Structural analysis of the multienzyme aminoacyl-tRNA synthetase complex: A three-domain model based on reversible chemical crosslinking

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Abstract

A subset of eukaryotic aminoacyl-tRNA synthetases (a-RS) are contained in a multienzyme complex for which little structural detail is known. Three reversible chemical crosslinking reagents have been used to investigate the arrangement of polypeptides within this particle as isolated from rabbit reticulocytes. Identification of the crosslinked protein pairs was accomplished by two-dimensional SDS diagonal gel electrophoresis. Seventeen neighboring protein pairs have been identified. Eight are seen with at least two reagents: K-RS:p38, D-RS:K-RS, R-RS dimer, K-RS dimer, K-RS:Q-RS, E/P-RS:K-RS, E/P-RS:I-RS, and Q-RS with one of the nonsynthetase proteins. Nine more are observed with one reagent: D-RS dimer, R-RS:p43, D-RS:Q-RS, D-RS:M-RS, K-RS:L-RS, I-RS:R-RS, D-RS:E/P-RS, I-RS:Q-RS, I-RS:L-RS. One trimeric association is seen: E/P-RS:I-RS:L-RS.

The observed neighboring protein pairs suggest that the polypeptides within the aminoacyl-tRNA synthetase complex are distributed in three structural domains of similar mass. These can be arranged in a U-shaped particle in which each "arm" is considered a domain and the third forms the "base" of the structure. The arms have been termed domain I (D-RS, M-RS, Q-RS) and domain II (K-RS, R-RS), with domain III (E/P-RS, I-RS, L-RS) assigned to the base. The smaller proteins (p38, p43) may bridge the domains. This proposed spatial relationship of these domains, as well as their compositions, are consistent with earlier studies. Thus, this study provides an initial three-dimensional working model of the arrangement of polypeptides within the multienzyme aminoacyl-tRNA synthetase complex.

Keywords: aminoacyl-tRNA synthetase complex; multienzyme complex; protein-protein interaction; protein structure; reversible chemical crosslinking

Aminoacyl-tRNA synthetases are a family of enzymes characterized by their role in coupling each amino acid with the correct tRNA for subsequent use in protein biosynthesis. In addition to this fundamental role in translation, these enzymes are widely studied as models for the evolution of modular proteins and for insight into the mechanisms of RNA-protein recognition. These enzymes also have additional cellular functions, such as synthesis of signaling dinucleotides. There are several recent reviews of these topics (Mirande, 1991; Schulman, 1991; Cusack, 1995).

One of the most intriguing and least understood properties of aminoacyl-tRNA synthetases from higher eukaryotes is the existence of a high molecular mass multienzyme complex of a subset

of these proteins (reviewed in Kisselev & Wolfson, 1994; Yang, 1996). As isolated from a variety of sources, this complex contains eight types of individual aminoacyl-tRNA synthetases and one bifunctional polypeptide. These are the enzymes specific for arginine (dimer of 70-kDa polypeptides), aspartic acid (dimer of 56-kDa polypeptides), glutamine (95 kDa), isoleucine (140 kDa), leucine (132 kDa), lysine (dimer of 77-kDa polypeptides), methionine (105 kDa), and the glutamyl/prolyl-tRNA synthetase (160 kDa). Three additional small proteins identified by their apparent masses (p43, p38, p18) are associated with the particle, but their functions have not yet been determined.

Information about the structure of this complex is sparse. The underlying basis of association has been demonstrated for several of the polypeptides to be the hydrophobic nature of extensions unique to the eukaryotic enzymes (reviewed in Mirande, 1991; Yang, 1996). In addition, specific sequence motifs have been identified in synthetase polypeptides and p18 that play a role in protein-protein interaction (Quevillon & Mirande, 1996; Rho et al., 1996). The overall shape and size of the particle are known from electron microscopy studies (Norcum, 1989). However, dissociation of the

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Abbreviations: a-RS, aminoacyl-tRNA synthetase, a is the one-letter amino acid abbreviation; *cis*-DDP, *cis*-dichlorodiammine platinum; EGS, ethylene glycobis(succinimidyldisuccinate); SMPT, 4-succinimidyldi-*o*-xycarbonyl-2-pyridyldithioluene.

particle (Dang & Yang, 1979; Norcum, 1991) into subcomplexes has given only a general idea of the distribution of polypeptides within the particle. Only one specific interaction has been demonstrated: crosslinking of arginyl-tRNA synthetase to p38 (Filonenko & Deutscher, 1994).

This article describes an extensive study of the structure of the multienzyme aminoacyl-tRNA synthetase complex as isolated from rabbit reticulocytes. Three reversible chemical crosslinking reagents have been used to identify neighboring protein pairs. A total of 17 specific crosslinks within the complex has been identified by SDS-diagonal gel electrophoresis. Within the constraints of these protein pairs and of previous data, the constituents of the aminoacyl-tRNA synthetase complex appear to fall into three distinct structural domains. Observed crosslinks between components of different domains also suggest their spatial arrangement. Finally, the positions of individual polypeptides within each section of the particle have been modeled based on intradomain crosslinks.

Results and discussion

Methodology

Nearest neighbor analysis using reversible bifunctional crosslinking reagents has proved useful for analysis of the topological arrangement within a variety of multisubunit proteins and even such highly complex macromolecules as the ribosome (reviewed in Wong, 1991). After reaction of a multiprotein particle with such reagents, crosslinked products can be identified by the appearance of new bands in electrophoretic band patterns. However, when particle components are of similar size, one-dimensional analysis is insufficient, even with immunochemical identification reagents. Thus, crosslinked pairs are typically determined by second-dimension analysis after chemical cleavage of the crosslinking reagent. One type of such analysis is SDS-PAGE in two dimensions (SDS diagonal gel electrophoresis), resulting in spots on vertical lines below a diagonal line of noncrosslinked polypeptides on the second-dimension gel. These spots correspond to the components of the protein pairs that are now migrating to the positions of their individual molecular sizes. These may be identified by comparison to the diagonal line of noncrosslinked protein or to a separate calibration lane (Traut et al., 1989). In this study of the aminoacyl-tRNA synthetase complex, the use of a variety of SDS-PAGE systems coupled with the high sensitivity of alkaline silver stain has allowed such direct visualization of protein spots and the subsequent determination of components within protein pairs.

Three bifunctional cleavable crosslinking reagents have proved of sufficient reactivity and specificity for analysis of neighboring protein pairs within the aminoacyl-tRNA synthetase complex. A classic antitumor agent, *cis*-dichlorodiammineplatinum (II), is also a highly reactive protein-protein crosslinker. Its stable coordination complexes with sulfhydryl groups are reversed with diethyldithiocarbamate (Gonias et al., 1984). The second reagent is ethylene glycobis (succinimidylysuccinate), which reacts with primary amines through *N*-hydroxysuccinimide ester groups. For identification of crosslinked proteins, nucleophilic cleavage of the reagent is performed under mild conditions with hydroxylamine (Abdella et al., 1979). Last, a heterobifunctional reagent has been used. This is 4-succinimidylyl-oxycarbonyl- α -(2-pyridyldithio)toluene, which bridges free amines and sulfhydryls. Crosslink cleavage is accomplished by reduction of the disulfide linkage with dithiothreitol (Thorpe et al., 1987).

One-dimensional analysis

As shown in Figure 1, when each of these crosslinking reagents is reacted with the aminoacyl-tRNA synthetase complex, a number of bands that are not seen in control samples (lane 2 in each panel) are visible in crosslinked samples (lane 3 in each panel). Distinct new crosslinked products are detected from about 80 to 450 kDa (Table 1). Smears of high mass material are also seen, especially after reaction with EGS. This is not unexpected due to the numerous reactive groups, especially lysines, present in most proteins.

Some of the crosslinked products are formed by only one of the reagents. For example, the lowest mass pairs of 78 and 87 kDa are seen only after reaction with EGS. However, other products of very similar size appear in the patterns of two crosslinkers, such as the 290-kDa band observed with both *cis*-DDP and EGS. Similarly, the approximately 260-kDa band is seen with SMPT and EGS. All three crosslinkers generate a band of 110–113 kDa. In the case of *cis*-DDP, there are two bands in this region as well as at 121 kDa, both sets of which are clearly resolved by electrophoresis on gradient gels (data not shown). That similar crosslinked products are formed from three reagents of disparate length and reactivity indicates multiple available side chains within the polypeptides. This in turn implies extensive interfaces between the individual components of the complex.

A first step in identifying the constituents of the crosslinked products is to list all possible protein pairs within the aminoacyl-tRNA synthetase complex. From this list, it is clear that many are of identical or very similar mass. For example, all of the following could be the 110–113-kDa products observed: K-RS:p38, K-RS:p43, R-RS:p38, R-RS:p43, D-RS:D-RS. Moreover, several possible crosslinked products are of masses similar to individual synthetases, and so are likely hidden within the bands corresponding to noncrosslinked polypeptides. Even use of alternative methods of polypeptide identification, e.g., mono-specific antibodies against each of the polypeptides within the particle, would not improve one-

Fig. 1. One-dimensional analysis of aminoacyl-tRNA synthetase complex reacted with either *cis*-DDP, SMPT, or EGS. Each panel shows crosslinked phosphorylase *b* kinase mass standards (lane 1), noncrosslinked control aminoacyl-tRNA synthetase complex (lane 2), and complex after reaction with the indicated crosslinker (lane 3). Mass in kDa is indicated on the left of each panel. On the right of each panel, constituent polypeptides of the aminoacyl-tRNA synthetase complex are labeled as identified previously (reviewed in Yang, 1996) and short bars indicate bands corresponding to crosslinked proteins.

Table 1. Crosslinked polypeptide bands observed in 1D analyses

Estimated M_r of new bands (kDa)	Crosslinker		
	<i>cis</i> -DDP	SMPT	EGS
78			X
87			X
110–113	X (doublet)	X	X
121	X (doublet)		
195–205		X	X
220–230	X		X
245			X
258–265		X	X
290	X		X
340–345	X		X
400			X
460			X

dimensional analysis sufficiently for identification of crosslinked pairs within the aminoacyl-tRNA synthetase complex. Therefore, all determinations of neighboring proteins in this study are from second-dimension electrophoretic analyses.

Crosslinking with *cis*-DDP

Figure 2 shows two crosslinking reactions of aminoacyl-tRNA synthetase complex with *cis*-DDP. Figure 2A and B show digitized images of second-dimension gels after cleavage of crosslinks, whereas Figure 2C and D include data analysis overlays. In each case, the lane on the left of the gel is noncrosslinked aminoacyl-tRNA synthetase complex used as a mass standard. In the center is the diagonal series of unreacted polypeptides within the reaction mixture, which also serve to calibrate the gel. The spots underneath this line represent the components of the crosslinked products that can now be identified from their positions relative to the standard lane. Assignment of a specific neighboring protein pair is based on this information and the estimated mass of the cross-linked product.

In the first experiment, five protein pairs can be identified (Fig. 3A). Line 1 is the densitometric tracing for the appropriate section of the standard lane, which, in this case, spans the region from M-RS through p38. In line 2, three off-diagonal spots appear that are derived from crosslinked products of 110–113 kDa. The corresponding protein pairs are the D-RS dimer (110 kDa) and K-RS:p38 (110 kDa). Lines 3 and 4 represent crosslinked pairs with masses of 125–135 kDa. The density peaks on line 3 indicate a D-RS:K-RS protein pair (127 kDa). Q-RS is also present, but this peak is likely due to streaking of the Q-RS spot that is included in line 4. Similarly, the apparent K-RS, R-RS, and D-RS in line 4 are

Fig. 2. Visualization of protein pairs crosslinked within the aminoacyl-tRNA synthetase complex by *cis*-DDP. **A,B:** Digitized images of second-dimension electrophoretic patterns after cleavage of crosslinks. A lane of noncrosslinked complex serves as mass standard on each gel. **C,D:** Same images overlaid with vertical analysis lines drawn through the standard (lanes 1) and the off-diagonal spots (lanes 2–6 and 2–8 in C and D, respectively). Two separate experiments are shown: I (A,C) and II (B,D).

Fig. 3. Densitometric analysis of crosslinked pairs formed by *cis*-DDP. Individual panels contain densitometric tracings along the corresponding lines shown in Figure 2. Only those peaks representing proteins in identified pairs are labeled. **A:** Experiment I. **B:** Experiment II.

due to spot widths from lines 3 and 5. Despite the strong Q-RS density in line 4, no partner is apparent. Possibilities include p38, p43, or perhaps two copies of p18. This cannot be resolved in this system. In contrast, line 5 is clearly the R-RS dimer (144 kDa). Two protein pairs of approximately 145 kDa are indicated by line 6. These are logically D-RS:Q-RS (149 kDa) and the K-RS dimer (149 kDa).

Analysis of the second *cis*-DDP experiment (Figs. 2C,D, 3B) demonstrates the reproducibility of observed crosslinks, confirms several of the protein pairs deduced from the first gel, and resolves additional ones. Note that, in this case, the starting point of the analysis lines is above the E/P-RS band in the standard lane (line 1), so that the higher-mass protein pairs can be assigned. Lines 2 and 3 again indicate K-RS:p38, D-RS:K-RS, and Q-RS with an unknown partner, so the densitometric tracings are not shown. Line 4 is confirmation of the R-RS dimer, whereas the peaks on line 5 are consistent with the D-RS:Q-RS pair observed in the first experiment. Line 6 indicates a K-RS:Q-RS pair of the appropriate 166-kDa size. In addition, this second experiment better resolves the higher-mass areas and so allows identification of additional protein pairs. From line 7, which corresponds to crosslinked products of approximately

230 kDa, the four components can be paired as I-RS:Q-RS (229 kDa) and E/P-RS:K-RS (232 kDa). Finally, the neighboring location of E/P-RS and I-RS is indicated from the components of the 296-kDa product of line 8.

Crosslinking with SMPT

Similarly, a representative experiment with SMPT is shown in Figure 4. Figure 4A is the digitized image of the second-dimension gel after reversal of crosslinks, and Figure 4B includes the data analysis lines. The pyridyldithio reagent was of limited use because an appreciable level of reducing agent was needed to adequately resolve the complex polypeptides on SDS-PAGE. Thus, most of the crosslinks were reversed during electrophoresis in the first dimension. However, two protein pairs are identifiable from the densitometric tracings (Fig. 4C,D). A strong K-RS off-diagonal spot is again in the 110-kDa area. Although no partner is evident, this would logically be p38, as seen with *cis*-DDP. The densitometric tracing for line 3 clearly shows the D-RS:K-RS pair.

Crosslinking with EGS

Lastly, a representative crosslinking experiment with EGS is shown in Figure 5. The number of off-diagonal spots in the digitized image of the second-dimension gel after cleavage of crosslinks indicates the high reactivity of this reagent. Analysis of crosslinked products reveals several of the same protein pairs seen with the other reagents, as well as suggesting several more neighboring polypeptides.

From the analysis lines (Fig. 5B) and their densitometric tracings (Fig. 6), the off-diagonal spot on line 2 is seen to be derived from 110–115-kDa crosslinked products and spans the positions of K-RS and R-RS. The protein pairs are assigned as K-RS:p38 = 110 kDa and R-RS:p43 = 113 kDa. In this particular experiment, the small proteins have been run off the gel, but these assignments have been confirmed by results obtained with additional crosslinking reagents, either in this study (Table 2) or by other investigators (Filonenko & Deutscher, 1994). Lines 3 and 4 represent pairs in the 135–145-kDa range, as can be seen because the top portion of the lines fall within the portion of the diagonal near I-RS (140 kDa). The first is composed of Q-RS, but again no specific partner can be assigned. The broad peak in the area of K-RS and R-RS is “spill-over” from the large spot in line 4. This is clearly composed of R-RS and K-RS, but they can be paired in any of several ways: the R-RS dimer = 140 kDa, R-RS:K-RS = 142 kDa; the K-RS dimer = 144 kDa. Additional data (Table 2) indicate that the crosslinks are between the polypeptides within each dimer. Line 5 contains spots corresponding to several RS proteins: M-RS, Q-RS, K-RS (or R-RS), and D-RS. These can be appropriately paired based on the approximately 160-kDa size of the crosslinked products as D-RS:M-RS = 159 kDa and Q-RS with either K-RS or R-RS, which would be 166 or 164 kDa. The K-RS:Q-RS assignment is likely correct because this pair was also observed by crosslinking with *cis*-DDP, as described above. Two protein pairs are also suggested by the peaks in line 6. These are K-RS:L-RS = 197 kDa and I-RS:R-RS = 205 kDa, both of which fit with the estimated 200-kDa size. The top of this line has an apparent peak in the area of E/P-RS, but this is also present in the control gel (data not shown) and so is caused by slight streaking of the on-diagonal spot. However, E/P-RS is evident as part of a 215-kDa protein pair in line 7 along with D-RS. Line 8 shows E/P-RS again in a

Fig. 4. Visualization of protein pairs crosslinked within the aminoacyl-tRNA synthetase complex by SMPT. **A:** Digitized image of the second-dimension electrophoretic pattern after cleavage of crosslinks. A lane of noncrosslinked complex serves as mass standard. **B:** Same image overlaid with vertical analysis lines drawn through the standard (lane 1) and off-diagonal spots (2-3). **C,D:** Densitometric tracings along the lines. Only those peaks representing proteins in identified pairs are labeled.

230-kDa pair, but it cannot be decided strictly on this gel whether its partner is R-RS or K-RS. However, the E/P-RS:K-RS pair was observed directly with *cis*-DDP. Clear protein pairs are seen in lines 9 and 10. These are I-RS:L-RS = 260 kDa and E/P-RS:I-RS = 295 kDa, respectively. The last crosslinked product that can be analyzed on this gel is from the approximately 400-kDa region and is seen from the peaks in line 11 to be a trimer of E/P-RS:I-RS:L-RS.

Summary of crosslinked protein pairs

Table 2 summarizes the neighboring protein pairs identified from SDS-diagonal gel analyses of aminoacyl-tRNA synthetase complex crosslinked with *cis*-DDP, SMPT, or EGS. