Neurospora Arginyl Transfer Ribonucleic Acid Ligase. Binding and Dissociation of Transfer Ribonucleic Acid†

Jeffrey A. Evans* and M. Nazario‡

ABSTRACT: The binding of several purified tRNAs to *Neurospora* arginyl-tRNA ligase and their dissociation from the complex were studied using nitrocellulose membrane filtration assays. The ligase was found to have a high affinity for arginyl-tRNA from *Neurospora* (K_a = 1.2 × 10^8 M^{-1}); the K_a for both yeast and *Escherichia coli* arginyl-tRNA was only about 10-fold lower. The results of competition experiments in which $tRNA^{Arg}$ and arginyl-tRNA competed for binding to the ligase indicated that the affinities of $tRNA^{Arg}$ from all three sources were similar to those of the respective arginyl-tRNAs. The K_a for periodate-oxidized $tRNA^{Arg}$ was 1.5 × 10^8 M^{-1}. The minimum dissociation rate constant of the homologous ligase–arginyl-tRNA complex was 13.0 × 10^{-2} sec^{-1}; this value is six times greater than the rate of esterification. Thus, unlike all other synthetases investigated, the dissociation of arginyl-tRNA from the ligase is not the rate-limiting step. The heterologous ligase–arginyl-tRNA complexes had dissociation rate constants similar to those of the homologous complex. Therefore, the reduced rate, and the absence of esterification of yeast and *E. coli* $tRNA^{Arg}$, respectively, can not be attributed to poor binding, or slow dissociation of arginyl-tRNA, from the ligase.

Aminoacyl-tRNA ligases and tRNAs provide a convenient system for the study of protein–nucleic acid interactions. Several techniques have been applied to study the interactions between aminoacyl-tRNA ligases and tRNAs, including nitrocellulose membrane filtration (Yarus and Berg, 1967), gel filtration (Lagerkvist *et al.*, 1966), sucrose density gradients (Yaniv and Gros, 1969), protection from thermal inactivation (Mitra *et al.*, 1970), electrophoresis (Okamoto and Kawade, 1967), and fluorescent quenching (Hélène *et al.*, 1969). Nitrocellulose membrane filtration (Yarus and Berg, 1967) has been used for the determination of association constants and dissociation rate constants of a few ligase–tRNA complexes.

In this report we present our results of studies on the rates of formation and dissociation of complexes between *Neurospora* arginyl-tRNA ligase with arginyl-tRNAs from *Neurospora, Escherichia coli,* and baker's yeast. These interactions were measured by the nitrocellulose membrane filtration technique using purified ligase and arginyl-tRNA. The studies were extended to include the interactions of the enzyme with $tRNA^{Arg}$. The effects of adding other substrates on several binding parameters were also examined. The dissociation of arginyl-tRNA from the enzyme is not the rate-limiting step in catalytic cycle.

Methods

Strain and Growth Conditions. The wild-type strain of *Neurospora crassa* 74A was used. The source of strain and the culture conditions were described previously (Hill and Nazario, 1973).

Chemicals. L-[U-^{14}C]arginine, L-[U-^{14}C]valine, L-[U-^{3}H]isoleucine, L-[U-^{3}H]lysine, and L-[U-^{3}H]serine were obtained from Amersham/Searle, New England Nuclear or Schwarz/Mann. [γ-^{32}P]ATP was from New England Nuclear. DEAE-cellulose was from Bio-Rad. SP-Sephadex[1] was purchased from Pharmacia. Bz-DEAE-cellulose and enzyme grade ammonium sulfate were from Schwarz/Mann. Dicyclohexylcabodiimide, ATP, and the napthoxyacetic acid ester of *N*-hydroxysuccinamide were purchased from Sigma. Millipore type HA (0.45 μ) nitrocellulose filters were from Millipore Corp. Arginyl adenylate was synthesized and assayed as described by Berg (1958) and was 87% pure.

Purification of $tRNA^{Arg}$. *N. crassa* tRNA was isolated from lyophilized mycelium of the wild-type 74A by the procedure of Nazario (1972). Baker's yeast tRNA was isolated by the procedure of Holley (1967) or purchased from Calbiochem. *E. coli* tRNA was isolated by the procedure of Zubay (1962). The isolated tRNAs from all three sources were chromatographed on Bz-DEAE-cellulose columns by the method of Gillam *et al.* (1967). The first major $tRNA^{Arg}$ species from *Neurospora* (Nazario, 1972) and from yeast and the main species of $tRNA^{Arg}$ from *E. coli* obtained by Bz-DEAE-cellulose chromatography were further purified separately by the derivatization procedure of Gillam *et al.* (1968) using the naphthoxyacetic acid ester of *N*-hydroxysuccinamide. The application of this procedure to the purification of *Neurospora* $tRNA^{Arg}$ is illustrated in Figure 1. The resolved fractions of naphthoxyacetylarginyl-tRNA were hydrolyzed by incubation with 1.8 M Tris-HCl (pH 8.0) for 1 hr at 35° to yield $tRNA^{Arg}$. The purified tRNAs from *Neurospora, E. coli,* and yeast were assayed with their respective ligases and found to contain 93, 92, and 37% $tRNA^{Arg}$, respectively. Arginine acceptor capacities and tRNA concentrations were determined as described previously (Nazario, 1972).

Periodate-oxidized $tRNA^{Arg}$ was prepared as described (Nazario and Evans, 1974) except that 93% pure *Neurospora* $tRNA^{Arg}$ was used.

Enzymes. The *Neurospora* arginyl-tRNA ligase used was purified 1800-fold by conventional procedures (Nazario and Evans, 1974). For the binding assays, the SP-Sephadex fraction IVb, which is 95% homogeneous on polyacrylamide gel electrophoresis, was used; fractions of lower specific activities

† From the Department of Biochemistry, University of Kansas Medical Center, Kansas City, Kansas 66103. *Received February 4, 1974.* This work was aided by Grants E-522 and NP-48A from the American Cancer Society. This paper was abstracted from a Ph.D. thesis to be submitted by J. E. to the University of Kansas in partial fulfillment of the requirements for the Ph.D. degree.

* Predoctoral trainee of the National Science Foundation under National Science Foundation Grant GZ-2367 to the University of Kansas.

‡ Deceased (June 1, 1974).

[1] Abbreviations used are: Bz-DEAE-cellulose, benzoylated-DEAE-cellulose; SP-Sephadex, sulfopropyl-Sephadex; BSA, bovine serum albumin; $tRNA^{Arg}_{ox}$, periodate-oxidized $tRNA^{Arg}$.

were occasionally used for the preparation of arginyl-tRNA. The *E. coli* arginyl-tRNA ligase was purified by the procedure of Muench and Berg (1966) through the poly(ethylene glycol) concentration step. The preparation thus obtained was chromatographed on SP-Sephadex column, under the conditions described for the *Neurospora* enzyme (Nazario and Evans, 1974). The fractions active in the esterification assay were pooled and dialyzed against 1 l. of 10 mM potassium phosphate (pH 7.0) containing 1 mM reduced glutathione and 55% glycerol. The dialyzed preparation had a specific activity of 6. For the purification of baker's yeast arginyl-tRNA ligase, the extraction and ammonium sulfate fractionation were carried out as described by Makman and Cantoni (1965). The enzyme was then purified further by chromatography on DEAE-cellulose and SP-Sephadex under the conditions used for the *Neurospora* ligase (Nazario and Evans, 1974). The active fractions from the SP-Sephadex column were pooled, dialyzed against 1 l. of 10 mM potassium phosphate (pH 7.0) containing 1 mM reduced glutathione and 55% glycerol, and stored at −20°. The dialyzed preparation had a specific activity of 26. This specific activity is more than five times greater than the highest specific activity reported so far (Makman and Cantoni, 1965).

Assay for Aminoacyl-tRNA Ligases. Each ligase was routinely assayed by measuring the initial rates of esterification (Nazario and Evans, 1974), of tRNA from the same source. One enzyme unit catalyzes the esterification of 1 μmol of arginine to tRNA per hr at 35°. Specific activities are units per milligram of protein. Protein was determined by the procedure of Lowry *et al.* (1951) using BSA as standard.

Esterification of tRNA with Arginine. The purified $tRNA^{Arg}$ from all sources was esterified with [^{14}C]arginine (specific activity of 130 Ci/mol). In a total volume of 1.0 ml, the reaction mixture contained 0.16 M Tris-acetate (pH 7.5), 0.01 M $MgCl_2$, 0.004 M ATP, 75 μM [^{14}C]arginine, 2–8 nmol of $tRNA^{Arg}$, and 0.5 unit of ligase. Incubations were at 35° for 15 min. Usually, the reaction was stopped with 0.2 ml of 2 M potassium acetate (pH 4.5) and 2.5 ml of cold 95% ethanol. When the amount of protein added exceeded 10 μg, the reaction was stopped by the addition of 1 ml of cold, water-saturated phenol and 0.2 ml of 2 M potassium acetate (pH 4.5). The aqueous phase was separated after centrifugation at 0°. The phenol layer was then reextracted with 1 ml of 1 mM potassium phosphate (pH 4.5). The combined aqueous layers were then precipitated with 2.5 volumes of 95% ethanol. The precipitated tRNA was kept at −20° for at least 2 hr and then collected by filtration on a 0.45 μ Millipore type HA filter. The tRNA was washed with 6 ml of cold 70% ethanol and air-dried. The tRNA was then eluted from the filter with 1 ml of 1 mM potassium phosphate (pH 4.5); aliquots (0.2 ml) of the eluate were stored at −20°. Esterification of the second species of $tRNA^{Arg}$ and tRNAs for valine, isoleucine, lysine, and serine from the first Bz-DEAE-cellulose chromatography of *Neurospora* tRNA was carried out as described above using the appropriate labeled amino acid and a dialyzed ammonium sulfate fraction (Nazario, 1972) as the source of aminoacyl-tRNA ligases.

Nitrocellulose Membrane Filtration Assays. The nitrocellulose membrane filtration technique of Yarus and Berg (1967) is rapid to perform, relatively economical from the standpoint of biological material needed, and yields reliable measurements of the interactions occurring in solution. Using this technique, conditions must be found which give optimal retention of complex formed and also low retention of free arginyl-tRNA. The conditions chosen should also stabilize the aminoacyl bond of arginyl-tRNA. Maximum relative efficiency in the detection of

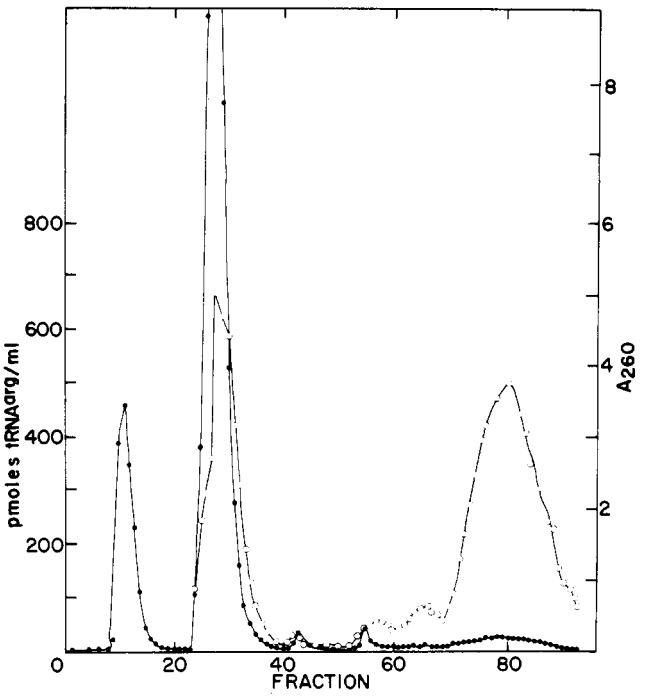

FIGURE 1: Resolution of napthoxyacetylarginyl-tRNA on Bz-DEAE-cellulose. A mixture of napthoxyacetylarginyl-tRNA, arginyl-tRNA, and tRNAs for other amino acids in 40 ml of 0.01 M sodium acetate (pH 4.0) containing 0.45 M NaCl was applied to a column (1.5 × 62 cm) of Bz-DEAE-cellulose previously equilibrated with the above buffer. The concentration of NaCl in the eluent was increased to 1.0 M at fraction 16. At fraction 33, ethanol was added to the eluent to give a final concentration of 4.7%. At fraction 45, the concentration of ethanol in the eluent was increased to 18%. Symbols indicate absorbance at 260 nm (●) and pmol of $tRNA^{Arg}$/ml (○).

complex formation was found at pH 6.0. At lower pH values, the apparent retention of complex was high, but simultaneously, a high retention of free arginyl-tRNA made those conditions prohibitive. With different buffers, a correlation was observed between relative efficiency of binding and relative retention of BSA, suggesting that the efficiency of complex detection is dependent upon the amount of ligase retained by the filter. When potassium phosphate (pH 6.0) was the buffer, up to 25 μg of BSA was linearly retained by one filter.

The amount of complex retained did not change when the concentration of Mg^{2+} was varied from 0 to 5 mM. This may be due to the presence of Mg^{2+} in arginyl-tRNA used for complex formation. It was found by atomic absorption spectroscopy that the arginyl-tRNA used in this study contained about 1.4 mM Mg^{2+}. Thus arginyl-tRNA contributed from 0.1 to 0.5 mM Mg^{2+} over the added concentration.

At pH 6.0 the esterification and ATP–pyrophosphate exchange reactions were inhibited by *p*-hydroxymecuribenzoate.[2] In view of these findings, reduced glutathione was added to the assay mixtures. The temperature was kept at 0° to stabilize the aminoacyl bond of arginyl-tRNA.

The standard incubation mixture contained 0.05 M potassium phosphate (pH 6.0), 2 mM $MgCl_2$, 0.1 mM Na_2EDTA, and 1 mM reduced glutathione. When either ATP or pyrophosphate was included in the incubation mixture additional $MgCl_2$ was added to yield a Mg^{2+}/ATP (or PP_i) ratio of 2.5.

In the association constant experiments the reaction mixtures contained, in a total volume of 0.3 ml, 12 μg of *Neurospora* arginyl-tRNA ligase. The amount of [^{14}C]arginyl-tRNA added varied from 0 to 300 pmol. Reaction mixtures were incubated for 30 sec at 0° following the addition of ligase

[2] Unpublished observations.

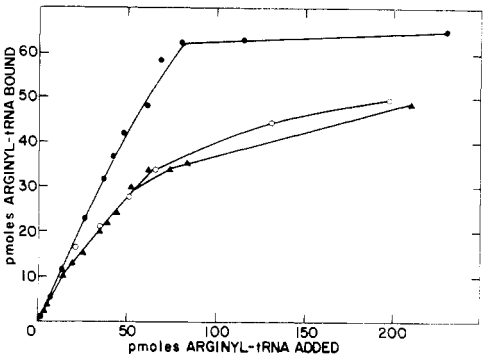

FIGURE 2: Saturation curves of *Neurospora* arginyl-tRNA ligase with homologous and heterologous arginyl-tRNAs. Each sample contained 12 μg of enzyme and arginyl-tRNA as indicated. The source of arginyl-tRNAs was as follows: (●) *Neurospora;* (○) yeast; (▲), *E. coli.*

(the last component added) and filtered at a flow rate of 10 ml/min on Millipore type HA 0.45 μ nitrocellulose filters.[3] The filters were previously soaked in wash fluid (0.05 M potassium phosphate (pH 6.0), 2 mM $MgCl_2$, 0.1 mM Na_2EDTA, and 1 mM reduced glutathione) for 4–15 hr at 0°. Following filtration, the filters were washed with 2 ml of wash fluid (at the same flow rate), dried for 12 min at 105°, and counted in 10 ml of a toluene-based scintillation fluid. The counts obtained above controls in which BSA replaced the ligase were assumed to represent ligase: [^{14}C]arginyl-tRNA complex retained by the filter. The amount of complex observed was converted to amount of complex in solution by taking into account the efficiency of the assay, as discussed by Yarus and Berg (1967).

The association constant (K_a) was obtained by plotting the data according to the following equation (Scatchard, 1949).

$$\frac{\text{fraction ligase bound}}{[\text{free arginyl-tRNA}]} = -K_a(\text{fraction ligase bound}) + nK_a$$

Therefore, when fraction ligase bound/[free arginyl-tRNA] is plotted against fraction ligase bound, the negative slope of the line equals the association constant (K_a) and the intercept on the abscissa equals n, the number of arginyl-tRNA binding sites on the ligase. Straight lines were drawn by least-squares regressions.

The competition experiments were carried out under conditions used for the determination of K_a, except that the concentration of the competing tRNA was varied from 0 to 25 times the concentration of arginyl-tRNA; the concentration of arginyl-tRNA was kept constant, at about twice the level of the ligase. The association constant of the competing tRNA was calculated according to Yarus and Berg (1967). The slope of the resulting line (Figures 5 and 6) equals the ratio of the association constant of the competing tRNA over the association constant of arginyl-tRNA.

For the dissociation rate constant experiments the standard incubation mixture was used (total volume 0.64 ml). One minute after the addition of the enzyme, a 1.5-fold excess of arginyl-tRNA was added. Thirty seconds later a 100-μl aliquot was removed, filtered, and washed. This sample gave the amount of complex at zero time. A 2.0–2.3-fold excess of $tRNA^{Arg}$ (over arginyl-tRNA) was immediately added and 100-μl aliquots were removed, filtered, and washed at intervals ranging from 15 sec to 15 min. The total amount of arginyl-tRNA exchanged for $tRNA^{Arg}$ was calculated as the difference between the zero time value and the plateau value (15 min). The dissociation rate constant (k_d) was calculated from the fraction of the total exchange occurring at 15 sec according to the equation used by Yarus and Berg (1969). At 15 sec (the limit of accurate manipulation) the system is close to the new equilibrium; the calculated k_d's represent minimum dissociation rate constants. In these calculations it was assumed that arginyl-tRNA and $tRNA^{Arg}$ had the same affinity for the enzyme (see Results).

[3] Millipore type HA (0.45 μ) filters lot 34537-13, used routinely, were found to give slightly higher efficiency of complex detection when compared either to Schleicher and Schuell B-6 nitrocellulose filters or to other lots of Millipore filters.

Results

Association Constants of Homologous and Heterologous Arginyl-tRNAs with Neurospora Arginyl-tRNA Ligase. The equilibrium association of arginyl-tRNA and arginyl-tRNA ligase can be quantitated by varying the concentration of arginyl-tRNA in the presence of a constant amount of ligase, until saturation is reached. The saturation curve of *Neurospora* arginyl-tRNA with the *Neurospora* ligase is illustrated in Figure 2. As the concentration of arginyl-tRNA added was increased the amount of arginyl-tRNA bound also increased until a plateau was reached with a maximum of 66 pmol of arginyl-tRNA bound when the amount of arginyl-tRNA added was 250 pmol or more (point not shown). Assuming a one to one stoichiometry of binding between arginyl-tRNA and the ligase the concentration of active enzyme can be calculated. Each assay contained 12 μg of enzyme protein, equivalent to 141 pmol (assuming 100% purity). Therefore 47% of the protein is active arginyl-tRNA ligase. By gel filtration, the amount of active enzyme was calculated to be 41% (Nazario and Evans, 1974).

The affinity of the ligase for arginyl-tRNA from the second *Neurospora* $tRNA^{Arg}$ species resolved by chromatography on Bz-DEAE-cellulose (Nazario, 1972), was found to be similar to that of the first $tRNA^{Arg}$ component. The specificity of these associations was examined by testing the ability of homologous noncognate aminoacyl-tRNAs to bind the arginyl-tRNA ligase. The aminoacyl-tRNAs for valine, isoleucine, lysine, and serine were tested and no binding to the arginyl-tRNA ligase was detected.

The *Neurospora* ligase does not esterify *E. coli* $tRNA^{Arg}$; yeast $tRNA^{Arg}$ is aminoacylated at a lower rate than the homologous tRNA (Nazario and Evans, 1974). It was of interest to examine the interaction of these heterologous tRNAs with the enzyme. Saturation curves for both heterologous arginyl-tRNAs are included in Figure 2. The association constants and the stoichiometry of binding were calculated from Scatchard plots of the results presented in Figure 2. With *Neurospora* arginyl-tRNA (Figure 3A) a K_a of 1.2×10^8 M^{-1} was estimated. The K_a's for the heterologous systems (Figure 3B) were found to be 1.6×10^7 and 1.2×10^7 M^{-1} for *E. coli* and yeast arginyl-tRNAs, respectively. Therefore, the *Neurospora* ligase had only about 10-fold less affinity for the heterologous arginyl-tRNAs than for the homologous arginyl-tRNA. The apparent number of arginyl-tRNA binding sites was 1.0, 0.9, and 0.8 per molecule of ligase for *Neurospora*, yeast, and *E. coli* arginyl-tRNAs, respectively.

We investigated the effect of adding ATP plus arginine on the association of arginyl-tRNA with the ligase (Figure 4). The addition of these substrates had only a small effect on the association lowering the K_a from 1.2×10^8 to 0.8×10^8 M^{-1}.

Competition between Arginyl-tRNA and $tRNA^{Arg}$. The association constant of *Neurospora* $tRNA^{Arg}$, the actual substrate for the esterification reaction, was estimated by measur-

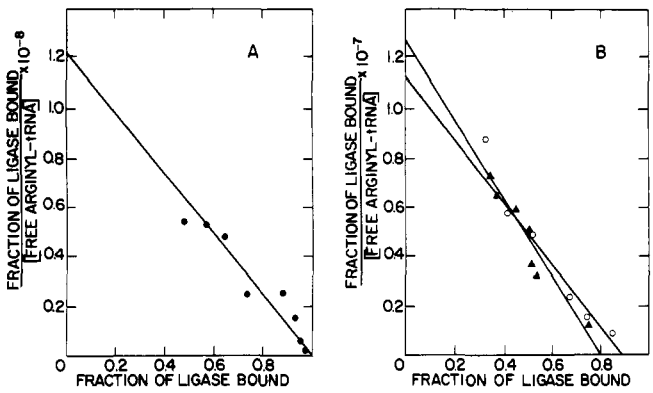

FIGURE 3: Scatchard plots of the saturation curves (Figure 2) for calculations of the K_a for arginyl-tRNA. Part A is for *Neurospora* arginyl-tRNA (●). In part B, the results for yeast (○) and *E. coli* (▲) arginyl-tRNAs are plotted.

ing the competition between arginyl-tRNA and $tRNA^{Arg}$ for binding to the ligase. We found that the ratio of the K_a for $tRNA^{Arg}$ over the K_a for arginyl-tRNA was 0.75 (Figure 5). In the presence of ATP the above ratio was increased to 1.0 (Figure 5). In another experiment (results not shown) it was found that $tRNA^{Arg}_{ox}$ was also a competitor of arginyl-tRNA with a K_a 1.3 times higher than the K_a for arginyl-tRNA.

Similarly, to estimate the binding of *E. coli* $tRNA^{Arg}$ we measured its ability to compete with *E. coli* arginyl-tRNA. The ratio of affinities obtained was 1.1 (Figure 6). Thus *E. coli* $tRNA^{Arg}$ does bind the *Neurospora* ligase with relatively high affinity, and the failure of the enzyme to esterify this tRNA can not be explained in terms of relative strength of binding.

The ratio of the K_a's for yeast arginyl-tRNA and yeast $tRNA^{Arg}$ was 1.0.

The association constants obtained in the heterologous binding experiments were checked by determining the K_a's of both *E. coli* and yeast $tRNA^{Arg}$ by competition with *Neurospora* arginyl-tRNA. The K_a values found were 2.0×10^7 and 1.2×10^7 M^{-1} for yeast and *E. coli* $tRNA^{Arg}$, respectively, and are

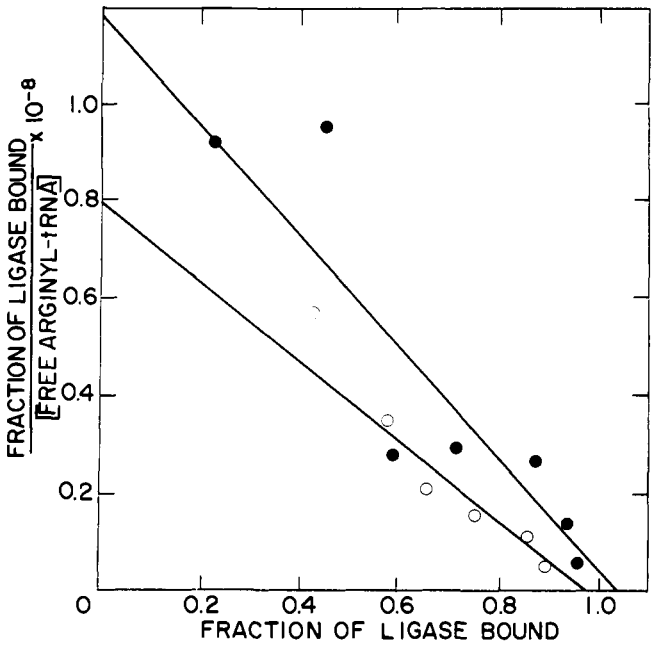

FIGURE 4: Effect of ATP plus arginine on the association of *Neurospora* arginyl-tRNA. The Scatchard plots represent: (●) assays in the absence of substrates; (○) assays in the presence of ATP (2 mM) plus arginine (40 μM).

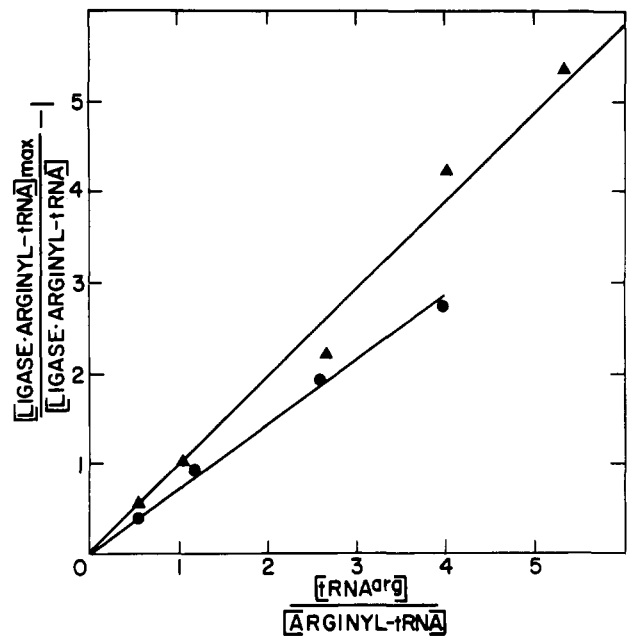

FIGURE 5: Effect of ATP on the competition between arginyl-tRNA and $tRNA^{Arg}$ for ligase binding. Competition was examined in the absence (●) or presence (▲) of 2 mM ATP. For further details, see the text.

therefore in good agreement with K_a's determined previously.

Association Constants and Kinetic Affinity Constants. Table I presents a summary of association constants obtained by nitrocellulose binding. These may be compared with the kinetic affinity constants, K_m and K_i, calculated from double-reciprocal plots (Nazario and Evans, 1974). In comparing the constants, it should be realized that the K_m's and K_i's were determined under conditions giving optimal rates of reaction while the K_a's were obtained under conditions which were optimal for detection of the ligase-arginyl-tRNA complex.

The K_m for *Neurospora* arginyl-tRNA and the K_i's for $tRNA^{Arg}$ and $tRNA^{Arg}_{ox}$ were about 0.1 μM in the reverse reaction, the enzymatic deacylation of arginyl-tRNA (Nazario

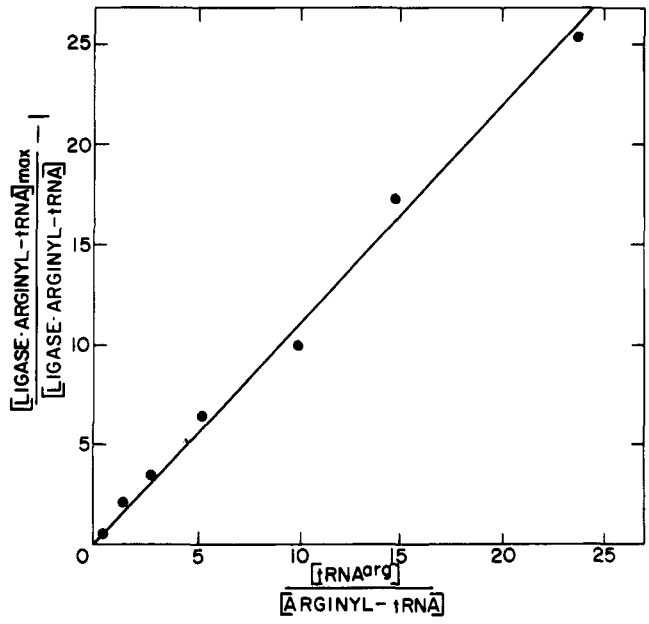

FIGURE 6: Competition between arginyl-tRNA and $tRNA^{Arg}$ (both from *E. coli*) for binding to the *Neurospora* ligase. For details, see the text.

TABLE I: Summary of Association Constants.

Source of tRNA	K_a (M^{-1})
N. crassa	
Arginyl-tRNA	1.2×10^8
$tRNA^{Arg}$	0.8×10^8
$tRNA^{Arg}_{ox}$	1.5×10^8
Baker's yeast	
Arginyl-tRNA	1.2×10^7
$tRNA^{Arg}$	1.2×10^7
E. coli	
Arginyl-tRNA	1.6×10^7
$tRNA^{Arg}$	1.8×10^7

[a] The K_a's for arginyl-tRNA were obtained directly from Scatchard plots of saturation curves. The K_a's for $tRNA^{Arg}$ and $tRNA^{Arg}_{ox}$ were obtained from competition experiments.

and Evans, 1974). These kinetic constants indicate affinities that are only one order of magnitude lower than the K_a for *Neurospora* arginyl-tRNA, obtained in the binding experiments.

In the esterification reaction, the K_m for *Neurospora* and yeast $tRNA^{Arg}$ and the K_i for *E. coli* $tRNA^{Arg}$ were 0.7, 0.8, and 3.9 μM, respectively (Nazario and Evans, 1974). While these values reflect high affinities, the K_a's for *Neurospora*, yeast and *E. coli* $tRNA^{Arg}$ suggest higher affinities (57-, 9-, and 69-fold greater, respectively).

Binding of Other Substrates. The binding of ATP and arginine to the ligase was examined by nitrocellulose membrane filtration. To improve sensitivity, the amount of active ligase was increased to 20 μg. No binding of [γ-^{32}P]ATP was observed either in the absence or presence of an excess of additional substrates. The concentration of [γ-^{32}P]ATP used (6 μM) was well below the K_m for ATP (0.2 mM); extremely high blank values prohibited the use of higher ATP concentrations.

Binding of [^{14}C]arginine was only detected in the presence of ATP and $tRNA^{Arg}$. We attributed that binding not to free arginine but instead to [^{14}C]arginyl-tRNA formed under those conditions. These findings correlate with the gel filtration experiment presented previously (Nazario and Evans, 1974) where neither ATP nor arginine binding was detected in the absence of $tRNA^{Arg}$.

Dissociation of the Arginyl-tRNA–Enzyme Complex. Upon addition of $tRNA^{Arg}$ to a system containing ligase–arginyl-tRNA and free arginyl-tRNA (time 0, Figure 7) rapid dissociation of arginyl-tRNA from the complex was observed and a new equilibrium value for the ligase–arginyl-tRNA complex was attained (Figure 7). Controls indicated that the total amount of trichloroacetic acid precipitable [^{14}C]arginyl-tRNA did not change throughout the experiment. Figure 7 also shows that on addition of either ATP alone or ATP plus arginine the new equilibrium level of complex was approached faster than in the absence of substrates or in the presence of arginine alone. The addition of ATP plus arginine also lowers the final equilibrium value of the ligase–arginyl-tRNA complex (Figure 7). This observation is probably related to the earlier finding that ATP increased the relative affinity of the ligase for $tRNA^{Arg}$ (Figure 5). The dissociation rate constants were calculated from the values observed at 15 sec. As can be seen, (Figure 7) at this point the new equilibrium state has almost been achieved and therefore the calculated k_d represents a minimum value. The (minimum) dissociation rate constants of arginyl-tRNA found under different conditions are shown in Table II. On addition of ATP or ATP plus arginine the k_d is increased from 8.4×10^{-2} to 13.0×10^{-2} sec^{-1}. This change is significant ($P < 0.01$) and is consistent with the increase in the ratio of the K_a of $tRNA^{Arg}$ to the K_a of arginyl-tRNA observed in the presence of ATP (Figure 5). Since the K_a for arginyl-tRNA is decreased by addition of ATP (Figures 4 and 5), the association rate constant must remain unchanged.

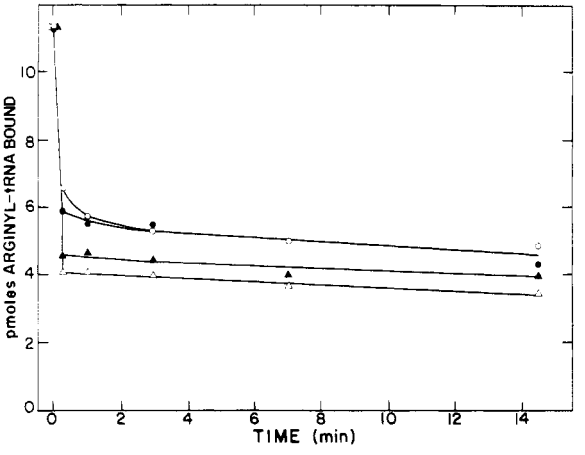

FIGURE 7: Dissociation curves of the ligase: arginyl-tRNA complex. The experiments were carried out as described in Methods. Additions to the complete system were as follows: (●) none; (○) 32 μM arginine; (△) 2 mM ATP; (▲) 32 μM arginine plus 2 mM ATP.

TABLE II: Effect of Substrates on the Dissociation of the Arginyl-tRNA–Enzyme Complex.

Source of tRNA	Other Substrates Present[a]	Dissociation Rate Constant[b] (10^{-2} sec^{-1})
Neurospora	None	8.4 ± 0.7 (7)
	32 μM arginine	8.1 ± 2.7 (3)
	2 mM ATP	13.1 ± 1.6 (3)
	2 mM ATP + 32 μM arginine	13.0 ± 1.8 (3)
	22 μM arginyl-AMP	7.4 (1)
	2 mM PP_i	11.6 ± 1.0 (2)
	2 mM AMP	Not measurable
E. coli	None	12.8 (1)
Baker's yeast	None	10.5 (1)

[a] Substrates were added, as indicated, to standard assay mixtures. [b] The dissociation rate constants were calculated from the fraction exchanged 15 seconds after the addition of $tRNA^{Arg}$ (see Figure 9). The dissociation rate constants are given as the rate constant ± std dev (number of determinations). For further details, see the Methods.

It is interesting to note (Table II) that while addition of ATP and arginine increased the dissociation rate constant, arginyl adenylate had no effect. Pyrophosphate (2 mM) was almost as effective as ATP (2 mM) in increasing k_d. In the presence of AMP, k_d could not be determined due to rapid deacylation of the arginyl-tRNA.

The heterologous arginyl-tRNAs studied were found to be bound tightly by the ligase (Figures 2 and 3B). The slow rate of esterification of yeast $tRNA^{Arg}$ and the lack of esterification of *E. coli* $tRNA^{Arg}$ could be due to slow dissociation of the respective arginyl-tRNAs formed. We examined this possibility by measuring the k_d's for yeast and *E. coli* arginyl-tRNA with the *Neurospora* ligase using the technique described for the ho-

mologous system. The k_d's found (Table II) were 12.8×10^{-2} and 10.5×10^{-2} sec^{-1} for *E. coli* and yeast arginyl-tRNA, respectively, thus eliminating the possibility that slow dissociation of arginyl-tRNA could account for the slow rate or lack of esterification of the heterologous tRNA's. Since the k_d's of the heterologous arginyl-tRNAs are similar to the k_d of *Neurospora* arginyl-tRNA, the association rate constants (k_a's) in the heterologous case must be about 10-fold lower than the k_a of the homologous system to account for the 10-fold difference in association constants found earlier.

Several authors (Yarus and Berg, 1969; Rouget and Chapeville, 1971; Charlier, 1972) have concluded that the dissociation of the aminoacyl-tRNA is the rate-limiting step. To examine this possibility we measured the turnover number of the *Neurospora* arginyl-tRNA ligase under binding conditions. Using *Neurospora* $tRNA^{Arg}$, the turnover number was found to be 2.0×10^{-2} mol of arginyl-tRNA formed per mol of enzyme per sec. This turnover number was calculated assuming that 41% of the total protein is active enzyme *i.e.*, it can be isolated complexed to arginyl-tRNA by gel filtration (Nazario and Evans, 1974). Obviously, the turnover number would be lower if we assume that 47% of the total protein is active enzyme (Figure 2). Therefore, in the absence of substrates, the k_d was at least four times faster than the rate of esterification and in the presence of substrates not less than six times faster. Clearly, the dissociation of arginyl-tRNA from the ligase is not the rate-limiting step.

The turnover number for the *Neurospora* ligase with yeast $tRNA^{Arg}$ as substrate under binding conditions was found to be 3.8×10^{-3} mol of arginyl-tRNA produced per mol of enzyme per sec. In this case, the rate of esterification was at least 30 times slower than the dissociation rate constant. Dissociation of the product, arginyl-tRNA, is not the rate-limiting step when either *Neurospora* tRNA or heterologous tRNAs are used as substrates.

Discussion

The *Neurospora* arginyl-tRNA ligase had a high affinity for *Neurospora* arginyl-tRNA ($K_a = 1.2 \times 10^8$ M^{-1}). The association constant for arginyl-tRNA is very similar to the association constants found by the nitrocellulose technique for isoleucyl- (Yarus and Berg, 1967), valyl- (Yaniv and Gros, 1969), leucyl- (Rouget and Chapeville, 1971), and tyrosyl- (Buonocuore and Schlesinger, 1972; Chousterman and Chapeville, 1973) tRNA of *E. coli* and isoleucyl-tRNA (Charlier and Grosjean, 1972) of *Bacillus stearothermophilus* with their respective ligases.

In addition, the 57-fold difference between K_a and the reciprocal of the K_m for $tRNA^{Arg}$ (estimated under standard assay conditions) is of similar magnitude to the differences found in several of the systems mentioned above. The report by Brenner *et al.* (1972) on the histidyl-tRNA ligase of *Salmonella typhimurium* is the only one where using the nitrocellulose membrane technique identical values were found for the kinetic affinity constants and the binding constant, measured under very similar conditions.

The addition of amino acid to the isoleucine enzyme (Yarus and Berg, 1969) or the addition of amino acid and ATP to the leucine enzyme (Rouget and Chapeville, 1971) of *E. coli* increased the dissociation rate constant of the aminoacyl-tRNA about 6-fold. Addition of isoleucine alone to the *B. stearothermophilus* enzyme (Charlier, 1972) increased the dissociation rate constant 20 times. In the isoleucine and leucine systems of *E. coli* this was also paralleled by a similar increase in the association rate constant, the K_a remaining essentially unchanged. In each of these cases the increase in k_d attained in the presence of other substrates made the rate of dissociation similar to the rate of esterification for the ligase. It was concluded that the dissociation of aminoacyl-tRNA, facilitated by the addition of new substrates, was the rate-limiting step for each of these enzymes' catalytic cycles.

The affinity of the enzyme for *Neurospora* arginyl-tRNA was reduced slightly in the presence of ATP (Figures 4 and 5); the effect of ATP was attributed to an increase in the dissociation rate constant of arginyl-tRNA (Table II) while its association rate constant was not affected. The minimum dissociation rate constant for *Neurospora* arginyl-tRNA was 6-fold faster than the rate of esterification. The arginyl-tRNA ligase system is unique in that the rate of the reaction is not limited by the rate of dissociation of the aminoacyl-tRNA.

We examined also two heterologous systems (*Neurospora* ligase and arginyl-tRNAs from *E. coli* and baker's yeast). The affinity of the ligase for the heterologous arginyl-tRNAs was about 10-fold lower than for *Neurospora* tRNA, while the dissociation rate constants were similar for all three systems. Therefore, the association rate constants for the heterologous complexes must be about 10-fold lower than for the homologous species. The minimum dissociation rate constants found were 30 times and immeasurably faster than the rate of arginyl-tRNA formation with yeast and *E. coli* $tRNA^{Arg}$ as substrates, respectively. Consequently, the conclusion that dissociation of arginyl-tRNA is not the rate-limiting step of the reaction (see above) applies also to the heterologous systems.

Acknowledgments

We thank Mr. R. Ochs for the magnesium determinations.

References

Berg, P. (1958), *J. Biol. Chem. 233,* 608.

Brenner, M., Lewis, J. A., Straus, D. S., DeLorenzo, F., and Ames, B. N. (1972), *J. Biol. Chem. 247,* 4333.

Buonocuore, V., and Schlesinger, S. (1972), *J. Biol. Chem. 247,* 1343.

Charlier, J. (1972), *Eur. J. Biochem. 25,* 175.

Charlier, J., and Grosjean, H. (1972), *Eur. J. Biochem. 25,* 63.

Chousterman, S., and Chapeville, F. (1973), *Eur. J. Biochem. 35,* 51.

Gillam, I., Blew, D., Warrington, R. C., von Tigerstrom, M., and Tener, G. M. (1968), *Biochemistry 7,* 3459.

Gillam, I., Millward, S., Blew, D., von Tigerstrom, M., Wimmer, E., and Tener, G. M. (1967), *Biochemistry 6,* 3043.

Hélène, C., Brun, F., and Yaniv, M. (1969), *Biochem. Biophys. Res. Commun. 37,* 393.

Hill, R., and Nazario, M. (1973), *Biochemistry 12,* 482.

Holley, R. W. (1967), *Methods Enzymol. 12A* 596.

Lagerkvist, U., Rymo, L., and Waldenstrom, J. (1966), *J. Biol. Chem. 241,* 5391.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265.

Makman, M. H., and Cantoni, G. L. (1965), *Biochemistry 4,* 1434.

Mitra, S. K., Chakraburtty, K., and Mehler, A. H. (1970), *J. Mol. Biol. 49,* 139.

Muench, K. H., and Berg, P. (1966), *in* Procedures in Nucleic Acid Research, Cantoni, G. L., and Davis, D. R., Ed., New York, N. Y., Harper & Row.

Nazario, M. (1972), *J. Bacteriol. 112,* 1076.
Nazario, M., and Evans, J. A. (1974), *J. Biol. Chem. 249* (in press).
Okamoto, T., and Kawade, Y. (1967), *Biochim. Biophys. Acta 145,* 613.
Rouget, P., and Chapeville, F. (1971), *Eur. J. Biochem. 23,* 443.
Scatchard, G. (1949), *Ann. N. Y. Acad. Sci. 51,* 660.
Yaniv, M., and Gros, F. (1969), *J. Mol. Biol. 44,* 17.
Yarus, M., and Berg, P. (1967), *J. Mol. Biol. 28,* 479.
Yarus, M., and Berg, P. (1969), *J. Mol. Biol. 42,* 171.
Zubay, G. (1962), *J. Mol. Biol. 4,* 347.

Isolation and Characterization of Porcine Pancreatic Kallikrein†

Martin Zuber and Edgar Sache*

ABSTRACT: Kallikrein was isolated from a defatted extract of partially autolyzed porcine pancreas. A 1300-fold purification was achieved using ammonium sulfate precipitation, calcium phosphate gel adsorption, gel filtration on Sephadex G-100, and DEAE-cellulose chromatography. Repeated DEAE-cellulose chromatography permitted the separation of two major, enzymically active kallikrein components (forms d_1 and d_2) exhibiting distinct mobilities during acrylamide–agarose gel electrophoresis. The latter experiments also revealed the presence of small amounts of an additional, slightly more anionic active kallikrein component (d_3), closely related to form d_2 under the conditions employed for ion-exchange chromatography. By isoelectric focusing, forms d_1 and d_2 could each be resolved into discrete enzymically active peaks (isoelectric points between 3.92 and 4.11). Kallikrein forms d_1 and d_2 both contain a small amount of carbohydrate, 4.6 and 3.2%, respectively. A different hexosamine content was found to be associated with each of these major forms, which may account for some of the observed differences in their elution behavior and electrophoretic mobility. Forms d_1 and d_2 have essentially the same sedimentation coefficient (2.83 S) and closely related amino acid compositions and molecular weights (about 33,000), as determined by gel filtration, sedimentation velocity, and amino acid composition. In both forms, electrophoresis in sodium dodecyl sulfate–polyacrylamide gel in the presence of β-mercaptoethanol separated several components, thus suggesting in each case a disulfide-linked polypeptide chain structure. The enzyme contains no detectable free sulfhydryl groups. Disulfide reducing agents cause loss of activity. Casein or azocoll is not hydrolyzed by kallikrein. In a series of esters, *N*-benzoyl-L-arginine ethyl (or methyl) ester proved to be the best substrate (pH 8.0). *N*-α-Carbobenzoxy-L-lysine benzyl ester is hydrolyzed at nearly a comparable rate. Hydrolysis of *N*-α-carbobenzoxy-L-lysine methyl ester was, however, much slower, that of *N*-α-benzoyllysine methyl ester being nearly negligible. These results show that the specificity is directed primarily toward the esters of arginine and to a lesser extent toward those of lysine. They also indicate that "secondary interaction" implying the nature of the *N*-acyl group of the substrate (position P_2) as well as that of the P'_1 group (according to the nomenclature of Schechter and Berger (1967)), *Biochem. Biophys. Res. Commun. 27,* 157) also contributes to the overall catalytic efficiency of kallikrein. The enzyme also reacts with *p*-nitrophenyl *p'*-guanidinobenzoate giving a burst, followed by a rapid production of *p*-nitrophenol. The enzyme is inhibited by the Kunitz bovine basic proteinase inhibitor. It is also inactivated by diisopropyl phosphorofluoridate, phenylmethanesulfonyl fluoride, diphenylcarbamyl chloride, *p*-aminobenzamidine, and a number of quaternary ammonium compounds. Tosyl-L-lysine or tosyl-L-phenylalanine chloromethyl ketones have no effect on enzyme activity. A slow but definite inhibition was obtained, however, with *N*-α-benzyloxycarbonyl-L-phenylalanine chloromethyl ketone. Following intravenous injection of the enzyme into a dog an immediate and marked fall in arterial blood pressure was observed. The enzyme was identified as porcine pancreatic kallikrein on the basis of its physical and chemical properties and hypotensive action.

It has been proposed that a relationship exists in a number of pathophysiological states between the fibrinolytic, blood coagulation, and vasoactive peptide systems (Eisen, 1964; Back, 1966). Whereas plasmin and thrombin play a key role in fibrinolysis and blood coagulation, respectively, the term kallikrein has been applied to a number of enzymes from various origins (*i.e.*, tissues, body fluids) which rapidly and specifically produce potent physiologically active peptides (kinins) from a plasma α_2-globulin substrate (kininogen). In addition to their strong vasodilatory and hypotensive properties, kinins also increase vascular permeability, produce pain, and modify the migration of leukocytes (Kellermeyer and Graham, 1968). Thus, functionally, a kallikrein is a kininogenase. Such activity, however, does not completely characterize kallikreins since a number of proteins, either proteolytic enzymes (Prado, 1970) or not (Fischer and Udermann, 1970) have also been found to release kinins from a plasma precursor. Kallikreins may thus represent a class of proteolytic enzymes of limited (and still ill defined) specificity for which, at least in the present state of knowledge, a plasma globulin constitutes the best known natural substrate.

In order to understand fully the role of kallikreins in a number of pathophysiological mechanisms, a better knowledge of these enzymes appears desirable particularly with respect to their catalytic activity and specificity. This requires that highly purified enzyme preparations should be readily available.

Porcine pancreas represents a convenient and relatively inexpensive source of glandular kallikrein (EC 3.4.4.21). Although

† From the Centre de Recherche, Institut Choay, 92120 Montrouge, France. *Received November 20, 1973.*