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Molecular Weights of Mitochondrial and Cytoplasmic Aminoacyl-tRNA Synthetases of Beef Liver and Their Complexes†

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ABSTRACT: In eukaryotes, multienzyme complexes containing five to nine aminoacyl-tRNA synthetase activities have frequently been reported. In this study, we report the existence, in bovine liver cytoplasm, of a multienzyme complex containing at least 16 activities which can be disrupted by homogenization to give rise to smaller complexes and noncomplexed synthetases. Determination of the size and component activity of these complexes and of the molecular weights of all 20 free synthetases suggests that the smaller complexes and free activities normally identified arise from the larger complex by well-defined stages during homogenization. We also show that similar, though not identical, complexes are found in bovine liver mitochondria and give the molecular weights of 16 mitochondrial synthetases.

The aminoacyl-tRNA synthetases, a class of enzymes playing a vital role in protein synthesis, have been extensively studied in recent years. Synthetases from many prokaryotic sources have been well characterized and appear to occur as independently active, nonassociated enzymes. For eukaryotes, on the other hand, Dang et al. (1982) have recently compiled over 70 references which suggest that the synthetases occur in one or more multienzyme complexes. All 20 synthetase activities may occur in a single supramolecular complex (Bandyopadhyay & Deutscher, 1971). Such a complex has been reported to contain tRNA, elongation factors, tRNA-modifying enzymes, and lipid (Soll & Schimmel, 1974). However, the existence, in vivo, of a complex containing all 20 synthetase activities is not universally accepted. Aggregation of its components following disruption of the cell (Irvin & Hardesty, 1972) or, alternatively, the presence of several homotypic complexes of similar type and behavior (Dickman & Boll, 1977) cannot be completely discounted.

If a supramolecular complex does exist, it is extremely fragile and apparently breaks down readily during purification to give rise to a fairly stable "core complex" containing seven to nine of the synthetase activities. The composition of this core complex is similar in a large range of tissues examined in many laboratories, but its size and composition do vary a little with the method of preparation. Again, there is not complete agreement that the core material is a heterocomplex. Dickman & Boll (1977), for example, claim that purification methods "mild enough not to break the types of bonds expected" alter the ratio of the components and hence suggest a mixture of homocomplexes. There have been a number of reports of the existence of homocomplexes containing multiple copies of a single synthetase (Pan et al., 1976; Dietrich et al., 1978; Zaccai et al., 1979) which have sometimes been quoted as evidence for the existence of a set of inseparable homocomplexes with similar properties rather than a heterocomplex. The homocomplex of cysteine studied by Pan et al. (1976), however, is much smaller than the heterocomplexes normally observed. The valyl-tRNA synthetase complex studied by the other workers appears to involve an electrostatic interaction between tRNA and enzyme and is readily disrupted by quite low ionic strengths while the core complex normally observed is stable to much higher ionic strengths. In fact, while it is impossible to disprove the existence of a set of homotypic complexes with very similar properties, there is an increasing weight of evidence in favor of the existence of a heterocomplex of defined structural composition and organization. Dang & Yang (1982) claim that the heterotypic multienzyme complex model is strongly favored over the homotypic complex model on the basis of cosedimentation and coelution of different synthetases from gel filtration, hydroxyapatite, and affinity resins with immobilized tRNA or amino acid. They write that "the sequential disassembly of the complex indicates specific association between synthetases", and the purification of a heterotypic core complex by Kellerman et al. (1979) indicates a fairly well-defined composition. The present study which presents evidence for complexes larger than the stable core complex usually observed, which also show correlation between the size and number of components and which also display sequential disassembly, provides further support for the heterotypic model.

There is, at present, little evidence whether, or by what intermediate stages, the stable core complex might arise from a supramolecular complex. Some results documenting such a process are provided by Dang & Yang (1979) in the form of a histogram tabulating the frequency of reports of the occurrence of a particular activity within a complex and thereby identifying "core" and "peripheral" activities.

Studies of the effects of methods of homogenization on the size and composition of the complex have produced contradictory results. Deutscher (1974) claims that the complex is very unstable to homogenization, though he suggests that homogenization may disrupt the lysosomes and increase proteolysis, rather than disrupting the complex directly. In contrast, Roberts & Olsen (1976) find that the ionic strength,

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the buffer, and the method of homogenization have no significant effect on the isolation of high molecular weight complexes, but it should be noted that the complexes they observe are much smaller than those reported by other workers. In general, these studies have not yielded as much information as they might. Several studies have provided sedimentation coefficients for small complexes of various compositions (Dang et al., 1982), but conversion of such values to the corresponding molecular weights is always unsatisfactory, the more so when the sedimentation coefficients have been measured in sucrose density gradients. Several studies using gel filtration methods [for example, see Charezinski & Borkowski (1981) and Roberts & Olsen (1976)] have not provided quantitative data, due either to the use of resins which do not resolve large intermediates or to the difficulty of calibrating the large molecular weight region of the elution profile. In the present study, we have taken advantage of the availability of four components of *Cherax destructor* hemocyanin with molecular weights of 450 000, 900 000, 1 760 000, and 3 520 000 and of an AcA 34 resin which gives sharp resolution of species with molecular weights from 12 000 to greater than 3.5×10^6 to determine the molecular weights of all the synthetases which elute as free activities and of several complexes of intermediate size. Knowledge of these values and shifts in the ratio of complexed to free activities is used to test the suggestion that the free activities arise from a larger complex during homogenization.

In addition to the probable existence of one or more high molecular weight complexes, the eukaryotic synthetases differ from the prokaryotic in that, while the prokaryotes usually contain one synthetase for each amino acid, the eukaryotes frequently contain several [for example, see Lea & Norris (1977) and Swamy & Pillay (1982)]. There is much evidence also that, in eukaryotes, cytoplasmic and organelle synthetases differ; thus, they carry out protein synthesis independently and vary in chromatographic behavior, size, immunological reactivity, and specificity for various tRNA isoacceptor species (Beauchamp et al., 1977) which, in turn, have frequently been assigned to different subcellular locations (Boguslawski et al., 1974). These comparisons have been carried out predominantly in lower eukaryotes (Krauspe & Parthier, 1974; Schneller et al., 1976) or plants (Lea & Norris, 1977). In the latter, the evidence for the existence of complexes is not convincing (Lea & Norris, 1977). On the basis of tRNA specificity, it is frequently stated that the organelle synthetases resemble those in prokaryotes [for example, see Krauspe & Parthier (1974)]. However, there seems to be no evidence to show whether the mitochondrial synthetases of mammalian organelles resemble the prokaryotic synthetases by appearing free or the eukaryotic synthetases by appearing in complexes. This study provides evidence for the existence of similar, though probably not identical, high molecular weight complexes within the mitochondria and cytoplasm of beef liver and compares the sizes of the dissociated activities which arise from both.

Materials and Methods

Uniformly ^{14}C-labeled amino acids of the specific activities shown in Table I were obtained from Amersham International Ltd. *Escherichia coli* tRNA and commercial calf liver tRNA were obtained from Boehringer Mannheim GMBH. Phenol used in the preparation of the remaining tRNAs was Peking brand laboratory grade. Molecular weight standards, bovine pancreatic ribonuclease, bovine liver glutamate dehydrogenase, and rabbit muscle aldolase, were from Sigma Chemical Co. as were adenosine 5′-diphosphate (ADP),[1] PEP, NADH, and lactate dehydrogenase for the assay of pyruvate kinase. PMSF was from Calbiochem and was dissolved in 2-propanol before addition to buffer solutions. DE52 cellulose was obtained from Whatman Ltd., and AcA 34 Ultrogel batch 3064 and AcA 22 were from LKB. PEG 6000 was obtained from Fluka AG and glass microfiber from Gelman.

Table I: Assay Conditions for Beef Liver Aminoacyl-tRNA Synthetases at pH 7.4

amino acid	concn (μM)	sp act. (mCi/mmol)	tRNA	time (min)	temp (°C)
His	1.46	342	yeast	2.5	37
Thr	1.0	100	calf 1	2.5	37
Ser	2.67	75	calf 2	10	37
Asn	2.0	100	calf 2	2.5	37
Trp	35.4	58	yeast	30	37
Ala	13.3	75	*E. coli*	10	37
Gly	20.0	50	calf 1	10	37
Tyr	4.44	225	yeast	30	37
Cys	15.15	47	*E. coli*	30	32
Pro	1.75	285	calf 1	2.5	37
Asp	2.0	100	calf 1	2.5	37
Phe	2.53	225	calf 1	2.5	37
Val	1.6	125	calf 2	2.5	37
Gln	100.0	50	*E. coli*	2.5	37
Glu[a]	8.0	125	commercial calf	30	37
Ile	1.33	150	*E. coli*	2.5	37
Lys	7.4	336	calf 2	2.5	37
Leu	3.3	150	calf 1	2.5	37
Met	3.3	61	calf 2	2.5	37
Arg	7.6	83	*E. coli*	2.5	37

[a] pH 6.7.

Homogenization of Tissue and Partial Purification of Aminoacyl-tRNA Synthetases. Bovine liver obtained from the slaughterhouse within 0.5 h of death was transported on ice, cut free of connective tissue, minced through a commercial mincer, and homogenized with 2 volumes of extraction buffer [Tris (50 mM), $MgCl_2$ (5 mM), glycerol (10%), DTT (1 mM), and PMSF (35 mg in 2 mL of 2-propanol/L of buffer), pH 7.4] at 4 °C by one of the following methods: (1) three passes of a hand-driven Potter-Elvehjem homogenizer; (2) 2 × 15 s in a low-speed, 250-mL glass Waring blender; (3) 3 × 15 s in a low-speed, 500-mL glass Waring blender; (4) 3 × 15 s in a high-speed, 3-L metal Waring blender.

The homogenate from method 1 was centrifuged for 20 min at 3000 rpm (GSA rotor) to remove cell debris, and then the supernatant was centrifuged for 10 min at 9500 rpm to give a predominantly cytoplasmic supernatant and a mitochondrial pellet. The mitochondrial pellet was washed twice in the same buffer by resuspension and recentrifugation. The homogenates for methods 2–4 were centrifuged for 40 min at 9500 rpm to remove cell debris and intact cells and leave a predominantly cytoplasmic fraction. The cytoplasmic fraction was partially purified and concentrated by PEG precipitation, and fractions precipitated by 2–5% and 5–7% PEG were redissolved in a minimum volume of extraction buffer and applied to the gel filtration columns. Alternatively, the cytoplasmic fraction, or a mitochondrial supernatant fraction (prepared from the mitochondria by their resuspension in extraction buffer and further gentle homogenization followed by recentrifugation

[1] Abbreviations: ADP, adenosine 5′-diphosphate; ATP, adenosine 5′-triphosphate; PEP, phosphoenolpyruvate; NADH, nicotinamide adenine dinucleotide, reduced; PMSF, phenylmethanesulfonyl fluoride; DTT, dithiothreitol; PEG, poly(ethylene glycol); Cl_3CCOOH, trichloroacetic acid; POPOP, 1,4-bis[2-(5-phenyloxazolyl)]benzene; Tris, tris(hydroxymethyl)aminomethane.

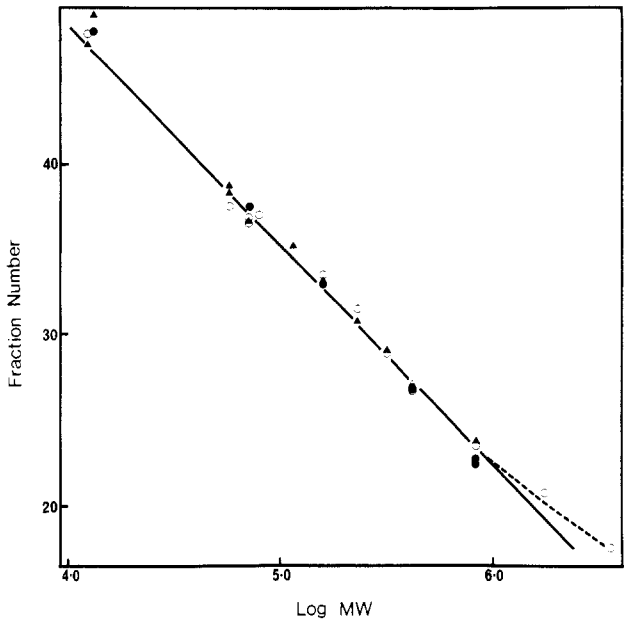

FIGURE 1: Calibration curve for AcA 34 columns (100 × 1.5 cm) equilibrated and eluted at pH 7.4 with (●) potassium phosphate (25 mM), glycerol (10%), and DTT (1 mM) or with Tris (50 mM), $MgCl_2$ (5 mM), glycerol (10%), and DTT (1 mM), without (▲) or with (○) PMSF (35 mg) in 2 mL of 2-propanol/L of buffer. The solid line is a linear least-squares regression fit to all of the points except the two of highest molecular weight. The dashed line is an extrapolation by eye through these last two points.

at 9500 rpm for 40 min), was further purified by chromatography on DEAE-cellulose. The fractions were applied to DE52 cellulose preequilibrated in extraction buffer, the resin was washed until the eluant was colorless, and then the synthetases were eluted with a minimum volume of extraction buffer containing NaCl (0.3 M). This high-salt eluant was further concentrated by filtration through an Amicon PM30 membrane before application to the gel filtration column.

Gel Filtration. AcA 34 resin preequilibrated in extraction buffer with or without PMSF or, alternatively, in potassium phosphate buffer (25 mM) and glycerol (10%), pH 7.4, was poured in columns (100 × 1.5 cm). Fractions of approximately 3 mL, measured by weighing, were collected. The columns were calibrated by the application of ribonuclease, glutamate dehydrogenase (monomer and hexamer), hemocyanin (monomer, hexamer, dodecamer, and 4× and 8× hexamer), and aldolase (subunit and dimer) in 0.5–1.0-mL fractions. Pyruvate kinase was measured as an internal standard by the method of Scopes (1977). Several applications of some standards were used to determine reproducibility of the elution volume, V_E. A plot of V_E vs. log M_r was fitted by an unweighted linear regression of all standards less than or equal to 840 000 in molecular weight (M_r). The line was extended by eye to include the larger standards which do not lie on the linear part of the plot. The calibration curve derived from experiments with three separate columns is shown in Figure 1.

Synthetase Assay. The eluant of the gel filtration columns was assayed individually for all 20 synthetase activities by a modification of the method quoted in Miles Biochemical Co. Catalogue (1979). Assay solution at pH 7.4 (120 μL) containing Tris-HCl (100 mM), magnesium acetate (10 mM), KCl (5 mM), ATP (4 mM), uniformly ^{14}C-labeled amino acids, and tRNA from yeast (4 mg/mL) or calf liver (2 mg/mL), as shown in Table I, and 5–40 μL of each fraction from the AcA column were incubated at the time and temperature shown in Table I. Apart from the variations shown, no further attempts were made to optimize assay conditions. Each reaction was stopped by pipetting 50-μL samples onto a 1.6-cm Whatman 3MM disk (or in the case of tryptophan and glutamine to a glass fiber disk) previously soaked in 10% Cl_3CCOOH and dried. The disks were washed in ice-cold 10% Cl_3CCOOH for 10 min, followed by 3 × 5 min washes with 5% Cl_3CCOOH and 3 × 5 min washes with 90% ethanol. The disks were dried, and the radioactivity was counted in a scintillation vial containing 5 mL of xylene/Triton POPOP scintillant.

Purification of tRNA and Hemocyanin Fractions. Yeast tRNA was prepared from 1 kg of commercial bakers' yeast by the method of Holley et al. (1961). Calf tRNA was prepared from 3.5 kg of calf liver by the method of Deutscher (1967). The tRNA precipitating in 0.54 volume of 2-propanol was called calf $tRNA_1$; that precipitating in 0.99 volume of 2-propanol was called calf $tRNA_2$. Later studies showed that, at least for $tRNA^{His}$, calf $tRNA_1$ contained more of the mitochondrial tRNA. Hemocyanin monomers and hexamers were obtained from *C. destructor* serum by the method of Jeffrey et al. (1976). Gel filtration of 0.5 mL of unfractionated serum from *C. destructor* allowed the determination of elution volumes of hemocyanin hexamer and dodecamer. Larger species of hemocyanin were prepared from serum by the removal of species less than or equal to a molecular weight of 840 000 on AcA 22 followed by concentration using an Amicon PM30 membrane. A molecular weight of 1.76×10^6 was measured for the major species in this preparation by sedimentation equilibrium. The next species observed was tentatively assigned a molecular weight of $(2 \times 1.76 \times 10^6$ by analogy with hemocyanin aggregation patterns in other arthropod species (Schutter et al., 1977).

Results

Effects of Homogenization on the Composition of Complexes from Cytoplasm. Table II shows the percentage of cytoplasmic aminoacyl-tRNA synthetases occurring as large (including intermediate) complex, core complex, or noncomplex activity as a function of severity of homogenization. On this basis, the 20 synthetases can be divided into 4 classes. Class 4 synthetases (Ile, Lys, Leu, Met, Arg, and Val) are seen at most as trace amounts of noncomplexed activity even with the most severe homogenization conditions. These activities appear as large (molecular weight approximately 1.47×10^6) or intermediate (molecular weight approximately 0.82×10^6) complexes with gentle homogenization conditions, or as a core complex [molecular weight $(0.53–0.68) \times 10^6$] with increasing severity of homogenization. The distribution of arginyl-tRNA synthetase, a typical example of this class, is shown as a function of severity of homogenization in Figure 2.[2] Class 3 synthetases (Pro, Asp, Phe, and Glu) show a marked shift from the position of the large complex to the noncomplexed position with increasing severity of homogenization but do show some activity as the intermediate complex under intermediate homogenization conditions. The distribution of

[2] It is unlikely that failure to detect the free form of the six class IV enzymes results from their being lost during DEAE-cellulose concentration. For five of these enzymes approximately 80–90% of the total activity binds to DEAE-cellulose and is thus present in the gel filtration experiment. The sixth, arginyl-tRNA synthetase, exceeds the capacity of the DEAE-cellulose column, but the enzyme failing to bind shows the same low proportion of free enzyme. Of the remaining activities only a small proportion of the total threonine and tyrosine activity binds to the resin, resulting in a probable overestimate of the ratio of complexed to noncomplexed activity in the original homogenate. Most of the remaining activities bind satisfactorily to DEAE-cellulose, and hence ratios shown in Table III probably reflect ratios in the original homogenate.

Table II: Percentage Activity in Complexed and Noncomplexed States of Cytoplasmic Aminoacyl-tRNA Synthetases Concentrated on DEAE-cellulose

Amino Acid	Homogenization	Large and Intermediate Complexes[a]	Core Complex[b]	Non-Complex
His	(1)-(4)	-	-	100
Thr	"	-	-	"
Ser	"	-	-	"
Asn	"	-	-	"
Trp	"	-	-	"
Ala	(1)	5	-	95
	(2)-(4)	-	-	100
Gly	(1)	5	-	95
	(2)-(4)	-	-	100
Tyr	(1)	20	-	80
Cys	(1)	50	-	50
Pro	(1)	70	-	30
	(2)	10	-	90
	(4)	5	-	95
Asp	(1)	75	-	25
	(2)	25	50	25
	(4)	-	50	50
Phe	(1)	90	-	10
	(2)	15	50	35
	(4)	-	20	80
Val	(1)	95	5	-
	(2)	15	65	20
	(4)	-	80	20
Gln	(1)	50	-	50
Glu	(1)	90	-	10
Ile	(1)	100	-	-
	(2)	20	80	-
	(4)	35	65	-
Lys	(1)	100	-	-
	(2)-(4)	30	70	-
Leu	(1)	100	-	-
	(2)	50	50	-
Met	(1)	100	-	-
	(2)-(4)	30	70	-
Arg	(1)	100	-	-
	(2)-(4)	30	70	-

[a] Molecular weight approximately (0.8-1.5) × 10^6. [b] Molecular weights approximately (0.4-0.8) × 10^6.

prolyl-tRNA synthetase, a typical example of this class, is shown as a function of severity of homogenization in Figure 2. Class 2 synthetases (Ala, Gly, Cys, and Tyr) appear only in the large complex or as noncomplexed forms, suggesting that the intermediate complex arises from the large complex by the stripping away of class 2 activities. Similarly, the core complex apparently arises from the intermediate complex by the removal of class 3 activities. Class 1 synthetases (Trp, Asn, Thr, Ser, and His) are not seen as part of the complex under even the most gentle homogenization used but may possibly belong to a supramolecular complex in vivo. In Figure 3, typical profiles for one member of each class of synthetases show the distribution of activities between large, intermediate, and core complexes and noncomplexed positions. Whether the samples were partially purified by PEG precipitation or by passage down a column of DEAE-cellulose made very little difference to the distribution of most of the activities, though it is possible that the PEG procedure produced marginally more disruption of the complexes. Cytoplasmic glutaminyl-tRNA synthetase proved difficult to assay and so is not included.

Occurrence of Mitochondrial Synthetases as Particle-Bound, Complex, or Free Activity. Table III shows the percentage of mitochondrial synthetase activity for 19 of the amino acids occurring in each of 3 fractions after gel filtration. Aspartic acid activity was not detectable in any fraction. Four of the class 1 activities (His, Thr, Ser, and Asn) which were

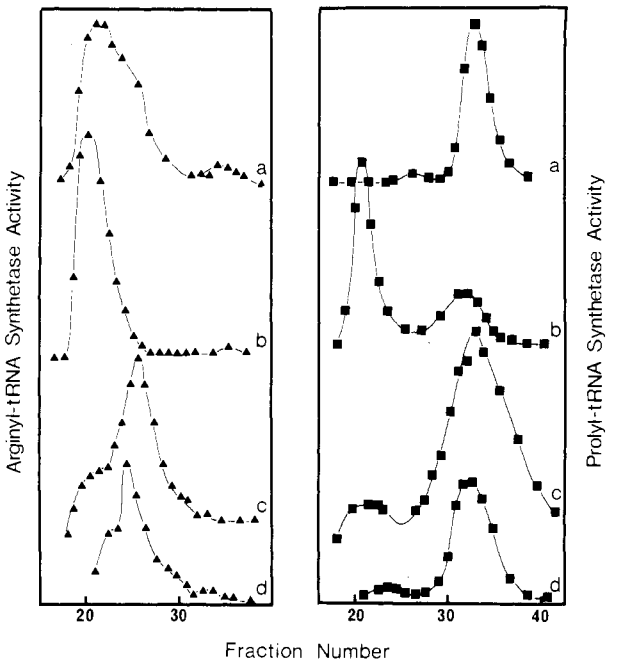

FIGURE 2: Chromatography of (▲) arginyl- and (■) prolyl-tRNA synthetases on an AcA 34 column (100 × 1.5 cm) equilibrated and eluted at pH 7.4 with Tris (50 mM), $MgCl_2$ (5 mM), glycerol (10%), DTT (1 mM), and PMSF (35 mg in 2 mL of 2-propanol/L of buffer). (a) Mitochondrial aminoacyl-tRNA synthetases. (b) Cytoplasmic aminoacyl-tRNA synthetases prepared by homogenization method 1. (c) Cytoplasmic aminoacyl-tRNA synthetases prepared by homogenization method 2. (d) Cytoplasmic aminoacyl-tRNA synthetases prepared by homogenization method 4.

Table III: Percentage Activity of Particle-Bound, Complexed, and Free Mitochondrial Aminoacyl-tRNA Synthetases from Beef Liver Concentrated on DEAE-cellulose

amino acid[a]	particle bound[b]	complexed[c]	free
His			100
Thr			100
Ser			100
Asn			100
Trp	15[d]		85
Ala			100
Gly		25[d]	75
Tyr			100
Cys	55[d]		45
Pro		*d*	100
Phe	100[d]		
Val			100[d]
Gln		40	60
Glu		100	
Ile		100	
Lys		70	30[d]
Leu		70	30[d]
Met		60	40[d]
Arg		95	5

[a] Amino acids arranged in order of increasing tightness of binding of cytoplasmic synthetase activity to complex. [b] Molecular weight approximately 2 × 10^6. [c] Molecular weight approximately (0.8-1.5) × 10^6. [d] Values anomalous with cytoplasmic values.

seen only in the free state in the cytoplasm were similarly noncomplexed in the mitochondrial system. Alanine activity, which occurred in only trace amounts in the cytoplasmic complex, also appeared as totally noncomplexed in the mitochondrial system. Tryptophan activity, the fifth of the class 1 activities, appeared predominantly as free activity in the mitochondria but, in addition, appeared as a species of apparent molecular weight 2.2 × 10^6. This high molecular weight species also contained half the cysteinyl activity and

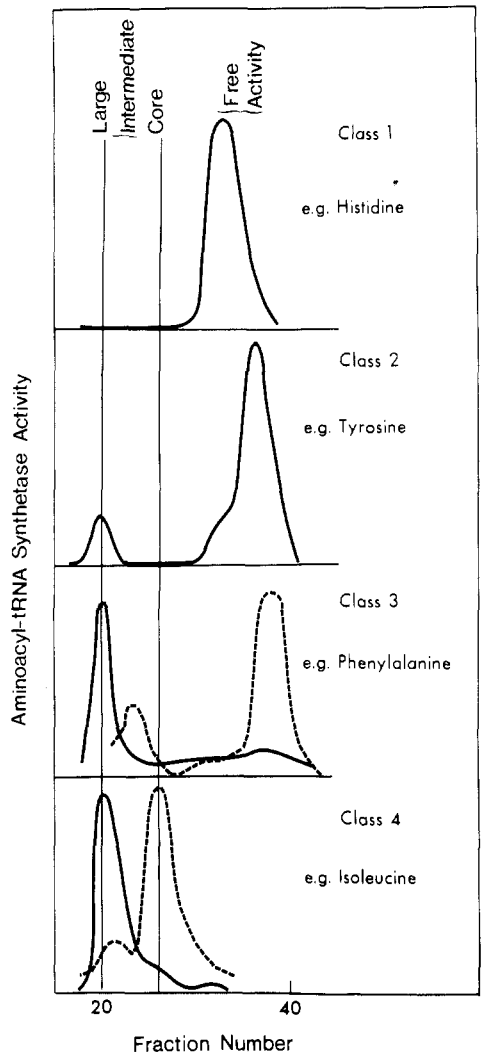

FIGURE 3: Distribution of typical aminoacyl-tRNA synthetases from four classes (as described in the text) between large, intermediate, and core complexes and noncomplexed activity during gel filtration as described in Figure 2. (—) Mild homogenization; (- - -) severe homogenization.

all of the phenylalanyl activity but none of the other synthetases. The molecular weight of this species is greater than that estimated for any complex we have observed, and, since it contains no other synthetase activities, it appears likely to be particle-bound activity rather than a further synthetase complex. The molecular weight is approximately that expected for synthetase activity bound to a 60S ribosomal subunit where phenylalanyl-tRNA synthetase has been postulated to bind (Irvin & Hardesty, 1972).

