: Summary of Inhibition Patterns for ATP–PP_i Exchange and Aminoacylation of $tRNA^{Phe}$.

Inhibitor	Variable Substrate				
	ATP–PP_i Exchange		Phe-tRNA Formation		
	Phenylalanine	ATP	Phenylalanine	ATP	$tRNA^{Phe}$
L-Phenylalaninol	Competitive (K_i = 6.0 μM)	Noncompetitive			
D,L-*p*-Fluorophenylalaninol	Competitive (K_i = 200 μM)	Noncompetitive			
D,L-APEP	Competitive (K_i = 40 μM)	Noncompetitive	Competitive (K_i = 180 μM)	Noncompetitive	Noncompetitive
Adenosine	Noncompetitive	Competitive (K_i = 15 μM)	Noncompetitive	Competitive (K_i = 63 μM)	Noncompetitive
$tRNA^{Phe}_{ox}$			Noncompetitive	Noncompetitive	Competitive (K_i = 0.12 μM)
L-Phenylalaninol-AMP	Competitive (K_i = 1.0 μM)	Competitive (K_i = 1.0 μM)	Competitive (K_i = 1.8 μM)	Competitive (K_i = 1.5 μM)	Noncompetitive

patterns with respect to all three substrates provides a unique solution for the order of substrate addition to the enzyme.

The initial products formed in the reaction sequence leading to Phe-tRNA are phenylalanyladenylate and PP_i. Although it is unlikely that the former is released from the enzyme, it is not known if the release of PP_i is obligatory for the addition of tRNA. As shown in Figure 8, when the concentration of $tRNA^{Phe}$ was varied at different fixed levels of ATP and phenylalanine, held at constant ratio, the double-reciprocal plots intersected to the left of the ordinate. If the addition of $tRNA^{Phe}$ were separated from the addition of ATP and phenylalanine by an obligatory irreversible step, *viz.*, release of PP_i, parallel lines would have been obtained. These results indicate that the overall reaction may proceed by a sequential pathway, and unlike the Thr-tRNA synthetase from rat liver (Allende *et al.*, 1970), the release of PP_i is not obligatory to binding of $tRNA^{Phe}$. It is to be noted that our results do not exclude the existence of PRS·Phe-AMP·$tRNA^{Phe}$ complexes which would arise from nonobligatory release of PP_i. In fact, the existence of such a complex is indicated by the observation (Hirsh, 1968) that the PRS·Phe-AMP complex isolated on Sephadex G-50 can transfer phenylalanine to $tRNA^{Phe}$. Simultaneous operation of Ping-Pong and sequential mechanisms has also been suggested for the citrate-cleavage enzyme from rat liver (Plowman and Cleland, 1967).

As shown in Figure 9, APEP is a competitive inhibitor with respect to phenylalanine ($K_i = 1.8 \times 10^{-4}$ M) and noncompetitive with respect to ATP and $tRNA^{Phe}$. Since parallel lines would have been obtained if addition of ATP or $tRNA^{Phe}$ were obligatory to binding of phenylalanine, this pattern establishes that phenylalanine must add to PRS either first or randomly. Similarly, adenosine, a competitive inhibitor with respect to ATP ($K_i = 6.3 \times 10^{-5}$ M), demonstrates noncompetitive inhibition when phenylalanine or $tRNA^{Phe}$ is the variable substrate (Figure 10), a pattern which indicates initial or random addition of ATP. In concert, these data are only in accord with a random addition of both phenylalanine and ATP. Further evidence for this was obtained by demonstrating that phenylalaninol adenylate was a competitive inhibitor with respect to both phenylalanine ($K_i = 1.8 \times 10^{-6}$ M) and ATP ($K_i = 1.5 \times 10^{-6}$ M) and noncompetitive with respect to $tRNA^{Phe}$. The existence of an enzyme form which may bind to either ATP or phenylalanine is only consistent with a random order of addition for these substrates.

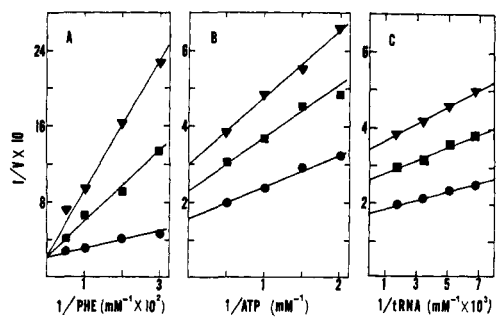


FIGURE 9: Inhibition of Phe-tRNA formation by D,L-APEP. (A) Varying phenylalanine in the absence of inhibitor (●) and at 0.5 mM (■) and 1.0 mM (▼) APEP; (B) varying ATP in the absence of inhibitor (●) and at 1.0 mM (■) and 2.0 mM (▼) APEP; (C) varying $tRNA^{Phe}$ in the absence of APEP (●) and at 2.25 mM (■) and 4.5 mM (▼).

