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Binding of Aminoacyl Transfer Ribonucleic Acid Synthetases to Ribosomes from Rabbit Reticulocytes†

James D. Irvin and Boyd Hardesty*

ABSTRACT: Some, but not all, of the aminoacyl-tRNA synthetases present in lysates of rabbit reticulocytes are recovered in high proportion with ribosomes collected by high-speed centrifugation. Up to 90% of the phenylalanyl-tRNA synthetase activity in the lysate is recovered in the ribosomal fraction and appears to have been physically bound to the ribosomes in intact cells. Synthetase activity for lysine and arginine is bound to the ribosome but also appears as a high molecular weight complex or aggregate that sediments at approximately 14 S. Aminoacyl-tRNA synthetases are removed from the ribosomes by 0.5 M KCl but may be rebound to the ribosomes or ribosomal subunits under suitable conditions in solutions of moderate ionic strength. The synthetases for phenylalanyl-tRNA, lysyl-tRNA, and arginyl-tRNA bind preferentially to the large subunit.

We have reported previously the existence of a factor in the salt-wash fraction from reticulocyte ribosomes which promotes binding of *N*-AcPhe-tRNA or Phe-tRNA to nitrocellulose filters or to ribosomes (McKeehan *et al.*, 1970). This factor was identified as Phe-tRNA synthetase on the basis of cochromatography of the activity for binding and the synthesis of Phe-tRNA plus the specific inhibitory effect of phenylalanine and ATP on *N*-AcPhe-tRNA binding. It was concluded that a phenylalanyl adenylate complex was formed on Phe-tRNA synthetase bound to ribosomes. Here, we extend these studies and demonstrate an interaction of other synthetases with ribosomes.

Experimental Procedure

Materials. CHEMICALS. All labeled amino acids were obtained from New England Nuclear, except for cysteine and tryptophan obtained from Amersham-Searle Co. Sephadex G-200 was purchased from Pharmacia.

SOLUTIONS. Solution A contained 2 mM Tris·HCl, pH 7.5, 1 mM β-mercaptoethanol, 1 mM EDTA, and 5% glycerol (v/v). Solution B contained 20 mM Tris·HCl, pH 7.5, 1 mM β-mercaptoethanol, 0.1 mM EDTA, and 0.25 M sucrose. Solution C contained 20 mM Tris·HCl, pH 7.5, 5 mM $MgCl_2$, and 1 mM β-mercaptoethanol.

Methods. The preparation of rabbit reticulocytes, regular ribosomes, ribosomal supernatant, rabbit liver tRNA, and aminoacyl-tRNA was as previously described (Hardesty *et al.*, 1971). In the procedure used, ribosomal pellets collected by centrifugation from lysates of reticulocytes are rinsed gently with solution B before the ribosomes are resuspended. This washing procedure removes loosely packed material from the surface of the ribosomal pellet.

RIBOSOMAL SUPERNATANT AMINOACYL-tRNA SYNTHETASES. Ribosomal supernatant protein was precipitated at 74% saturation of ammonium sulfate (466 g of ammonium sulfate per l.) without pH adjustment in the cold and isolated by centrifugation for 15 min at 15,000 rpm in the Sorvall SS-35 rotor. The precipitate was dissolved in 0.01 the original supernatant volume of solution A and dialyzed overnight against about 50 volumes of the same solution at 4°. The dialyzed enzyme fraction was stored at −90°. Generally, the final protein concentration of this fraction was about 50 mg/ml. The hemoglobin concentration was about 8 mg/ml.