In the mitochondria, valyl- and prolyl-tRNA synthetases occur entirely as free activity, in contrast to their cytoplasmic activities which are predominantly complexed. In addition, three (Lys, Leu, and Met) out of the five most tightly complexed cytoplasmic activities occur in significant amounts as free activity in the mitochondria. The distribution of the mitochondrial forms of arginyl- and prolyl-tRNA synthetases is shown in Figure 2.

Although slightly more homogenization was required to break open the mitochondria, the proportion of noncomplexed activity seems greater than would be expected from the results with the cytoplasmic system. In addition, although some of the complexed activities occur as a well-defined peak of molecular weight 1.47×10^6, most occur as fairly broad peaks over the complete range of molecular weights shown in the cytoplasm for large, intermediate, and core complexes, suggesting that the mitochondrial complex contains fewer and less tightly bound activities.

Molecular Weights of Cytoplasmic and Mitochondrial Synthetases. Table IV shows the molecular weights of the free synthetases obtained from both cytoplasm and mitochondria. In several cases, where two bracketed numbers are shown, the noncomplexed activity of a particular synthetase could be partially resolved into two peaks, suggesting an association or dissociation of the enzyme. In such cases, we have used the larger molecular weight observed in the calculation of theoretical complex molecular weights for reasons given under Discussion. With the exception of cysteinyl- and valyl-tRNA synthetases, where the cytoplasmic molecular weights appeared unusually large, molecular weights estimated for both cytoplasmic and mitochondrial enzymes for each amino acid were the same within experimental error. Table IV also shows all the molecular weights quoted for aminoacyl-tRNA synthetases in the compilation of Joachimiak & Barciszewski (1980).

Discussion

Figure 1 shows a compilation of data from the calibration of three separate AcA columns eluted respectively in phosphate, Tris–magnesium, and Tris–magnesium–PMSF/2-propanol buffers. The data were fitted with an unweighted linear regression to all points with molecular weights less than or equal to 840000, and the observed V_E values were used to obtain a corresponding calculated molecular weight for each of the standard proteins. For 20 out of the 29 calibration points, the difference between this calculated value and the known molecular weight was less than 10%. For the remaining points, the difference was less than 20%. Our observations of the reproducibility of measurement of the synthetase molecular weights are in good agreement. For the 12 synthetases where replicate measurements were obtained, two-thirds show variations of less than 10% and the remainder variations of less than 20%. This degree of accuracy is consistent with that expected from gel filtration determinations. Of course, since a small number of proteins has been reported to give misleading values of molecular weight due to interaction with gel filtration resins, it is desirable to confirm the values by a different method if possible. Table IV includes such a comparison with all the molecular weights quoted for aminoacyl-tRNA synthetases from a variety of species and tissues in the compilation by Joachimiak & Barciszewski (1980). Comparison of values for mitochondrial and cytoplasmic molecular weights from the present study with mammalian and nonmammalian values previously reported allows several generalizations. First, with the exception of cysteinyl- and valyl-tRNA synthetases, molecular weights for cytoplasmic and mitochondrial synthetases, determined in the present study, are similar. With the cysteinyl enzyme, the mitochondrial value is consistent with that quoted in the literature for nonmammalian cysteinyl-tRNA synthetases, while the cytoplasmic value is similar to that quoted for the enzyme from a mammalian source. The molecular weight measured for the valyl enzyme from beef liver mitochondria reflects several nonmammalian values quoted, but the cytoplasmic value is not similar to valyl-tRNA synthetase values quoted in the literature or to any other aminoacyl synthetases, possibly suggesting an aggregation of smaller components. Second, of the remaining activities, aspartyl-, methionyl-, arginyl-, alanyl-, glycyl-, and tyrosinyl-tRNA synthetases show both cytoplasmic and mitochondrial values from this study lying within the quite narrow range of values quoted in the literature. For trypto-

TABLE IV: Molecular Weights of Free Aminoacyl-tRNA Synthetases from Beef Liver Cytoplasm and Mitochondria and their Comparison with Values in the Literature