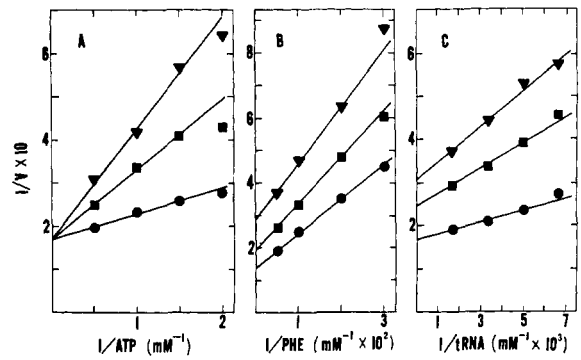


FIGURE 10: Inhibition of Phe-tRNA formation by adenosine. (A) Varying ATP in the absence of adenosine (●) and at 0.1 mM (■) and 0.2 mM (▼); (B) varying phenylalanine in the absence of adenosine (●) and at 0.45 mM (■), and 0.9 mM (▼); (C) varying $tRNA^{Phe}$ in the absence of adenosine (●) and at 0.45 mM (■) and 0.9 mM (▼). Replots of slopes and/or intercepts *vs.* concentration of adenosine were linear.

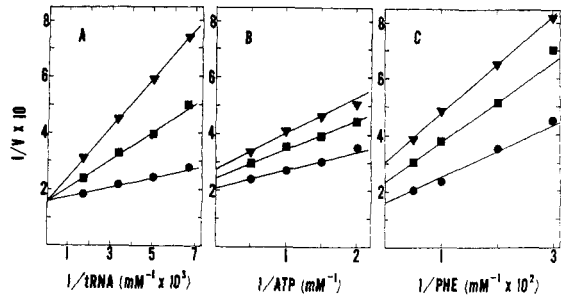


FIGURE 11: Inhibition of Phe-tRNA formation by $tRNA^{Phe}_{ox}$. (A) Varying $tRNA^{Phe}$ in the absence of $tRNA^{Phe}_{ox}$ (●), and at 0.3 μM (■) and 0.6 μM (▼); (B) varying ATP in the absence of $tRNA^{Phe}_{ox}$ (●), and at 0.18 μM (■) and 0.35 μM (▼); (C) varying phenylalanine in the absence of $tRNA^{Phe}_{ox}$ (●), and at 0.35 μM (■) and 0.7 μM (▼). Replots of slopes and/or intercepts *vs.* $tRNA^{Phe}_{ox}$ concentration were linear.

As parallel lines were not obtained when $tRNA^{Phe}$ was varied in any of the above experiments, it may be surmised that binding of $tRNA^{Phe}$ is not obligatory for addition of either ATP or phenylalanine; $tRNA^{Phe}$ may add to PRS randomly, or subsequent to the addition of both ATP and phenylalanine. Kinetic evidence for random addition of $tRNA^{Phe}$ was obtained by demonstrating that periodate oxidized $tRNA^{Phe}$ ($tRNA^{Phe}_{ox}$), a competitive inhibitor of $tRNA^{Phe}$ ($K_i = 1.2 \times 10^{-7}$ M), is noncompetitive with respect to both ATP and phenylalanine (Figure 11); if binding of ATP and phenylalanine were obligatory to addition of $tRNA^{Phe}$ parallel lines would have been obtained. Supporting evidence for a random order of binding of $tRNA^{Phe}$ is also provided by the observation (D. V. Santi and J. Kasperek, unpublished results) that PRS·$tRNA^{Phe}$ complexes may be isolated on nitrocellulose membranes (Yarus and Berg, 1967) in the presence or absence of ATP and phenylalanine.

The inhibition data presented above are summarized in Table III. It is to be emphasized that although our data allow the assignment of a completely random sequence of substrate addition to PRS, we are unable to assess the importance of preferred pathways. Studies on substrate induced changes in kinetic parameters of other substrates are in progress.

Acknowledgments

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