SALT-WASHED RIBOSOMES. Regular ribosomes suspended in solution B were washed by a procedure modified from that of Miller and Schweet (1968). The ribosomal solution containing 20 mg/ml of ribosomes was adjusted to 0.5 M KCl by addition of 4 M KCl with stirring at 0°. The mixture was stirred for 15 min, and then centrifuged for 2.5 hr at 60,000 rpm in the 65 rotor (Beckman Instruments, Inc.). The supernatant from the centrifugation was removed and treated to give the salt-wash fraction as described below. The ribosomal pellet was rinsed three times with solution B, dissolved in the same solution, and adjusted to 20 mg of ribosomes per ml using a 1 mg/ml extinction coefficient for 260-

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TABLE I: Aminoacyl-tRNA Synthetases from Reticulocytes. Enzyme Distribution between Ribosomes and Supernatant Solution.[c]

Synthetase	Mg^{2+} and K^+ Concentration		Ribosomes Activity[a]/mg of Ribosomes	Salt-Wash Activity[a]/mg of Protein	Supernatant Activity[a]/mg of Protein	% Activity with Ribosomes
	mM Mg^{2+}	mM K^+				
Ala	6	5	<1	3	43	<1
Arg	6	5	357	936	77	41 (41)[b]
Asn	6	4	10	16	18	8
Asp	14	14	5	13	<1	54
Cys	6	0	35	91	12	30
Glu	2	0	30	66	12	30
Gln	6	8	13	33	4	35
Gly	6	0	N.D.	2	2	<1
His	12	0	29	81	13	45
Ile	6	4	42	132	39	14
Leu	6	0	10	56	7	19
Lys	6	0	232	636	58	37 (38)[b]
Met	6	8	36	223	20	36
Phe	14	14	314	557	9	90 (83)[b]
Pro	10	4	2	22	2	21
Ser	6	0	47	86	69	11
Thr	10	4	1	7	13	12
Try	6	4	39	83	13	48
Tyr	2	4	<1	2	<1	6
Val	6	8	29	179	52	15

[a] Picomoles of aminoacyl-tRNA formed per minute. [b] Values from a separate experiment in which synthetase activity was determined in reaction mixtures containing 10^{-5} M of the indicated amino acid. [c] Aminoacyl-tRNA synthetase activities in the different cell fractions. The rate of synthetase activity was determined as reported in Materials and Methods from crude ribosomes, salt-wash, and supernatant fraction. The activities obtained shown for regular ribosomes and supernatant fraction were adjusted to activity units per 1 ml of packed reticulocytes and the percentage activity present in the ribosome fraction calculated. The yield of ribosomes and soluble protein per milliliter of packed reticulocytes was 4.6 and 26 mg, respectively. The values listed for Mg^{2+} and K^+ are optimal concentrations for aminoacyl-tRNA formation under standard assay conditions with enzymes from the supernatant fraction.

mμ light of 11.3 (Ts'o and Vinograd, 1961). The ribosomal solution was again adjusted to 0.5 M KCl then the ribosomes were sedimented through 30% sucrose (w/v) in solution B which contained 0.5 M KCl. Centrifugation was for 2.5 hr at 60,000 rpm in the 65 rotor. The pellet was rinsed and dissolved as before then stored at 4° or frozen at −90° until it was used. Salt-washed ribosomes prepared in this manner contain amounts of tRNA that are below detectable levels for aminoacyl-tRNA formation under the conditions used in this study.

RIBOSOMAL SALT-WASH ENZYME FRACTION. Protein of the crude salt-wash fraction as obtained above was precipitated in the cold with 70% saturation ammonium sulfate formed by addition of saturated ammonium sulfate solution to the 0.5 M KCl supernatant. The precipitate was collected by centrifugation for 15 min at 15,000 rpm in the Sorvall SS-34 rotor. The precipitate was dissolved in one-half the original volume in solution A and dialyzed overnight in the cold against the same solution. This procedure yields about 1 mg of protein in the salt-wash fraction per 10 mg of ribosomes. The final protein concentration for the salt-wash fraction generally was about 5 mg/ml. The corresponding concentration of nucleic acid was 0.15 mg/ml. Protein and nucleic acid concentrations were estimated from the absorption of 260- and 280-mμ light as described by Warburg and Christian (1942). Formation of aminoacyl-tRNA in the absence of added tRNA was below detectable levels with the amounts of salt-wash protein used as an enzyme source in the work reported below. Generally, hemoglobin comprised less than 1% of the total protein in this fraction. The enzyme activity for aminoacyl-tRNA synthetases in the salt-wash fraction is given in Table I. The activity levels for arginine, lysine, and phenylalanine are representative for a large number of salt-wash enzyme preparations in which these values have been determined. Salt-wash preparations used in this study had specific activities within 50% of those listed in Table I.

