A Three-dimensional Working Model of the Multienzyme Complex of Aminoacyl-tRNA Synthetases Based on Electron Microscopic Placements of tRNA and Proteins*

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It has become evident that the process of protein synthesis is performed by many cellular polypeptides acting in concert within the structural confines of protein complexes. In multicellular eukaryotes, one of these assemblies is a multi-enzyme complex composed of eight proteins that have aminoacyl-tRNA synthetase activities as well as three non-synthetase proteins (p43, p38, and p18) with diverse functions. This study uses electron microscopy and three-dimensional reconstruction to explore the arrangement of proteins and tRNA substrates within this “core” multisynthetase complex. Binding of unfractionated tRNA establishes that these molecules are widely distributed on the exterior of the structure. Binding of gold-labeled tRNA places leucyl-tRNA synthetase and the bifunctional glutamyl-/prolyl-tRNA synthetase at the base of this asymmetric “V”-shaped particle. A stable cell line has been produced that incorporates hexahistidine-labeled p43 into the multisynthetase complex. Using a gold-labeled nickel-nitrilotriacetic acid probe, the polypeptides of the p43 dimer have been located along one face of the particle. The results of this and previous studies are combined into an initial three-dimensional working model of the multisynthetase complex. This is the first conceptualization of how the protein constituents and tRNA substrates are arrayed within the structural confines of this multiprotein assembly.

It has been known for many years that a “core” complex of aminoacyl-tRNA synthetases can be isolated from all multicellular eukaryotes (1). Its characteristic composition is three non-synthetase proteins and eight polypeptides having nine aminoacyl-tRNA synthetase activities. With the enzymes abbreviated as the three-letter amino acid name plus RS for the enzyme, the components are ArgRS, AspRS, GluProRS, GlnRS, IleRS, LeuRS, LysRS, MetRS, p43, p38, and p18. Several of these are known dimers, so the total polypeptide count in the multisynthetase complex is at least fifteen.

This is a particularly intriguing protein assembly as all of the enzymes catalyze the same reaction, albeit with individual and highly specific substrates. The reaction catalyzed is the covalent attachment of an amino acid to either the 2’- or 3’-hydroxyl of the 3’-terminal adenosine of tRNA. Recent increased interest in these enzymes has been generated by studies indicating that they have roles in a variety of other cellular processes (2–4). Such activities range from cytokine-mediated chemotraction to priming of reverse transcription of the human immunodeficiency virus, type 1 genome. These enzymes are also viewed as targets in the development of new antimicrobial agents (5). The multisynthetase complex also contains three non-synthetase proteins. The first of these, p18, has been shown to interact with EF-1γ, which may facilitate transfer of aminoacyl-tRNAs to the ribosome (6). Data from yeast two-hybrid screens (7), in vitro binding assays (8), and deletion analysis (9) indicate that p38 functions as a scaffolding protein and appears to be essential for complex assembly. Of particular interest is protein p43, which also appears to have multiple functions. Possibilities include tRNA binding, modulation of aminoacylation activity, a cytokine precursor, as well as providing structural integrity to the multisynthetase complex (10–12).

To understand how its component function within the multisynthetase complex, knowledge of its blueprint is necessary. That is, accurate description of the topology of the complex is necessary for the study of its assembly, disassembly, and, perhaps, the formation of subcomplexes. Placement of the protein components within particular areas of the structure was begun some time ago through use of chaotropic salts to prepare and characterize subcomplexes (13). Then, a number of nearest-neighbor protein interactions within the isolated complex were detected via chemical cross-linking (14). Using an in vivo approach, a number of potential component interactions have been identified by yeast two-hybrid genetic screens (15, 16). Surface plasmon resonance measurements were used to examine interactions of individual proteins from the complex (8). Additional information regarding the approximate location of one of the aminoacyl-tRNA synthetases and of p43 was provided by immunoelectron microscopy (12, 17).

As a result of the above studies, two working models of the distribution of polypeptides within the multisynthetase complex are now extant. The first model is derived from combination of the chemical cross-linking data with two-hybrid analyses (4). The complex is presented as two domains with GlnRS and ArgRS in one section and all of the other enzymes in a second, larger domain. The auxiliary proteins are at the heart of the complex with p38 interactions responsible for connecting the two domains. The second model divides the multisynthetase complex into three domains (14). The supporting data include characterization of sub-complexes, reversible chemical cross-linking experiments, and electron microscopy. The AspRS dimer, MetRS, and GlnRS are placed in one “arm” of the complex. A second arm is composed of the LysRS and ArgRS dimers. These two arms extend from a “base” composed of GluProRS, IleRS, and LeuRS. The auxiliary protein p43 is perceived as a bridge that connects the domains and stabilizes the structure. In this working model, other arrangements of the proteins are possible within the constraints of the data. Although these models are
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good start toward an understanding of the structure and function of the multisynthetase complex, they present only a two-dimensional view of the particle. However, three-dimensional reconstructions of both rabbit and human multisynthetase complex are now available (18). In both, the particle is shaped as an asymmetric “V.” Its most striking features are a deep central cleft and multiple “windows” into the interior. These reconstructions are of sufficient resolution to show distinct protrusions and sub-domains of the particle, which are likely to correspond to individual proteins within the structure.

The work presented herein is a first step toward the goal of extending to three dimensions the placement of proteins and tRNAs within the multisynthetase complex. Such information in turn is fundamental in providing answers to the questions posed above regarding how the multisynthetase complex actually works. This initial approach examines the distribution of tRNA binding sites on the particle and uses this information to position the corresponding aminoaeryl-tRNA synthetase. By use of its cognate tRNA labeled with a gold reporter molecule, the position of the LeuRS active site is placed at a specific location within the three-dimensional structure of the human multisynthetase complex as calculated from electron microscopic images. The positions of the C termini of the p43-His<sub>s</sub> dimer within the particle have been identified by use of a gold-labeled probe designed to interact with the hexahistidine tag. Thus, we now have the first conceptualization of the positions of tRNAs and specific proteins within the context of the “core” multiprotein complex of eukaryotic aminoaeryl-tRNA synthetases. Moreover, this study describes the first incorporation of a tagged protein component into the particle.

MATERIALS AND METHODS

Cell Lines—Human erythroleukemia K562 cells were grown under standard conditions (18). Control human embryonal kidney 293-F cells and those expressing p43-His<sub>s</sub> were maintained in Freestyle<sup>TM</sup> 293 Expression Medium according to the manufacturer’s instructions (Invitrogen).

To create the latter cell line, total cellular RNA was isolated from 293-F cells (Promega SV Total RNA Isolation System<sup>TM</sup>), and the p43 open reading frame was amplified by reverse transcription-PCR (Promega Access RT-PCR System<sup>TM</sup>). The primers used for the amplification were CCCACTGTGCAATGGCAAATAATGAGCTGTCTCTTTGAAAG and CCCACTGGAATCCTTGATGGCTGTCTGTTTG. The gene was inserted into the pEF6/V5-His3 expression vector (Invitrogen) in-frame with the coding sequences for tRNAs and specific proteins within the context of the “core” multiprotein complex of eukaryotic aminoaeryl-tRNA synthetases. Moreover, this study describes the first incorporation of a tagged protein component into the particle.

Isolation of Multisynthetase Complex—For the tRNA binding experiments, the core multisynthetase complex was isolated from K562 cells as previously described (18). For samples used in localization of p43, a new isolation method using lysine-agarose chromatography was developed. This procedure is scaled to the smaller amounts of cellular material used, is rapid, and requires fewer steps. Approximately 1 g (wet weight) of control or transfected 293F cells was suspended in hypotonic lysis buffer containing 10 mM Hepes, pH 7.2, 5 mM β-mercaptoethanol, 1 mM N<sub>e</sub>-p-tosyl-L-arginine methyl ester hydrochloride, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor-EDTA mixture (Roche Applied Science). After gentle Dounce homogenization one-fifth volume of 5 × column buffer (250 mM Hepes, 25 mM Mg Acetate, 2.5 mM EDTA, 5 mM dithiothreitol, 50% glycerol, pH 7.2) was added. The lysate was centrifuged for 10 min at 13,000 × g to remove insoluble cell debris and then loaded onto a 2-ml lysine-Sepharose 4B column (Amersham Biosciences) that had been pre-equilibrated with 1 × column buffer.

The column was successively washed with three column volumes of 25 mM lysine in 1 × column buffer then one column volume each of 1 × column buffer alone, 1 mg/ml yeast tRNA (Sigma) in 1 × column buffer, and 2 mg/ml tRNA in 1 × column buffer. Partially purified multisynthetase complex was then eluted with 10 mg/ml tRNA in 1 × column buffer.

For specific isolation of multisynthetase complex containing p43-His<sub>s</sub>, lysate was loaded onto a 1-ml HIS-Select<sup>TM</sup> nickel affinity column (Sigma) that had been pre-equilibrated with 1 × column buffer. After washing with 3 column volumes of the same buffer, protein was eluted with a gradient of 0 to 200 mM imidazole in column buffer.

Labeling of tRNA—Total yeast tRNA (Sigma) and Escherichia coli tRNA<sup>Es</sup> were commercially prepared (Subriden RNA, Rolling Bay, WA). After evaluation of a variety of methods for tRNA labeling, it was found that modification of the 5′-end could be accomplished with consistency and efficiency while retaining the most specificity of binding to the appropriate polypeptide within the aminoaeryl-tRNA synthetase complex. A typical reaction mixture contained 50 μg of tRNA in 50 μl of 0.1 M imidazole, pH 6, 5, 6 mg of N<sub>S</sub>-(3-dimethylaminopropyl)-N′-ethyldi-carbodiimide hydrochloride, and 6 nmol of monoamino-Nanogold<sup>TM</sup> (Nanopros). After 2 h at room temperature on a rotating wheel, excess labeling reagent was removed by gel filtration, and the tRNA was collected by precipitation at −20 °C with the addition of 0.1 volume of 3 M sodium acetate, pH 5, and 3 volumes of cold 100% ethanol. Extent of labeling was calculated using A<sub>260</sub> for the Nanogold and A<sub>260</sub> for tRNA. Labeling efficiency was routinely 80–90%.

Labeling of Multisynthetase Complex with tRNA—Covalent labeling of multisynthetase complex with total yeast tRNA or gold-labeled E. coli tRNA<sup>Es</sup> was accomplished by use of 5′-end oxidation of the tRNAs and reaction with protein in the presence of mild reductant (19). Briefly, oxidized tRNAs were prepared by incubation in 3 mM sodium periodate for 10 min at room temperature in the dark, collected by ethanol precipitation as above, and stored at −20 °C in 10 mM sodium acetate, pH 5.5, 10 mM magnesium acetate. Reaction mixtures for tRNA linkage to multisynthetase complex contained 1–15 pmol of protein, 15–25 molar excess of oxidized tRNA, 5 μM sodium cyanoborohydride, 5 mM Hepes, pH 7.2, 5% glycerol. Reactions were for 20 min at 30 °C. For analysis by electron microscopy, nonspecific binding was minimized by addition of NaCl to a concentration of 0.1 M and removal of excess tRNA by HPLC<sup>3</sup> gel filtration (BioSep SP<sub>C</sub>5 column, Phenomenex). Derivatized complex was eluted isocratically with 25 mM Hepes, pH 7.2, 100 mM NaCl.

Analytical Methods—SDS-PAGE analysis was on 7.5% gels (20). Proteins and tRNA-protein adducts were visualized using alkaline silver stain (21). The method for immunodetection has been previously described (18). The anti-V5 antibody (Bethyl) was used in dilution ranging from 1:5,000 to 1:10,000. The anti-p43 antibody (US Biological) was generated from a peptide corresponding to the N-terminal region of the p43/EMAPII cleavage site (7) and used in a 1:4,000 dilution. Detection was achieved with the SuperSignal West Pico Chemiluminescent System<sup>TM</sup> (Pierce). For electron microscopy, samples from control and transfected cells were incubated for 15 min with 50 μl of nickel-nitroliacetic acid attached to Nanogold (Ni-NTA-Nanogold<sup>TM</sup>, Nanopros, Inc.), filtered through a 0.45-μm polyvinylidene difluoride membrane.

<sup>3</sup>The abbreviations used are: HPLC, high-performance liquid chromatography; aaRS, aminoaeryl-tRNA synthetase; xxxRS, where xxx is the three letter amino acid code and RS is aminoaeryl-tRNA synthetase; Ni-NTA, nickel-nitrioltriacetic acid.
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(Millipore) and excess labeling reagent removed by HPLC size fractionation. Samples of 300–400 μl were applied to a Biosep-Sec-S 4000 column (Phenomenex) equilibrated with 100 mM NaCl, 25 mM Hepes, pH 7.2. The same buffer was used for isocratic elution of protein at a flow rate of 0.35 ml/min with collection ~100-μl fractions. Absorbance was simultaneously monitored at 260, 280, and 420 nm.

Electron Microscopy—Samples for electron microscopy were negatively stained with methylamine vanadate (NanoVan™, Nanoprobes, Inc.) as previously described (18). Using a LEO 912 microscope at 100 kV with the inline energy filter, micrographs were taken on Kodak SO-163 film with minimum focus at nominal magnification of 50,000 × and an approximate defocus of 2 μm. The negatives were then digitized on an Agfa Duoscan flatbed scanner at an optical resolution that provided 4.01 Å/pixel on the image scale.

Image Analysis—Three-dimensional structures of multisynthetase complexes were calculated using SPIDER software (22). For each of the structures presented herein completely new data sets were obtained except for 3520 images that were previously used for the initial calculation of the structure of the human cytoplasmic multisynthetase complex from K562 cells (18). All data sets were obtained by manual particle selection. The number of images selected per micrograph ranged from ~150 to 250. Actual numbers of images in the initial data sets and those used for the reconstruction calculations are given under “Results” where each is described.

Through the use of a series of SPIDER (22) commands, each reconstruction calculation began with reference-free rotational and translational alignment of the images. The Eulerian orientation angles were then obtained by mapping each image to the appropriate primary reference structure as specified below. That is, the reference was projected at 10° angular intervals, which provided 194 views. Each image was compared with each projection and classified as best fitting a particular projection based on the correlation coefficient. The data set was then limited to those images with acceptable correlation coefficients. The cutoff varied among data sets, but a value near or above 0.8 was typical. An initial three-dimensional structure was then calculated by iterative back projection. Then, a second round of projection mapping was used to refine each initial structure. That is, 194 projections of the new structure were calculated again at 10° angular intervals. The appropriate angular orientations of each image in the data set were again obtained based on highest correlation coefficient with respect to a particular projection. The second round of iterative back projection differed from the first in that the number of images in each class was limited to a consistent number of images with the highest correlation coefficients to prevent any adverse effects of overrepresentation of particular views (23).

Two primary reference structures were used in the first round of projection mapping for the various data sets analyzed in this study. The first was that previously calculated from images of human multisynthetase complex from K562 cells negatively stained with methylamine vanadate (18). This provided the projections to which images were mapped from the data sets with bound total tRNA and with labeled tRNA<sub>Mεa</sub>. The second primary reference was a refinement of the previously published structure (18) accomplished by a 40% increase in the data set using new images of the particle as isolated from 293-F cells. This was used for calculation of structures for complex isolated by the new lysyl-agarose method, those containing p43-His<sub>6</sub>, and with bound Ni-NTA-Nanogold (Nanoprobes, Inc.).

The positions of bound Nanogold-tRNALeu and of bound Ni-NTA-Nanogold were determined by calculation of difference maps between the control and labeled reconstructions. Because the images were of negatively stained samples, this required the counterintuitive subtraction of the labeled structure from the control.

Resolution limits were determined from the 50% point of the Fourier shell correlation between reconstructions calculated from half data sets. Reconstructions are displayed after filtration to the appropriate resolution. The reconstruction surface representation thresholds were determined from the number of voxels corresponding to 100% of the predicted mass of the particle as adjusted for variations in partial specific volume. Specifically, the unlabeled particle mass is 1.2 MDa (1).<sup>a</sup> To account for the gold particle (25) and tRNALeu, the mass value used for the threshold of the reconstruction from the sample labeled with gold-tRNALeu was 1.24 MDa. For that labeled with total yeast tRNA, a value of 1.3 MDa was used, which assumes an average of four bound tRNAs of 25 kDa each. The number of bound tRNAs was estimated from the values in Table One. For the particle containing p43-His<sub>6</sub>, 5 kDa was added due to the extension of p43 and 144 kDa for Ni-NTA-Nanogold. Values of partial specific volumes used were 0.73 for protein, 0.6 for tRNA, and 0.25 for the gold cluster. Presentations of surfaces, projections, and overlays were prepared using the WEB portion of the SPIDER package and Explorer (Numerical Algorithms Group).

RESULTS

Positioning of Multiple Bound tRNAs—As a way to obtain a general idea of how the multisynthetase complex accommodates the simultaneous binding of multiple tRNAs, a reconstruction was calculated after reaction with a mixture of tRNAs. Table One shows the extent of covariant binding of tRNAs to enzymes within the complex after reacting it with 3'-end oxidized total yeast tRNA in the presence of a mild reducing agent. These values were calculated by comparison of the densities of each band of the polypeptide in control and labeled samples after separation by SDS-PAGE. Because approximately three-fourths of Glu-RS, Arg-RS, Ile-RS, Lys-RS, and Met-RS all were >40%. The signal generated in such cases of partial labeling is reduced because of the contribution of unlabeled particles in the data set. Thus, any new areas of density seen in the corresponding reconstruction are a minimal representation of actual tRNA binding sites.

Calculation of three-dimensional reconstructions of both control and labeled multisynthetase complex (Fig. 1, A and B) were from initial data sets of 9,244 and 12,225 images obtained by manual selection.

| TABLE ONE |
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| **Extent of labeling of the multisynthetase complex with oxidized yeast tRNA** |
| Aminoacyl-tRNA synthetase | Percent labeling % |
| Aspartyl | 7 |
| Arginyl and lysyl<sup>a</sup> | 42 |
| Glutamyl/prolyl<sup>b</sup> | 77 |
| Glutaminyl | 28 |
| Isoleucyl | 47 |
| Leucyl | 72 |
| Methionyl | 41 |

<sup>a</sup> These two proteins could not be sufficiently separated via electrophoresis to determine individual levels of labeling.  
<sup>b</sup> This is a bifunctional enzyme containing the two listed activities.
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refined structures were calculated from 8,839 and 11,153 images, respectively. These were the images that best matched each angular projection of the reference as based on highest correlation coefficient. The limits were 50 and 70 images per projection, respectively. As shown below in Fig. 4A, this limit provided complete and even angular coverage. That is, each circle corresponds to one of the 194 reference projections, and the diameter represents the number of images that mapped to that projection. Thus, there should be no artifactual effects from either missing or overrepresented angular information.

From the values in TABLE ONE, it was assumed that an average of four tRNAs was bound and so the visualization threshold for the labeled volume was based on a mass of 100 kDa more than the control. Because protein accounts for the vast majority of the structures, the contribution of tRNA to the partial specific volumes was small. However, this was also taken into account in determining the threshold value. The resolution limits of the volumes were matched by filtering both to that of the labeled structure, which is 29 Å as determined using the Fourier shell correlation coefficient plot (Fig. 4C).

In the labeled structure (Fig. 1A), numerous densities were visible that were not present in that of the control (see Fig. 1B). All were unique to the labeled volume. That is, expansion of the control volume by lowering the visualization threshold did not reproduce the additional densities of the labeled structure. Thus, these new structural features correspond to sites of tRNA binding. The three orientations of the volumes shown were chosen to provide the clearest views of the areas of notable difference between the labeled and control structures. In the “front” view (top row), two areas of tRNA binding were visible (arrows). Area 1 consists of a ~4.5 nm extension from the left of one of the upper domains. Area 2 is a distinct alteration in the shape of the bottom of the base. In the “back” view (middle row), a major new density (area 3) that measures ~5.5 nm extends downward from a protrusion on the second upper domain. Also, a gap of about 3.5 nm in the edge of base in the control volume was filled in the labeled volume (area 4). The “top” view (bottom row) emphasizes the exterior positions of the tRNAs bound to the upper domains (areas 1 and 3). It was also apparent when looking down through the center of the particle in this orientation that the bottom of the cleft is considerably narrower than in the control structure (area 5). This suggests that at least some portion of the tRNAs extend into this portion of the multisynthetase complex.

These data do not provide for exact assignments of specific tRNAs to the new density areas within the labeled volume and the corresponding location of the cognate enzyme. However, by combining the information in TABLE ONE, previous results, and the new data, it is possible to make some reasonable predictions. For example, interactions between LeuRS and GluProRS have been demonstrated by chemical cross-linking (14) and genetic analyses (15, 16). Together with IleRS, these same two enzymes form a distinct sub-complex of the multisynthetase particle (13). In terms of the three-domain model of the multisynthetase complex, electron microscopic studies have placed GluProRS and IleRS within the base of the structure (17). Combination of all of this information makes it logical to place LeuRS in the same section. Thus, based on the greater than 70% labeling of LeuRS and GluProRS, areas 2 and 4 as shown in Fig. 1 were expected to correspond to a combination of the tRNA_Leu, tRNA_Pro, and tRNA_Glu.

By similar reasoning, the combination of cross-linking and protein interaction studies suggests that one upper domain of the structure contains ArgRS and LysRS and so the remaining enzymes are in the other domain. The auxiliary protein p43 has been shown to interact with ArgRS (11) and is near the center of the structure (12). Thus, the general location of tRNA_Asp is likely to be near area 3, which may also include tRNA_Lys. Finally, it is likely that binding of tRNA_Met provided the density seen in area 1.

Locating a Specific Enzyme within the Multisynthetase Complex

Using a Labeled tRNA—Precise localization of the individual proteins within the three-dimensional structure of the multisynthetase complex underlies a complete understanding of the means by which it functions. Toward this goal, we have used tRNA_Leu, which has been labeled on the 5’-end with a 1.4-nm gold particle (Nanogold), as a means to define the precise location of its corresponding enzyme. As demonstrated by SDS-PAGE analysis (Fig. 2), oxidized E. coli tRNA_Leu reacted almost exclusively with LeuRS. That is, the polypeptide corresponding to this enzyme disappeared almost completely (dashed arrow), and a larger species corresponding to the tRNA-protein adduct appeared (solid arrow). It is also apparent that the only nonspecific binding was to the GluProRS enzyme. In this experiment, ~20% of this polypeptide reacted with tRNA_Leu.

The labeling efficiency of LeuRS with oxidized Nanogold-tRNA_Leu was routinely 60–75%. As noted above, the only apparent nonspecific binding was to GluProRS. This was variable and ranged from 20 to 50%. This level of labeling efficiency and specificity is not ideal. How-
ever, coupled with the strong electron scattering of the gold label it appeared sufficient to allow definition of the position of the LeuRS active site within the multisynthetase complex. This was expected even in negatively stained samples because of the use of methylamine vanadate (25).

