*MULTIPLE PHENYLALANYL-TRANSFER RIBONUCLEIC ACID SYNTHETASE ACTIVITIES IN THE CYTOPLASM OF NEUROSPORA CRASSA**

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Abstract.—Several tRNA's specific for a particular amino acid have been shown to exist in multiple, or isoaccepting, forms. There is considerable interest in establishing whether multiple aminoacyl-tRNA synthetases also exist. We present evidence that the cytoplasm of *Neurospora crassa* contains three chromatographically separable phenylalanyl-tRNA synthetases distinct from mitochondrial phenylalanyl-tRNA synthetase. In addition to differences in chromatographic properties the three enzymes exhibit different affinities, in Tris-Cl buffer, toward purified species of valine and alanine tRNA's isolated from *Escherichia coli*. The two major chromatographic fractions have very similar sedimentation characteristics, which makes a monomer-dimer relationship unlikely.

Extracts of *Neurospora crassa* have been shown to catalyze the aminoacylation of phenylalanine to three species of *Escherichia coli* tRNA.[1] Barnett and coworkers[2–5, 7] and Imamota *et al.*[6] showed that the acylation of these tRNA's was due to the presence of two distinct phenylalanyl-tRNA syntases in *N. crassa*.‡ One of these enzymes is confined to the mitochondria and can aminoacylate *E. coli* $tRNA^{Phe}$ or mitochondrial *N. crassa* $tRNA^{Phe}$, but is not reactive toward other *E. coli* tRNA's or cytoplasmic *N. crassa* tRNA. The other enzyme, present in the cytoplasm, is active toward *E. coli* valine and alanine tRNA's and *N. crassa* cytoplasmic tRNA, but has little activity toward *E. coli* phenylalanine tRNA and reacts very slowly with *N. crassa* mitochondrial tRNA.

We report here the presence of multiple phenylalanyl-tRNA synthetases in the cytoplasmic fraction of *N. crassa*, designated *N. crassa* Syn^{Phe} *A*, *B*, and *C*. The three enzymes can be separated by hydroxylapatite chromatography and have different affinities for different tRNA substrates in Tris-Cl buffer. *N. crassa* Syn^{Phe} *A* and *C* have identical or very similar sedimentation characteristics, which tends to rule out a simple monomer-dimer relationship between the enzyme forms.

Materials and Methods.—Enzymes: The purification of the *N. crassa* Syn^{Phe} enzymes will be described in detail elsewhere. Purification was routinely carried out in the presence of 2-mercaptoethanol and glycerol, beginning with the ammonium sulfate precipitate of accumulated postmitochondrial supernatants from homogenates of *N. crassa* wild-type strain OR23-la grown, harvested, and prepared as described by Barnett and Brown.[7] We are indebted to these workers and to Dr. J. L. Epler for these supernatants and for fresh mycelia. Briefly, purification was as follows: precipitates were back-extracted with ammonium sulfate, and after another ammonium sulfate precipitation the enzymes were further purified by a batch hydroxylapatite treatment, DEAE-cellulose column chromatography, and finally hydroxylapatite column chromatography as described in Figure 1. After this step the *A* enzyme is purified about 60 times and the *C* enzyme