AMINOACYL-TRNA SYNTHETASE ASSAY. Unless indicated in the text, enzymatic activity was determined in 0.5-ml reaction mixtures containing 100 mM Tris·HCl, pH 7.5, 2×10^{-5} M [^{14}C]amino acid (specific activity 100 mCi/mmole for all except cysteine, specific activity 58 mCi/mmole, and tryptophan, specific activity 52 mCi/mmole), 2 mM ATP (previously adjusted to pH 6.5 with KOH), 20 mM β-mercaptoethanol, 100 μg of rabbit liver tRNA, and KCl and $MgCl_2$ as indicated for each aminoacyl-tRNA synthetase as given in Table I. The source and amount of enzyme used is indicated in the text. Reaction mixtures were incubated at 37° for 4 min unless otherwise indicated and precipitated with about 5 ml of cold 5% trichloroacetic acid, and the precipitate collected immediately on Millipore nitrocellulose filters. The filters were washed with at least an additional 20 ml of 5% trichloroacetic acid solution. The precipitated aminoacyl-tRNA

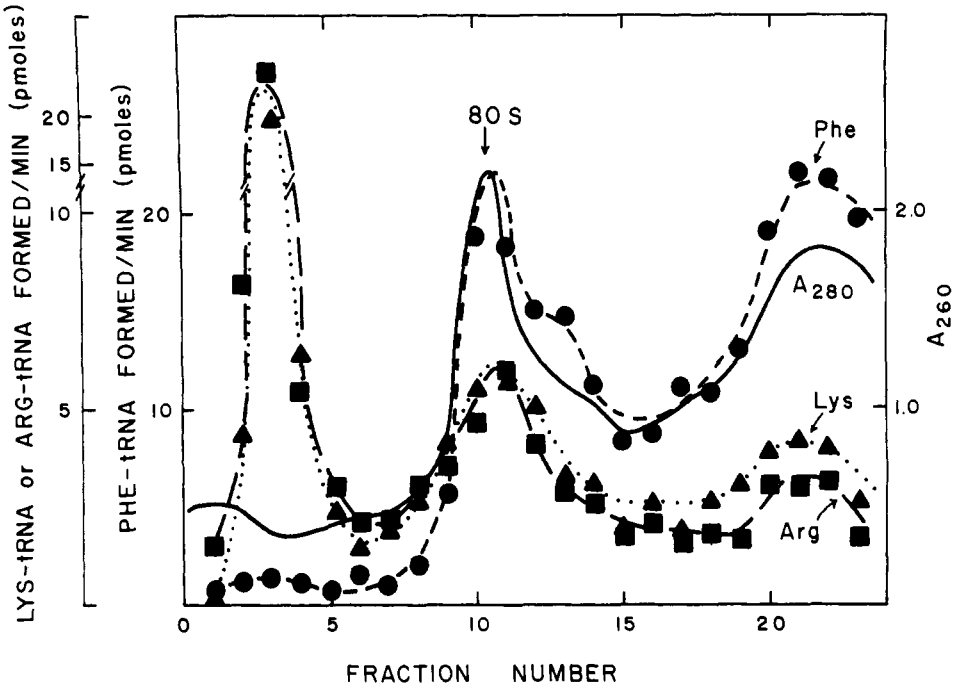

FIGURE 1: Activity profile from sucrose gradient centrifugation for Lys-tRNA, Arg-tRNA, and Phe-tRNA synthetase in the ribosomal fraction. One mg of crude ribosomes, dissolved at 4 mg/ml in 0.25 ml containing 20 mM Tris·HCl, pH 7.5, 25 mM KCl, 5 mM $MgCl_2$, and 1 mM β-mercaptoethanol, was layered on a 9-ml 10–30% linear sucrose gradient above a 2-ml layer at 45% sucrose. All sucrose solutions contained the same salt concentrations as the sample. The gradient was centrifuged for 1.5 hr at 40,000 rpm in a SW 41 rotor (Beckman Instruments, Inc.). The gradient was fractionated into 0.5-ml fractions with an Isco gradient fractionator. The fractions were diluted to 1.0 ml with solution B and aminoacyl-tRNA synthetase activity determined on 0.25-ml aliquots as described in Materials and Methods. Total enzymatic activity per fraction is reported in the figure.