That this was indeed the case is shown in Fig. 3, where gold particles can be seen associated with the multisynthetase particle after reaction with oxidized Nanogold-tRNALeu (A). The three-dimensional structure of the derivatized complex was calculated as described above except that 9,390 images were used for the initial structure and the data set for the refined structure was limited to the 50 best images at each projection angle, which gave a total of 8,231. A difference map of three-dimensional reconstructions of control and multisynthetase complex labeled with Nanogold-tRNALeu showed two distinct sites of new density. That the information in the difference map is real is indicated by the complete and even angular coverage of the data set used for the reconstruction (Fig. 4 B). The resolution cutoff was again 29 Å, as determined by the Fourier shell correlation coefficient plot (Fig. 4C).

The new densities are best seen (Fig. 1B) when the difference map (orange) is shown as an overlay on the control volume (yellow). Site 1 (solid arrow) is located in the base of the particle and can be seen on the front face (top row). Site 2 (dotted arrow) is best seen on the volume when it is presented in the back view (middle row). This area of new density is also located in the base of the particle and lies within a small depression. From its size and when seen in projections of the reconstruction (data not shown), this second site appears to be composed of two adjacent gold particles. Both sites can be clearly seen in the top view (bottom row), which also demonstrates that the gold-labeled area 1 extends into the bottom of the central cleft.

The significant areas of the difference map were determined by calculating six reconstructions for both labeled and unlabeled samples using randomly selected data sets containing 75% of the total number of images. A Student’s t test was applied using the average and variance of the six reconstructions for each sample to obtain the areas of high significance (26). These coincide with the areas seen in the difference map and emphasize the “doublet” in site 2 (data not shown).

The appearance of two tRNA binding sites is not unexpected in light of the observed nonspecific binding of Nanogold-tRNALeu to GluProRS (Fig. 2). Through combination of the observed labeling efficiencies with the polypeptide composition of the multisynthetase complex, it is possible to determine the relationship of tRNA binding sites to enzyme...
location with reasonable certainty given that the currently accepted stoichiometry of LeuRS and GluProRS within the multisynthetase complex is one copy of each (1). This is routinely the case in our samples (17). However, GluProRS is a bifunctional polypeptide with two tRNA binding sites, which are clearly in close proximity. Moreover, the protein has a domain between the two active sites that has the property of general tRNA binding (27). Thus, two out of the three possible tRNA binding sites within GluProRS were likely to be occupied with sufficient frequency to produce the dual spots comprising site 2 (Fig. 1B). In contrast, LeuRS is a monomer and its labeling by Nanogold-tRNALeu was much higher than that of GluProRS. Thus, it is logical that the single density at site 1 corresponded to the active site of LeuRS.

These observations add to the considerable evidence that both LeuRS and GluProRS are near one another in the multisynthetase complex and that portions of the polypeptides are in the base of the structure. The extent of labeling of these enzymes with a mixture of tRNAs was routinely greater than 70% (TABLE ONE). The results using Nanogold-tRNALeu indicate that the binding sites for these two enzymes are near the bottom of the structure. Taken together, these data suggest that binding to LeuRS is responsible for the narrowing of the bottom of the central cleft seen with binding of unfractionated tRNA (Fig. 1, arrow 5). Similarly, the gap on the back of the structure that is filled by tRNA binding (Fig. 1, arrow 4) likely identifies the position of GluProRS.

Incorporation of p43-His<sub>6</sub> into the Multisynthetase Complex—Turning now to direct placement of one of the non-synthetase polypeptides...
within the multisynthetase complex, we have used a combination of appending a short peptide tag onto p43 and use of a small electron dense reporter to locate the tag within the core multisynthetase complex by electron microscopy. That is, for this study native p43 was replaced with histidine-tagged p43. Ni-NTA–Nanogold, which reacts specifically with the hexahistidine sequence, was used to report the position of tagged protein in electron microscopic images. To replace native protein with tagged p43, a stable cell line was established that expresses a p43–V5-His$_6$ fusion protein having a 5-kDa peptide extension at the C terminus. The 14-amino acid V5 epitope from paramyovirus was incorporated within the extension to facilitate detection of the aminoacyl-tRNA synthetase complex throughout the isolation process.

Immunoblot analysis of lysate from transfected cells indicates that at least 75% of cellular p43 was tagged (Fig. 5A, lanes 1 and 3). It is not known whether the lower protein band in the lysate from transfected cells (lane 3) was endogenous unlabeled p43 or a cleavage product of p43–His$_6$. Immunoblots of protein eluted from a nickel-agarose column showed that the hexahistidine sequence was sufficiently exposed to allow isolation of tagged protein from crude lysate (Fig. 5B, lanes 3 and 4). To demonstrate that p43–His$_6$ is associated with high molecular mass material, eluants from the nickel-agarose column were fractionated by gel filtration HPLC. Immunoblot analysis with anti-V5 antibody demonstrated that tagged p43 elutes with material of ~1 MDa, which was in the fractions that characteristically contained the multisynthetase complex (data not shown).

Isolation of Multisynthetase Complex via a Novel Method and Verification of Normal Ultrastructure—Because the studies reported below each began with much less cellular material than our usual preparations of multisynthetase complex (14), it was of importance to develop an appropriately scaled isolation method that was rapid and required only a few steps. Briefly, lysate from ~1 g of wet weight of control or transfected 293F cells was applied to a 1.5-mL column of lysine-agarose, washed with 25 mL lysine, and then step-eluted with a solution of total yeast tRNA. Material that eluted with 10 mg/mL tRNA contained tagged p43 as detected by immunoblot with anti-V5 antibody. Isolated protein was then fractionated by gel filtration HPLC. SDS-PAGE analysis of fractions that eluted in the position corresponding to a mass of ~1 × 10$^6$ Da exhibited the band pattern characteristic of the multiprotein particle (14). Both control and tagged complex contained additional non-stoichiometric proteins as compared with those from the standard procedure. However, final purification could be done in silico. That is, multisynthetase complex particles were easily identified in negatively stained electron micrographs and could be appropriately selected for calculation of three-dimensional reconstructions.

To ensure that the ultrastructure of multisynthetase complex obtained by this new isolation procedure was the same as that isolated by conventional means its three-dimensional structure was calculated. The data set for the refined structure contained 10,976 images out of a total of 13,232. The limit was the 70 best matches to each of the reference projections. An angular plot indicated complete and even coverage. A resolution limit of 33 Å was determined from the Fourier shell correlation coefficient plot (data not shown).

For appropriate comparison, the structure of sample prepared by conventional means (Fig. 1B) was filtered to match the lower resolution of the sample obtained by the new method. Both reconstructions (data not shown) have the same ultrastructural features. That is, they are the same overall size and have an asymmetric V-shape. Both have similar protrusions from the body of the particle and the characteristic deep central cleft. Thus, the simplified isolation method as applied to small quantities of cells and the lengthy method used for larger amounts of material provide multisynthetase complex that are indistinguishable at this level of resolution.

Placement of p43-His$_6$ within the Three-dimensional Structure of the Multisynthetase Complex—To locate p43–His$_6$, within the three-dimensional structure of the multisynthetase complex it was planned that Nanogold-Ni-NTA would be used as a reporter molecule that bound specifically to the hexahistidine sequence. Thus, it was necessary to confirm that this reagent could bind to the tagged protein within the context of the entire particle. For this purpose, multisynthetase complex containing p43–His$_6$ was prepared using the lysine-agarose procedure and then reacted with the nickel-containing gold cluster. Subsequent fractionation of the mixture by gel filtration HPLC demonstrated colution of Nanogold-Ni-NTA and the multisynthetase complex (Fig. 5C). That is, the characteristic UV absorbance at 420 nm of the gold cluster was seen at 6–7 min. As verified by SDS-PAGE, this corresponds to the elution time of the multisynthetase complex (data not shown). Nanogold-Ni-NTA alone eluted in a peak centered at 12 min, which corresponds to its mass of ~70 kDa. Additional verification of binding of the reporter molecule to p43–His$_6$, within the intact multiprotein particle was provided by electron microscopy (Fig. 3B). The gold particles were consistently superimposed on characteristic images of the multisynthetase complex in electron micrographs.

The location of p43 within the multisynthetase complex was determined from the difference between three-dimensional structures of control and labeled samples. Independent reconstructions were calculated of the particle isolated from non-transfected cells and from cells containing the p43–His$_6$ construct. Before electron microscopic examination, both samples were reacted with Nanogold-Ni-NTA, and excess labeling reagent was removed by gel filtration HPLC.

The refined control structure was calculated from 10,976 out of 13,232 images, with the limit based on the 70 best matches to each reference projection. For the labeled structure, the data set consisted of 8,902 images out of 10,836, with the limit set at the 50 best matches to each reference projection. For both, the angular coverage was complete and even, and the resolution curves were almost identical to each other and to those shown in Fig. 4. The resolution limit of both structures was 32 Å.

An overlay of the difference map between the control reconstruction and that containing bound Nanogold-Ni-NTA showed distinct areas of density change (Fig. 1C). Four areas of intense density difference between the two volumes are seen as discrete oval spots. Spot 1 lies mostly outside the structural envelope at about the midpoint of one “side” of the reconstruction. Spots labeled ‘2’ and ‘3’ lie along the back of the structure in one of the upper domains. A fourth density difference is also mostly outside the normal ultrastructure at the particle’s base. These areas could be locations of the C termini of p43.

The C-terminal domain of p43 is a general tRNA binding domain (7). This suggests that reasonable conclusions about the correspondence of the areas of gold labeling to positions of p43 could be aided by consideration of sites of tRNA binding to the multisynthetase complex as well as results from previous studies. When compared with the reconstruction that contains covalently bound tRNAs (Fig. 1A), all of the putative p43 locations are near areas of tRNA binding. Previous immunoelectron microscopy data (12) rules out spots 1 and 4 as normal sites of p43. However, it is possible that they represent extra copies of the protein due to its overexpression. A similar situation was observed with insertion of an extra copy of methionyl-tRNA synthetase into the complex due to altered growth conditions (28). In contrast, spot 2 in the upper arm of the particle is a location in which the presence of p43 has been predicted by its interactions with other components of the complex.
from the upper domains of the structure. Thus, many different portions of the surface of the structure are used. This would clearly aid in the simultaneous functioning of the multiple enzymes. There is more than enough space to accommodate the substrates for all nine aminoacyl-tRNA synthetases in the particle. Moreover, there appears to be ample free space to accommodate interaction with additional proteins to form larger enzyme assemblies.

The coordination of data regarding the sites of tRNA binding as well as positions of specific aminoacyl-tRNA synthetases and of the auxiliary protein p43 can be maximized by translation of our two-dimensional working model of the multisynthetase complex into three dimensions. This model (Fig. 6A) organizes the nine synthetases and three auxiliary proteins into three domains (14). The 27-Å three-dimensional structure of the multisynthetase complex calculated from negatively stained images is shown in its front view in B (18). Circles corresponding to each of the three domains of the working model are drawn on the structure. This correspondence is based on data from which the original model was derived as well as additional evidence presented in this study, which locates enzymes within the three-dimensional structure using labeled tRNA and p43-His$_{6}$.

Specifically, Fig. 6 (C and D) combines difference maps showing the sites of general tRNA binding (blue) and locations of bound Nanogold-Ni-NTA. These emphasize that p43 is located at or near tRNA binding sites. Crystal structures reveal that the C-terminal or EMAPII (endothelial monocyte-activating polypeptide) domain of p43 forms a truncated dimeric oligonucleotide-binding fold. This serves as a nonspecific RNA binding domain (29, 32), which may function in trans to recruit tRNAs for specific enzyme(s) within the multisynthetase complex. This would result in enhancement of catalytic efficiencies (7, 33). Such an effect on aminoacylation has been observed in yeast with the p43 homolog Arc1p, which forms a ternary complex with MetRS and GluRS (34, 35). It has also been suggested that Arc1p is involved in nuclear export of tRNAs and their sequestration in the cytoplasm (24, 36).

Moreover, this study has shown that the C termini of the p43 dimer are located on the back of the complex in domain II (Fig. 6C). LeuRS and GluProRS have been located in the base of the particle, which is designated as domain III. As emphasized in the side view (Fig. 6D), these enzymes appear to be on opposite faces of the structure. The three-domain model places ArgRS in domain II (Fig. 6D). Evidence that this is appropriate comes from data showing that ArgRS is stabilized within the complex by the N-terminal portion of p43 (10). In addition, it is thought that a major function of complex-associated p43, a known tRNA-binding protein, is to facilitate enzymatic activities within the complex, in particular ArgRS (11). However, when working with individual purified proteins (10) no catalytic enhancement of ArgRS due to association with p43 was seen. Nonetheless, it is logical to position ArgRS in the same general area as p43. Yeast two-hybrid analysis (7) also suggests an interaction of p43 with GlnRS. The three-domain model places this enzyme in domain I. The area spanned by p43 approaches the "boundary" of this region, and so it is possible that the proposed location of GlnRS is correct. That one edge of the p43 dimer does appear to connect two domains also supports the hypothesis that this protein may play a structural role in the multisynthetase complex. Overall, the three-dimensional data available to date are consistent with the domain compositions proposed in the two-dimensional working model.

The data presented in this study are by no means complete. However, a firm conceptual basis in three dimensions is provided in which tRNAs are arranged around the particle exterior and that certain of the acceptor ends lie near or within the central cleft. Internal protein topology is also increasingly defined within specific domains of the particle.
Moreover, portions of the aminoacyl-tRNA synthetases are exposed that can provide space for interaction with other proteins. These can be either the known auxiliary polypeptides within the core particle as well as with additional proteins such as other aminoacyl-tRNA synthetases or intracellular anchors. A clear picture of how these interactions occur awaits placement of additional proteins within the three-dimensional structure of the multisynthetase complex and improvement of precision within high resolution structures. Nonetheless, we now have specific insight into how the multisynthetase particle can function as an "aminoacylation machine."

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