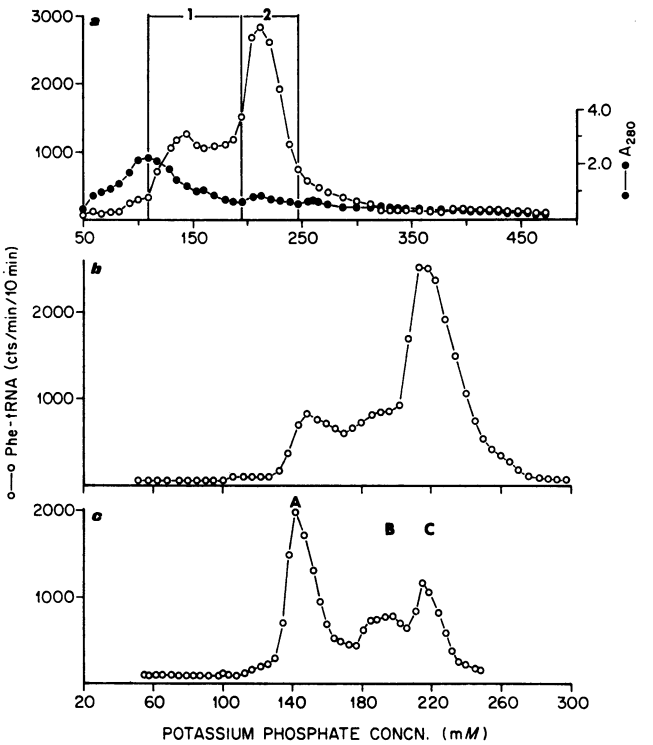

FIG. 1.—Hydroxylapatite column chromatography of *N. crassa* Syn^{Phe} (*a*) Enzyme was partially purified as outlined in *Materials and Methods*, and 735 A_{280} units were added to the column (3.5 × 3.5 cm). Both enzyme and column were in 0.05 *M* potassium phosphate, pH 7.9, 0.01 *M* 2-mercaptoethanol, and 30% propylene glycol. Elution was carried out by a linear phosphate gradient at pH 7.9, 0.05–0.50 *M*. Total volume was 1 liter. Five-ml fractions were collected; absorbence at 280 mμ and enzyme activity were measured for every third fraction. Ten μl of enzyme were diluted into 140 μl of the assay mixture: 50 m*M* Tris-Cl, pH 8.0, 15 m*M* magnesium acetate, 10 m*M* 2-mercaptoethanol, 0.5 m*M* ATP; the assay mixture also contained 1 μc L-[^{14}C]phenylalanine, 170 μg bovine serum albumin, and 0.64 A_{260} units *E. coli* tRNA per ml. The tRNA was enriched in $tRNA^{Val}$ and $tRNA^{Ala}$ and free of $tRNA^{Phe}$. Incubations were for 10 min at 20°. Portions (0.1 ml) were removed and Phe-tRNA determined. (*b*) Enzyme was prepared from fresh mycelia as outlined in *Materials and Methods*, except that propylene glycol was present in place of glycerol. A hydroxylapatite column was prepared (3.5 × 3.5 cm), and 640 A_{280} units were added. Elution and assays were as described in Figure 1*a*, except that the gradient was 0.05–0.30 *M* phosphate, and 3.5-ml fractions were collected. (*c*) Fraction 1 from Figure 1*a* was combined with similar regions from other chromatograms and rechromatographed under the conditions described in Figure 1*b*.

about 900 times. Throughout purification a unit of activity was defined as the amount of enzyme necessary to form one pmole of L-[^{14}C]phenylalanyl-$tRNA^{Val + Ala}$ (*E. coli*) per minute at 20° under the conditions described in Figure 1. Activity was estimated by extrapolation of Phe-tRNA formed, per minute, to zero time, because rates decrease with time. Under these conditions the rate of Phe-$tRNA^{Val + Ala}$ (*E. coli*) formation is proportional to enzyme concentration for any time of incubation up to 2 hr.

An unfractionated *E. coli* tRNA synthetase fraction was prepared from *E. coli* B (Witkin strain) according to the procedure of Kelmers *et al.*,[8] and crystalline inorganic pyrophosphatase was purchased from Worthington Biochemical Corp.

tRNA: Purified and partially purified species of *E. coli* tRNA's were prepared by reversed-phase and hydroxylapatite chromatography of tRNA isolated from *E. coli* B (Witkin strain) according to Weiss, Pearson, and Kelmers[9, 11] and were generously provided by them. Unfractionated *N. crassa* tRNA was prepared by standard procedures; other unfractionated tRNA's were gifts of Drs. W. E. Barnett and L. C. Waters.

Assays: L-[^{14}C]aminoacyl-tRNA was assayed by the filter paper disc method of Bollum.[11] Inorganic phosphate was measured by the method of Sumner.[12]

Reagents: L-[^{14}C]phenylalanine (366 μc/μmole), L-[^{14}C]alanine (117 μc/μmole), and L-[^{14}C]valine (209 μc/μmole) were purchased from New England Nuclear Corp. Hydroxylapatite was obtained from Bio-Rad Laboratories. All other reagents were also obtained commercially and used without further purification.

Results.—Purification of *N. crassa* Syn^{Phe} from a postmitochondrial supernatant fraction invariably resulted in separation of enzymic activity into two partially resolved peaks when chromatographed on hydroxylapatite (Fig. 1*a*). A distinct shoulder was apparent preceding the second peak when gradients were expanded by lowering the phosphate concentration (Fig. 1*b*). When the last peak of activity was removed this shoulder was shown to be a chromatographically separable fraction (Fig. 1*c*). Extensive rechromatography yielded single peaks of activity corresponding to each of the original peaks and indicated no redistribution of activity into multiple activity peaks, even after storage for up to six months. Furthermore, peak fractions invariably emerged from the hydroxylapatite columns at phosphate concentrations predicted from previous chromatograms and from chemical measurements.

When postmicrosomal supernatants of homogenates of fresh mycelia were chromatographed on hydroxylapatite without prior purification, multiple enzyme activities were also found. However, under these conditions enzyme peaks eluted at lower phosphate concentrations than when they were carried through the entire fractionation procedure.

To determine whether some sort of stable monomer-dimer relationship existed between *A* and *C* forms of *N. crassa* Syn^{Phe}, we performed sucrose gradient centrifugation experiments. Using various conditions, including the phosphate and propylene glycol concentrations at which either *A* or *C* eluted from hydroxylapatite, it could be clearly shown that *A* and *C* have identical sedimentation rates. Both sedimented somewhat more rapidly than rabbit-muscle lactate dehydrogenase, which places their approximate molecular weights close to those reported for other phenylalanyl-tRNA synthetases, 180,000.[13, 14]

Using the peak fractions of the chromatogram shown in Figure 1*c*, we found that the three enzymes were qualitatively similar in their specificity to various unfractionated tRNA's. None of the three showed significant aminoacylation of *N. crassa* mitochondrial tRNA, but all were very active in the aminoacylation of cytoplasmic *N. crassa* tRNA. Furthermore, since assays during purification were carried out using a mixture of *E. coli* alanine and valine tRNA's free of phenylalanine tRNA, the possibility that one of the contaminating enzymes was mitochondrial phenylalanyl-tRNA synthetase was eliminated. Using unfractionated *E. coli* tRNA, it was shown that the three enzymes aminoacylated widely different amounts of tRNA: The *A* enzyme was capable of producing three times more Phe-tRNA than the *B* enzyme and two times more than the *C*

enzyme. Doubling the enzyme concentration in each case did not affect the amount of Phe-tRNA formed.

Using partially purified species of *E. coli* tRNA, a differential affinity among the enzymes was indicated on the basis of their rates of reaction. For example, in ten minutes the *A* form acylated seven times more *E. coli* $tRNA^{Val}$ than the *B* enzyme, and three times more than *C*. *N. crassa* Syn^{Phe} *A* was also more effective than *B* or *C* in aminoacylation of *E. coli* $tRNA^{Ala}$, but here only about three and two times more effective, respectively. In each case more (*E. coli*) Phe-$tRNA^{Ala}$ was formed than Phe-$tRNA^{Val}$ (*E. coli*). None of the three enzymes were able to aminoacylate appreciable amounts of *E. coli* $tRNA^{Phe}$.

To examine in greater detail the specificity of the three enzymes toward *E. coli* tRNA's, it was necessary to normalize the activities of the three enzymes. To do this, unfractionated cytoplasmic *N. crassa* tRNA was used at saturating concentrations, and an enzyme unit was redefined as the amount of enzyme required to form 1 pmole of Phe-tRNA per minute at 20° under conditions as described in Figure 1. The concentrations of several purified and partially purified alanine and valine tRNA's isolated from *E. coli* by reversed-phase and hydroxylapatite chromatography were adjusted to approximately 390 pmoles of acceptor sites per milliliter by determining their maximal acceptance using an *E. coli* synthetase fraction and their respective amino acids. Incubation of these amounts of *E. coli* tRNA with equal activities of *A*, *B*, and *C* demonstrated distinct differences among the fractions (Table 1). The *A* enzyme formed twice as much *E. coli* Phe-tRNA as the *C* enzyme and about three times as much as the *B* enzyme, either with a mixture of isoaccepting forms of *E. coli* $tRNA^{Val}$ and *E. coli* $tRNA^{Ala}$ or with a single isoaccepting form of *E. coli* $tRNA^{Val}$. However, with *E. coli* $tRNA^{Ala-3}$, another single isoaccepting form,[15] the *A* and *C* enzymes formed nearly equal amounts of Phe-tRNA.