was then counted by liquid scintillation spectrometry. Enzyme activity is expressed in the units of pmoles of aminoacyl-tRNA formed per minute. Unless otherwise indicated, enzyme concentrations were adjusted to give nearly linear synthesis of aminoacyl-tRNA for at least 5 min. Decrease in the amount of enzyme fraction used resulted in a proportional decrease in aminoacyl-tRNA formed. When required, enzyme fractions were diluted in solution C shortly before they were added to reaction mixtures. Generally, enzyme dilution involved the addition of 100 μg of salt-wash protein and solution C to give a final volume of 1.0 ml. Aminoacyl-tRNA synthesis was determined with aliquots of these solutions containing the indicated amounts of salt-wash protein. Mg^{2+} and K^+ optima were determined separately for each aminoacyl-tRNA synthetase using the supernatant enzyme fraction.

Results

Synthetases Associated with Ribosomes. The relative proportions of the total aminoacyl-tRNA synthetase activity recovered with ribosomes or in the soluble fraction for 20 common amino acids is presented in Table I. For these experiments ribosomes and soluble fractions were obtained from a known volume of washed reticulocytes packed by low-speed centrifugation then lysed as indicated under Methods. After lysis the cellular debris was removed by centrifugation and discarded before the high-speed centrifugation by which ribosomes were pelleted. An aliquot of either the ribosome or supernatant fraction was used as a source of aminoacyl-tRNA synthetase. The amounts and percentage of the total activity in the two fractions were calculated on the basis of the original volume of packed cells from which they were derived. The results show that some of the synthetases occur in relatively high proportion in the ribosome fraction. This has been 80–90% of the activity for phenylalanine as determined in a number of experiments. Aminoacyl-tRNA synthetase activity in the ribosome fraction for the other amino acids ranges downward from about 50% of the total activity recovered. Activity for alanine, aspartic acid, glycine, serine, and tryptophan may not be bound to ribosomes in that about 10% or less of the total synthetase activity for these amino acids is recovered with ribosomes. It should be noted that these values, based on enzymatic activity, provide only an indication of the relative amounts of enzyme protein that may be associated with ribosomes in intact reticulocytes. The cells are lysed by hypotonic shock. During lysis the cellular contents undergo a dilution into a solution of relatively low ionic strength for 2 min until additional KCl and sucrose are added to the lysate. We estimate that intercellular potassium with the sodium from the solution in which the cells were suspended before lysis will produce concentrations of about 15 mM potassium and 30 mM sodium in the cell lysate during this period. The corresponding value for magnesium is 3 mM. Changes in the distribution of enzymes might occur during this period or at other times during the isolation procedure. In addition, a portion of some of the aminoacyl-tRNA synthetases appear to occur in a relatively large aggregate or complex. Some portion of this complex is precipitated during high-speed centrifugation of the lysate and is present in preparations of ribosomes. This aggregate is apparent in the sucrose gradient activity profile for lysine and ariginine shown in Figure 1. This figure represents material, primarily ribosomes, that were pelleted by centrifugation from the cell lysate from which cellular debris had been removed by low-speed centrifugation. Ribo-