	Gel filtration data			Compilation data[a]			
Amino Acid	Subcellular Localization	Molecular Weight (x 10^{-3})	Number of detn.	Source	Molecular Weight (x 10^{-3})	Subunit Structure	Number of detn.
His	Mito	154	(1)	Non-mammalian	80-120	α_2	(3)
	Cyto	153 ± 1%	(3)				
		70[b]	(1)	Mammalian	122	α_2	(1)
		124					
		multiples of 45[c]	(5)				
Thr	Mito	206	(1)	Non-mammalian	117-170	α_2	(6)
	Cyto	195 ± 10%	(5)				
		166[b]		Mammalian	170	α_2	(1)
Ser	Mito	50	(1)	Non-mammalian	88-120	α_2	(7)
		162					
	Cyto	166 ± 8%	(3)	Mammalian	90-122	α_2	(2)
		102[c]	(1)				
Asn	Mito	189	(1)	Non-mammalian	100-127		(2)
	Cyto	175 ± 7%	(4)				
Trp	Mito	91	(1)	Non-mammalian	70-110	α_2	(4)
					200		(1)
	Cyto	91	(1)	Mammalian	108-150	α_2	(5)
		170					
Ala	Mito	162	(1)	Non-mammalian	130-173	α_2	(3)
	Cyto	141 ± 9%	(3)		380	α_4	(1)
Gly	Mito	189	(1)	Non-mammalian	120	α_2	(1)
	Cyto	202 ± 11%	(3)		205-227	$\alpha_2\beta_2$	(3)
		170[b]	(1)				
Tyr	Mito	88	(1)	Non-mammalian	47		(1)
					86-122	α_2	(6)
	Cyto	93 ± 10%	(3)	Mammalian	65		(1)
					118-150		(3)
Cys	Mito	82	(1)	Non-mammalian	61		(1)
					160		(1)
	Cyto	262	(1)	Mammalian	240	α_2	(1)
Pro	Mito	162	(1)	Non-mammalian	94-125	α_2	(2)
	Cyto	155 ± 14%	(4)				
		110[c] ± 2% aggregates	(3)				
Asp	Cyto	117 ± 4%	(3)	Non-mammalian	113-129	α_2	(5)
		57					
Phe	Mito	particle bound	(1)	Non-mammalian	65		(1)
					180-276	$\alpha_2\beta_2$	(11)
	Cyto	60 ± 18%	(3)	Mammalian	130	$\alpha\beta$	(1)
					287	$\alpha_2\beta_2$	(1)
Val	Mito	162	(1)	Non-mammalian	100-170	α	(11)
	Cyto	361 ± 14%	(3)	Mammalian	110		(1)
Gln	Mito	138	(1)	Non-mammalian	69	α	(1)
					126		(1)
Ile	Cyto	162 ± 2%	(2)	Non-mammalian	102-125	α	(5)
Glu				Non-mammalian	42-62	α	(5)
					102	$\alpha\beta$	(1)
	Cyto	111	(1)	Mammalian	180		(1)
Lys	Mito	162	(1)	Non-mammalian	104-150	α_2	(6)
	Cyto	180	(1)	Mammalian	122-129	α_2	(2)
Leu				Non-mammalian	90-128	α	(15)
	Cyto	165	(1)	Mammalian	108		(1)
					182	α_2	(1)
Met	Mito	79	(1)	Non-mammalian	65-110	α	(5)
					153-170	α_2	(4)
	Cyto	82	(1)	Mammalian	78-90	α^2	(3)
Arg	Mito	100	(1)	Non-mammalian	63-85	α	(7)
					140	α_2	(1)
	Cyto	95	(1)	Mammalian	89		(2)

[a] Joachimiak & Barciszewski (1980). [b] Abnormally low molecular weights measured in the presence of PMSF. [c] Abnormally low molecular weights measured in the absence of PMSF.
For gel filtration data, bracketed values were observed on a single elution profile.

phanyl-tRNA synthetase, this is true for the nonmammalian values quoted while the wider range of mammalian values quoted may reflect the association we have observed.

Third, for histidinyl-, threonyl-, seryl-, and prolyl-tRNA synthetases, we observed occasional molecular weight values which were significantly smaller than the range of values normally observed. In several cases (footnote *c* in Table IV), these values were obtained from gel filtration studies run in the absence of PMSF, and, in general, we assume the smaller values to be the molecular weights of proteolytic fragments. Reports in the literature of molecular weights for these four synthetases were more varied but frequently were similar to the lower molecular weight values we observed, suggesting that proteolysis may be a particular problem with these enzymes. In studies with histidinyl-tRNA synthetase, we have shown that it is possible to purify a stable, active form of the enzyme with a molecular weight of 155 000. Kane et al. (1978), however, report purification of this enzyme with a molecular weight of 122 000, a form we have also observed to be relatively stable. In addition, we have prepared active enzyme which appears even smaller than this. There are several reports in the literature of other similar responses to proteolysis. Kisselev & Favorova (1974), for example, in discussing data in the literature for the methionyl-tRNA synthetase for *E. coli* suggest that the enzyme may be described as a dense nucleus resistant to proteolysis surrounded by loose polypeptide susceptible to proteolysis and unimportant for enzymic activity. Similarly, they point out that active lysinyl-tRNA synthetase from yeast can be prepared with a molecular weight of 140 000 in the presence of PMSF and with a molecular weight of 117 000 in the absence of PMSF, while tryptophanyl-tRNA synthetases from beef pancreas have a molecular weight of 108 000–120 000 or 80 000–85 000 when prepared in the presence or absence of PMSF, respectively. For the remaining synthetases (Lys, Asn, Ile, Glu, and Leu), the molecular weights determined in this study for both cytoplasmic and mitochondrial enzymes are generally larger than the wide range of values quoted in the literature, again suggesting that proteolysis may be a marked problem under some conditions.

It is interesting to note that for alanyl-tRNA synthetase, one of the few cases where the primary structure of the polypeptide chain is known (Putney et al., 1981), the monomeric molecular weight of 95 000 obtained is slightly larger than both our estimates (71 000 and 81 000) and the other estimates quoted in the literature for prokaryotes (65 000–86 000), showing again the ability of many of the synthetases to remain active in the presence of severe proteolysis. Mirande et al. (1982b) speculate about the fact that a number of the eukaryotic synthetases contain a polypeptide domain which can readily be removed by proteolysis with no loss of activity or subunit structure, resulting in a protein of similar size and subunit structure to the analogous synthetase in prokaryotes. They claim that the functional significance of this domain is unknown and that it cannot be directly correlated with the ability to form stable, high molecular weight complexes since it also occurs in synthetases which do not occur in stable complexes. If, as the evidence in the present study suggests, such a stable core complex arises during homogenization from a larger complex containing many more of the synthetase activities, such a criticism no longer applies. Rather, the release of proteolytic enzymes during disruption of the cell and the resulting severing of active domains from the inactive domains which once bound them into a complex could well account for the appearance of complexes of decreasing size and number of components during homogenization.

This study provides several further pieces of evidence for the existence of a heterocomplex of at least 16 synthetase activities in eukaryotes. First, an increase in severity of homogenization causes the expected shift in size and composition of the observed complexes, confirming and extending Dang & Yang's (1979) classification of activities into core and peripheral activities. (We observe also a third class of intermediate activities and thus three classes of complexes, containing core, core plus intermediate, or core plus intermediate plus peripheral activities.) Second, the molecular weights measured for the largest complex we observed and the two of intermediate size are consistent with what is expected from their apparent composition. Thus, if the molecular weights are calculated for theoretical complexes which contain one molecule of each of the activities observed in a particular complex, theoretical molecular weights of 1.83×10^6, 1.22×10^6, and 0.85×10^6 are obtained. Where an apparent association–dissociation reaction was observed in the noncomplexed synthetase, we have used the larger molecular weight in the calculation. This is because in the one or two cases where the synthetase released from the complex has an α_2 structure, it appears that this is the form included in the complex, and it is this which gives rise, for example, to two active sites for lysine per complex molecule (Dang & Yang, 1982). Similarly, Mirande et al. (1982a) suggest that the α_2 subunit structure observed for many free synthetases reflects the subunit structure of the same enzyme in the complex.

The theoretical molecular weights of 1.83×10^6, 1.22×10^6, and 0.85×10^6 may be compared with the measured molecular weights of 1.47×10^6, 0.82×10^6, and 0.68×10^6, respectively. The smaller measured molecular weights are consistent with the observed appearance of some of each activity as free enzyme. Obviously, a range of complex molecules, with slightly varying composition, occurs within each class of complex. Whether the order of loss of activities within a particular class is strictly specified requires finer resolution of the intermediate complexes than is presently possible, but further investigation of this region of the molecular weight range should provide valuable information about the arrangement of the components within the heterocomplex. Thus, all the available evidence suggests that the noncomplexed activities arise from a 16-membered complex via several well-defined, intermediate stages. It is possible that an even larger eukaryotic supramolecular complex containing all 20 activities exists in vivo.

Finally, while the mitochondrial synthetases of beef liver may show marked similarities to prokaryotic synthetases and marked differences from the synthetases of eukaryotic cytoplasm, these differences do not reside in the failure of mitochondrial synthetases to form complexes. In both the mitochondria and cytoplasm of eukaryotic cells, our experiments provide strong evidence that most of the aminoacyl-tRNA synthetases occur in a large heterotypic complex.

Registry No. His-tRNA synthetase, 9068-78-4; Thr-tRNA synthetase, 9023-46-5; Ser-tRNA synthetase, 9023-48-7; Asn-tRNA synthetase, 37211-76-0; Trp-tRNA synthetase, 9023-44-3; Ala-tRNA synthetase, 9031-71-4; Gly-tRNA synthetase, 9037-62-1; Tyr-tRNA synthetase, 9023-45-4; Cys-tRNA synthetase, 37318-56-2; Pro-tRNA synthetase, 9055-68-9; Asp-tRNA synthetase, 9027-32-1; Phe-tRNA synthetase, 9055-66-7; Val-tRNA synthetase, 9023-47-6; Gln-tRNA synthetase, 9075-59-6; Ile-tRNA synthetase, 9030-96-0; Glu-tRNA synthetase, 9068-76-2; Lys-tRNA synthetase, 9031-26-9; Leu-tRNA synthetase, 9031-15-6; Met-tRNA synthetase, 9033-22-1; Arg-tRNA synthetase, 37205-35-9; aminoacyl-tRNA synthetase, 9028-02-8.

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Binding of Spin-Labeled Carboxyatractylate to Mitochondrial Adenosine 5′-Diphosphate/Adenosine 5′-Triphosphate Carrier As Studied by Electron Spin Resonance†

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ABSTRACT: The spin-label 2,2,5,5-tetramethyl-1-oxy-3-pyrroline-3-carboxylic acid was attached to the inhibitor carboxyatractylate of the mitochondrial ADP/ATP carrier. Being closely linked to the inhibitor, the spin-label should reflect the mobility of the carboxyatractylate. When bound to the carrier in mitochondria, spin-labeled carboxyatractylate reveals a most unusual hyperfine splitting of 72 G. A second spectral component with a hyperfine splitting of 62 G is also mainly due to carrier-bound inhibitor. A similar spectrum with somewhat reduced hyperfine splitting was observed with the detergent-solubilized protein, whereas reincorporation into phospholipid membranes yielded almost the same spectra as in mitochondria. The carrier-bound spin-label is concluded to be highly immobilized. The less immobilized spectral component is discussed in terms of strongly anisotropic label motion. In addition, the unusual splitting is interpreted to indicate the highly polar environment of the nitroxide. The interpretations are supported by the temperature dependence, which indicates a reversible progressive spin-label mobilization up to 50 °C. Membrane-impermeable reducing agents showed that the spin-label is easily accessible from the aqueous phase.

The ADP/ATP carrier, being the most abundant integral protein of the inner-mitochondrial membrane, should be an interesting example for spin-labeling studies on membrane protein. A highly specific and tightly binding ligand is the inhibitor carboxyatractylate (CAT)[1] (Klingenberg, 1976). A

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[1] Abbreviations: ATR, atractylate; CAT, carboxyatractylate; CATSL, spin-labeled CAT (for structure, see Chart I); ESR, electron spin resonance; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid.

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