These results may indicate either that there are isoaccepting forms of both *E. coli* $tRNA^{Val}$ and *E. coli* $tRNA^{Ala}$ unreactive with one or another of the three enzymes, or that there are marked differences in the affinities or rates of turnover between the enzymes and the various isoaccepting forms of tRNA. To distinguish between these alternatives, a reversed-phase chromatogram of *E. coli* tRNA was surveyed with equivalent enzyme units (as defined with *N. crassa* tRNA) of the *A* and *C* enzymes. Isoaccepting forms of $tRNA^{Val}$ and $tRNA^{Ala}$ were located with an *E. coli* synthetase preparation and the appropriate amino acids. Figure 2 shows the results of this experiment. The largest difference seen (fraction 84) is most likely associated with $tRNA^{Val}$; however, a minor

TABLE 1. *Maximal aminoacylation of purified E. coli tRNA's by N. crassa* Syn^{Phe} *activities.**

Enzyme	*E. coli* tRNA			
	Val-2	Val total	Ala-3	Ala total
E. coli (unfractionated)	393	420	386	360
N. crassa Syn^{Phe}				
A 5 units	107(27)†	86(21)	356(92)	182(51)
B 5 units	28(7)	25(6)	260(67)	63(18)
C 5 units	45(12)	44(10)	312(81)	99(28)

* pmoles Phe-tRNA formed in 2 hr/ml tRNA under assay conditions as described in Figure 1.
† Numbers in parentheses represent per cent of *E. coli* aminoacylation.

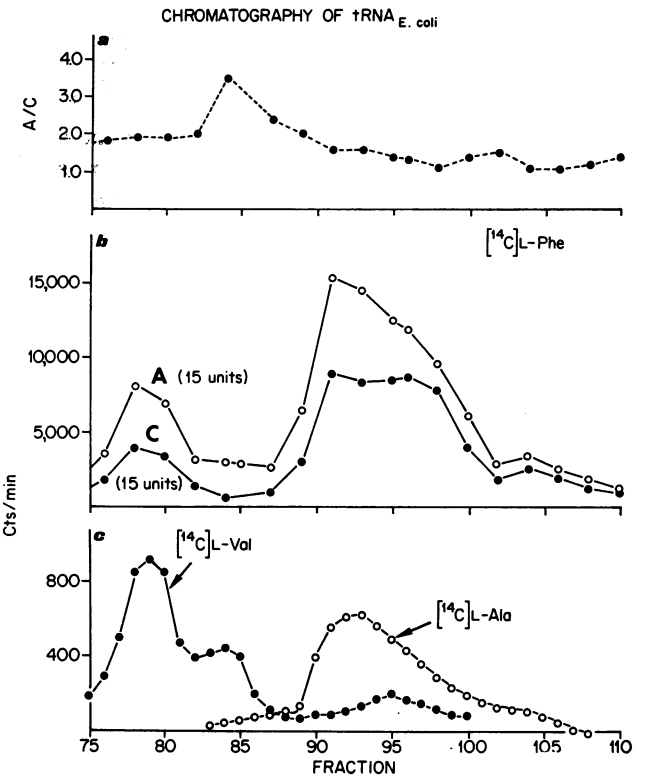

FIG. 2.—Reactivity of equivalent activities of *A* and *C* *N. crassa* Syn^{Phe} with the isoaccepting forms of *E. coli* alanine and valine tRNA's. (*a*) Ratio of total Phe-tRNA formed by the *A* enzyme to that formed by the *C* enzyme across the $tRNA^{Val}$ and $tRNA^{Ala}$ region of a reversed-phase chromatogram of *E. coli* tRNA. (*b*) Activity profiles of equivalent *A* and *C* enzyme units (defined using *N. crassa* tRNA) across the same region as in *a*. (*c*) Isoaccepting forms of $tRNA^{Val}$ and $tRNA^{Ala}$ assayed using unfractionated *E. coli* tRNA synthetases, L-[^{14}C]valine, and L-[^{14}C]alanine. In *b*, assays were carried out as described in Figure 1, except that incubation mixtures contained 0.05 ml of the various tRNA fractions in the final 0.15-ml volume, and incubations were for 30 min.

To remove the NaCl from the tRNA, each column fraction was precipitated with ethanol, and the tRNA was collected on millipore membranes and dissolved in 0.01 *M* Tris-Cl, pH 8.1, and 0.01 *M* magnesium acetate.

$tRNA^{Ala}$ occurs in the same region and cannot be ruled out as contributing to the three- to four-fold greater reactivity of *A* relative to *C* (Fig. 2*a*). As was observed above and shown in Table 1, the *A* enzyme is more active toward all species of reactive *E. coli* tRNA, and $tRNA^{Ala}$ appears to be acylated to a greater extent than $tRNA^{Val}$ for most isoaccepting forms. Therefore, all isoaccepting forms of $tRNA^{Val}$ and $tRNA^{Ala}$ that could be resolved by the chromatography are reactive with all three of the enzymes. The differences among the enzymes may then be the result of differential affinities, which is also in agreement with experiments reported by Holten and Jacobson.[15]

A comparison of the *A* and *C* forms of phenylalanyl-tRNA synthetase was

made on the basis of their K_m's and V_{max}'s (Table 2). Although they were similar, the K_m of the *C* form was somewhat less than that of the *A* form when reacting with tRNA^{Val} and tRNA^{Ala} of *E. coli*. The K_m in the presence of inorganic pyrophosphatase was determined, since $10^{-6}-10^{-7}$ *M* pyrophosphate has been shown to inhibit the *A* and *C* forms of the enzyme;[16] as expected, the K_m decreased for both forms when the pyrophosphatase was present, whereas the V_{max} was not altered. It appears that the two- to three-fold difference in V_{max} when the *A* and *C* forms react with *E. coli* tRNA^{Val} or *E. coli* tRNA^{Ala} must be the basis for the differences between these two enzymes. This conclusion is based on the assumption that the turnover of the two enzymes is the same when reacting with *N. crassa* tRNA.

As a control, the *A*, *B*, and *C* enzymes were assayed for possible ATP:tRNA adenylyltransferase activity. No such activity was found in the *A*, *B*, or *C* fraction using purified *E. coli* tRNA^{Val}, *E. coli* tRNA^{Ala}, or *E. coli* tRNApCpC

TABLE 2. *Kinetic parameters of N. crassa Syn^{Phe} A and C with purified E. coli tRNA's.*

N. crassa Syn^{Phe}	*E. coli* tRNA^{Val}		
	V_{max}	K_m	K_m(+ Inorganic pyrophosphatase)
A	88.3	13.9×10^{-6}	6.8×10^{-6}
C	26.4	7.4×10^{-6}	5.0×10^{-6}
	E. coli tRNA^{Ala}		
	V_{max}	K_m	K_m(+ Inorganic pyrophosphatase)
A	67.4	5.3×10^{-6}	3.0×10^{-6}
C	34.5	4.6×10^{-6}	2.7×10^{-6}

Incubations, in 0.3 ml, were as described in Figure 1, except that tRNA concentrations were varied and, when appropriate, two units of inorganic pyrophosphatase were present. Reactions were started by introduction of 40 units of enzyme (as defined using *N. crassa* tRNA), and 0.1-ml portions were removed at 3 and 6 min for determination of Phe-tRNA. Michaelis constants (moles/liter) and maximum velocities (pmoles Phe-tRNA/min per 0.3 ml) were calculated from Lineweaver-Burke plots. Initial velocity values were obtained by extrapolation of velocity versus time increment plots to zero time. The *E. coli* tRNA^{Val} (1200 pmoles Val-tRNA^{Val} (*E. coli*) formed per A_{260} by *E. coli* synthetase) was assumed to be 100% pure, and *E. coli* tRNA^{Ala} (740 pmoles Ala-tRNA^{Ala} (*E. coli*) formed per A_{260} by *E. coli* synthetase) 50% pure, for these calculations.

as acceptor for [^{14}C]ATP. Furthermore, the addition of excess purified *E. coli* ATP:tRNA adenylyltransferase to either *A*, *B*, or *C* during Phe-tRNA^{Val} (*E. coli*) formation failed to stimulate any of the three fractions. Therefore, the greater amounts of Phe-tRNA formed with either of the purified *E. coli* tRNA's by the *A* enzyme relative to the *B* and *C* fractions cannot be due to contamination of the *A* form with ATP:tRNA adenylyltransferase.

Discussion.—Several laboratories have reported the presence of multiple forms of aminoacyl-tRNA synthetases, but in most cases the experimental evidence is not rigorous, as has been pointed out in a recent review by Novelli.[17] Since this review appeared, multiple aminoacyl-tRNA synthetases have been reported in additional systems. Strehler *et al.*[18] have described the presence of multiple forms of Syn^{Leu} prepared from rabbit heart muscle and separable by a nonlinear gradient on DEAE-cellulose. These workers present evidence that two of the chromatographically separated enzyme forms have different specificities toward isoaccepting forms of leucine tRNA. In this study, the possibilities of mito-

chondrial leucyl-tRNA synthetase contamination, altered forms of the same enzyme, or the possibility of a monomer-dimer interconversion remain open. The presence of two Syn^{Leu} activities from rat liver, as described by Vescia,[19] may be interpreted in these ways, as can the report by Favorova *et al.*[20] concerning the presence in rat liver of two glycyl-tRNA synthetases.

In this report we do not establish the presence of genetically distinct forms of cytoplasmic phenylalanyl-tRNA synthetases in *N. crassa*. We have demonstrated that the differences in chromatographic behavior between *N. crassa* Syn^{Phe} *A*, *B*, and *C* are accompanied by differences in affinity toward purified *E. coli* tRNA's. But it is difficult to rule out, at present, the possibility of alterations having occurred to a single *N. crassa* Syn^{Phe} during preparation, perhaps as a result of enzyme action. However, such modifications would necessarily have to be extremely reproducible and very specific to account for the data we have accumulated. The strongest argument against altered or partially denatured forms constituting *N. crassa* Syn^{Phe} enzymes *A*, *B*, and *C* are the data obtained after normalizing the activity of the three enzymes to *N. crassa* tRNA. When normalized amounts of activity are used with standardized amounts of *E. coli* tRNA, large differences are seen. It is therefore apparent that the enzymes differ, although at this time it is unclear whether this difference is a result of differences in primary structure or in conformation.

Böck[21] has recently demonstrated the presence of subunits in *E. coli* Syn^{Phe}, a finding anticipated by Stulberg[15] and also by Makman and Cantoni[14] for the yeast enzyme. Cassio and Waller[22] have shown that *E. coli* Syn^{Met}, under the proper conditions, dissociates to subunits, which retain their activity. In our studies, neither the sucrose density gradient experiments nor the rechromatography experiments have indicated a breakdown to subunits and subsequent reassociation to a different form.

In extending these studies we will investigate further a recent finding that, in the presence of dimethylsulfoxide in Tris-Cl buffer or in cacodylate buffer at pH 6.3, the *C* enzyme is able to aminoacylate *E. coli* $tRNA^{Val}$ completely.[23] This finding substantiates our view that decreased Phe-$tRNA^{Val}$ (*E. coli*) formed by the *C* form of *N. crassa* Syn^{Phe} relative to that produced by the *A* form is a consequence of differences in kinetic parameters and not of preferential aminoacylation of certain isoaccepting forms of *E. coli* $tRNA^{Val}$.

We conclude that *N. crassa* contains in its cytoplasm at least three phenylalanyl-tRNA synthetases distinguishable by chromatographic properties and by their affinities in Tris-Cl buffer toward a variety of tRNA substrates.

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‡ The abbreviations used are: *N. crassa* Syn^{Phe}, phenylalanyl-tRNA synthetase (L-phenylalanine : tRNA ligase [AMP] EC 6.1.1.4); *E. coli* $tRNA^{Phe}$ (or $tRNA^{Val}$, etc.), phenylalanine (valine, etc.) tRNA from *E. coli*; Phe-tRNA (or Val-tRNA, etc.) phenylalanine (valine, etc.) esterified (aminoacylated) to tRNA.

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