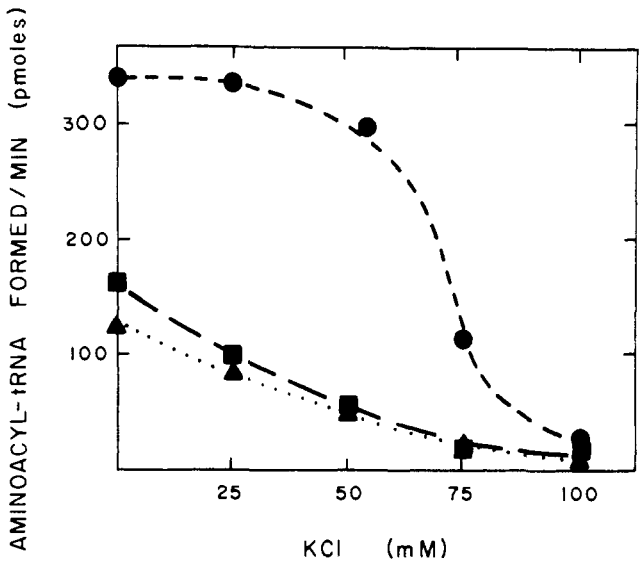

FIGURE 2: Effect of KCl concentration on the dissociation of synthetase from ribosomes. One mg of crude ribosomes was dissolved in 0.25 ml containing 20 mM Tris·HCl, pH 7.5, 5 mM $MgCl_2$, 1 mM β-mercaptoethanol, and KCl as indicated. The samples were layered on 10–30% linear sucrose gradients of 5 ml containing the same salt concentrations as their respective sample. The gradients were centrifuged for 2.0 hr at 60,000 rpm in a SW 65 rotor (Beckman Instruments, Inc.). The top 95% of the gradients were removed by aspiration. The remaining portion of the gradient solution was diluted to 1.0 ml with solution B and the ribosomes were resuspended by gentle homogenization. Aliquots from 0.02 to 0.1 ml were assayed for aminoacyl-tRNA synthetase activity. The values presented for aminoacyl-tRNA formed were corrected to 100% recovery of ribosomes. In all cases, recovery of ribosomes was greater than 80%. (●) Phe-tRNA; (▲) Lys-tRNA; (■) Arg-tRNA.

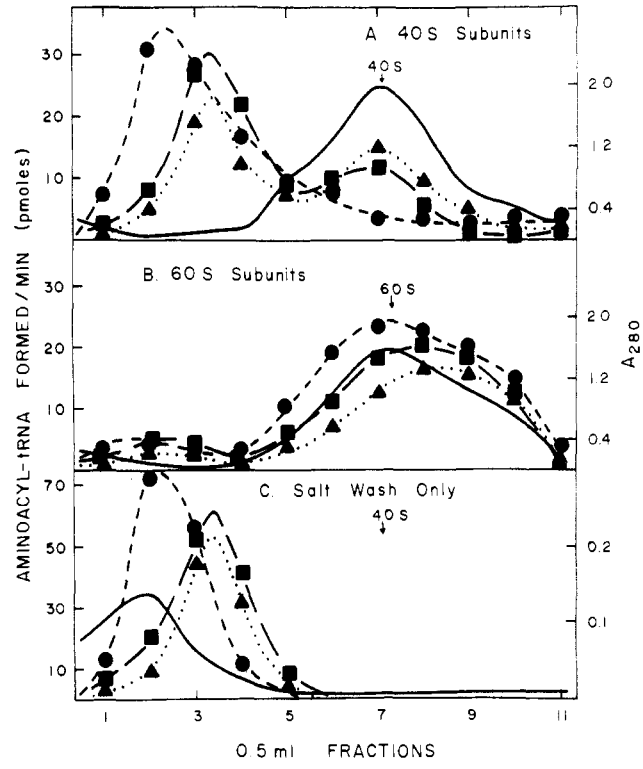

FIGURE 3: Sucrose gradient centrifugation of ribosomal subunits previously incubated with salt-wash enzyme fraction. Ribosomal subunits, 0.5 mg when added, were mixed with 250 μg of salt-wash protein in 0.25-ml samples containing 20 mM Tris·HCl, pH 7.5, 25 mM KCl, 5.0 mM $MgCl_2$, and 1.0 mM β-mercaptoethanol. Ribosomal subunits were prepared as previously described (Obrig *et al.*, 1971) and repurified by centrifugation through 10–30% linear sucrose gradients containing 20 mM Tris·HCl, pH 7.5, 50 mM KCl, 5.0 mM $MgCl_2$, and 1.0 mM β-mercaptoethanol for 8 hr in the SW 25.2 rotor (Beckman Instruments, Inc.). The samples were layered on 5 ml, 10–30% linear sucrose gradients containing the same salt concentrations as the samples. The gradients were centrifuged 1.5 hr at 60,000 rpm in the SW 65 rotor (Beckman Instruments, Inc.) for the gradients containing salt wash alone and the small or 40S subunit. Centrifugation time was reduced to 1.0 hr for the gradient containing the large or 60S subunit. The gradients were fractionated and assayed as described in Figure 1. (●) Phe-tRNA; (▲) Lys-tRNA; (■) Arg-tRNA.

somal pellets are rinsed to remove loosely packed protein rich in Arg-tRNA and Lys-tRNA synthetase activity from the surface of the pellet, then resuspended as previously described (Hardesty *et al.*, 1971). A portion of this resuspended material was layered directly on sucrose gradients containing 25 mM KCl, 5 mM $MgCl_2$, and 20 mM Tris·HCl, pH 7.5. After centrifugation, fractions were collected and the distribution of enzymatic activity and ribosomes determined. About one-third to one-half of the total synthetase activity observed for lysine and arginine appears in a discrete peak that sediments more slowly than smaller ribosomal subunits. We estimate the sedimentation coefficient of material in this peak to be approximately 14 S as determined by sucrose gradient analysis. Nearly all of the activity for these synthetases that is not present in this 14S peak appears to be associated with the ribosomes. This is not the case with Phe-tRNA synthetase activity, which is very low in the 14S region of the gradient. A small amount of Phe-tRNA synthetase activity is found near the top of the gradient, however, 90% or more of this activity follows the distribution of 80S ribosomes and polysomes in the gradient. These data appear to indicate that under these conditions Phe-tRNA synthetase is not associated with the enzymes for lysine and arginine in an enzyme aggregate or complex.

Also shown in Table I is the synthetase activity removed from ribosomes by sedimentation from 0.5 M KCl. The material removed from the ribosomes by this procedure is called the salt-wash fraction. This procedure performed as described under Methods removes nearly all of the synthetase activity from the ribosomes. The synthetases for phenylalanine, lysine, and arginine are particularly abundant in the salt-wash fraction. These three enzymes were chosen for further investigation as described below. The effect of KCl concentration on removal of Phe-tRNA, Lys-tRNA, and Arg-tRNA synthetase from ribosomes is shown in Figure 2. For these experiments regular ribosomes were sedimented through sucrose gradients under conditions similar to those used for the experiments depicted by Figure 3 except that the KCl concentration in the gradients were adjusted to the indicated levels and the centrifugation time was extended to 2 hr. This centrifugation brings 80S ribosomes to the bottom of the tubes. Material of about 40 S or less remains in solution. Synthetase activity was determined in aliquots of the resuspended ribosomes. Under these stringent conditions for separation of free enzyme or enzyme complex from ribosomes, most of the synthetase activity for phenylalanine, arginine, and lysine is removed from the ribosomes with 100 mM KCl. The data may reflect an equilibrium between free enzyme and enzyme bound to the ribosomes that is affected by KCl concentration.

Binding of Synthetases to Ribosomes and Ribosomal Sub-

units. Synthetase activities for lysine, arginine, and phenylalanine present in the dialyzed salt-wash fraction will rebind to ribosomes from which they were removed. Regular ribosomes pelleted by centrifugation from 0.5 M KCl solution used in preparation of the salt-wash fraction were resuspended in solution B and washed a second time as indicated in Methods. Aliquots of these ribosomes were incubated with portions of the dialyzed salt wash under conditions similar to those described for Figure 1. The profiles indicate binding of the enzymes to 80S ribosomes and polysomes (data not shown).

Binding of Lys-tRNA, Arg-tRNA, and Phe-tRNA synthetases from the supernatant fraction was also tested under similar conditions. It was found that all three of the enzymes bound to the ribosomes, although somewhat less efficiently than observed for the salt-wash synthetases. This reduction in binding might be due to interference from other proteins present at high concentrations in the supernatant fraction. ATP, tRNA, and amino acids have little if any effect on the binding of these synthetases to ribosomes. A small increase in the activity for Lys-tRNA synthetase recovered with 80S ribosomes has been observed with tRNA and ATP, however, this may be due to an increase in enzyme stability rather than to a direct effect on binding of the enzyme.

The ability of the synthetases to bind to ribosomal subunits was tested in experiments similar to those used for intact ribosomes. Centrifugation time was adjusted to position the subunits near the center of the gradients. The broad distribution of subunits as indicated by absorption observed in these gradients is related to the relatively large amounts of subunits used and, with 60S subunits, to the effect of the salt-wash fraction. Large or small ribosomal subunit preparations used in these studies were virtually free of subunits of the other type and gave nearly symmetrical peaks of about 40 S or 60 S in the absence of the salt-wash fraction. A description of the procedure used is presented with Figure 3 which represents the results from these experiments. Phe-tRNA synthetase is bound efficiently to the large subunit but is recovered in only small amounts with the 40S ribosomal subunit after sucrose gradient centrifugation of this component. Some activity for lysine and arginine is associated with this subunit in these gradients, however, most of the activity for these enzymes is found in the 14S region of these gradients. These results are in contrast to those observed with 60S subunits. With the 60S subunit nearly all the synthetase activity recovered for each of these three amino acids is found in fractions that contain the subunit. We have observed repeatedly that the activity for these synthetases appears to sediment slightly faster than the bulk of the 60S material. This might reflect an increase in sedimentation due to binding of synthetases and other components of the salt wash to this subunit.

Discussion

A consideration of primary importance is whether or not the phenomena described here reflect similar interactions that occur in intact cells. We believe that they may. Several laboratories have reported aminoacyl-tRNA synthetase activity associated with high molecular weight components from mammalian systems. Munro and his colleagues observed high levels of aminoacyl-tRNA synthetase activity in the postmicrosomal fraction from rat liver (Hird *et al.*, 1964). Vennegoor and Bloemendal (1970) reported a large component, fraction X, in the postmitochondrial fraction from rat liver that restored *in vitro* amino acid incorporation into peptides to levels obtained when the total cell sap was used as an enzyme source. They presented evidence that fraction X sedimented at about 28 S. In lysates fractions from rabbit reticulocytes, Lys-tRNA and Arg-tRNA synthetase activity sediments at about 14 S under the conditions used here. Although such values are highly dependent upon the conditions used and are subject to considerable error, it seems likely that reticulocyte component is smaller than fraction X.

Deutscher and his colleagues have also reported a high molecular weight complex of aminoacyl-tRNA synthetase in the postmitochondrial fraction from rat liver (Bandyopadhyay and Deutscher, 1971). It was concluded that all 18 liver aminoacyl-tRNA synthetases examined, as well as a large fraction of the cellular tRNA, were present in this complex. This appears not to be the case with the 14S component from reticulocytes in that at least Phe-tRNA synthetase does not sediment with the component even though it binds very efficiently to ribosomes. Furthermore, appreciable amounts of this enzyme may remain associated with ribosomes under conditions in which much of the activity for Lys-tRNA and Arg-tRNA synthetase are removed (see Figure 2, 50 mM KCl). It is not certain that the enzymes for lysine and arginine are physically associated in the same enzyme aggregate or complex, however, it seems likely that this is the case. Ribosomes bearing such a complex plus other synthetase might sediment appreciably faster than comparable ribosomes without these components. We have observed repeatedly that the synthetase activities for lysine, arginine, and phenylalanine appear to sediment slightly faster than the bulk of the 80S ribosomes as indicated by ultraviolet adsorption (see Figure 1).

The question of whether or not these interactions occur in intact cells involves consideration of changes in these relations that may occur during cell rupture and fractionation. Interactions between proteins, nucleic acids, and other cellular components frequently involve electrostatic binding and generally are dependent upon their ionic environment. Drastic changes in this environment, particularly from conditions of relatively high to very low ionic strength and reduction of the K^+ to Mg^{2+} ratio might lead to interaction between the synthetases, aggregation, or interaction with ribosomes, binding, of the type described here and by other workers. On the basis of the work presented here, it is impossible to be certain that artifactual changes of this type have not occurred during fractionation. However, the observed binding of the aminoacyl-tRNA synthetases to the ribosomes under the conditions studied may reflect weaker interactions which may occur at higher ionic strengths.

Work to be presented elsewhere indicates an effect of ribosomes on the activity of some synthetases for formation of aminoacyl-tRNA at K^+ and Mg^{2+} concentrations similar to those that occur in intact cells. These observations of the effect of ribosomes in enzymatic activity support the possibility that a physical interaction between ribosomes and some synthetases may be of physiological significance.

Acknowledgments

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Some Effects of Environment on the Folding of Nicotinamide–Adenine Dinucleotides in Aqueous Solutions†

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ABSTRACT: Nicotinamide-adenine dinucleotide (NAD) and dihydronicotinamide-adenine dinucleotide (NADH) exist in solution in a folded form in which the two bases, adenine and nicotinamide or dihydronicotinamide, are stacked over each other. This study was undertaken to investigate the forces that cause NAD and NADH to fold. Several statistical methods for the analysis of data involving the variation of the base proton chemical shifts with temperature were examined. The statistical method yielding the best results was used to obtain the parameters in the folding model. These values were then used as the initial estimates for a direct least-squares fit to the nonlinear equation relating the chemical shifts and the temperature. The values of the parameters calculated in this manner depend upon which proton is used to make the estimate. From this it was concluded that the folding reaction is not a two-state process. This means that unless the proportion that the intermediate folded forms contribute to the experimental chemical shift is known, it is not possible to calculate the usual thermodynamic parameters from the variation of chemical shifts with temperature. Instead, what one obtains is a ratio of the sum of weighted concentrations of all forms which are not completely folded to the sum of weighted concentrations of all forms that are not completely unfolded. The "thermodynamic parameters" (*i.e.*, $\Delta H'$, $\Delta F'$, and $\Delta S'$) that one would calculate from this ratio are difficult to relate to the usual definitions of the equilibrium constant, ΔF, ΔH, and ΔS obtained from a two-state process. It is clear from the data, however, that NAD and NADH behave differently when their behavior in 7 M methanol or 1 M urea is compared to their behavior in water. For example, $\Delta H'$ and $\Delta S'$ for folding are smaller for NAD when the reaction proceeds in 1 M urea or 7 M methanol instead of water, but larger for NADH when the reaction occurs in 1 M urea instead of water.

It is now well established (Meyer *et al.*, 1962; Czerlinski and Hommes, 1964; Jardetzky and Wade-Jardetzky, 1966; Miles and Urry, 1968; Sarma *et al.*, 1968; Cross and Fisher, 1969; Cattrall *et al.*, 1969) that oxidized or reduced nictoinamide-adenine dinucleotide (NAD or NADH) can assume a conformation wherein the nicotinamide ring and the adenine ring of a single molecule are in (nearly) parallel planes. Jacobus (1971) has raised cogent questions about the arguments of others which Jacobus feels were constructed to be "appealing in light of research objectives." While it is certainly true that published chemical shift data alone "cannot be interpreted to define unambiguously the molecular geometry of pyridine dinucleotides," it is also true that the combination of nuclear magnetic resonance (nmr), ultraviolet, and fluorescence spectral data makes it highly probable that intramolecular stacking interactions exist between the adenine and the nicotinamide or 1,4-dihydronicotinamide rings of NAD or NADH. These intramolecular interactions are reflected by hypochromic and hyperchromic effects in the ultraviolet, the ability of adenine to transfer energy to the 1,4-dihydronicotinamide ring of NADH at rates in excess of 10^{11} transfers/sec (Scott *et al.*, 1970), and significant changes to higher field in the chemical shifts of the nmr spectrum of NAD or NADH. The nmr effect is due to the fact

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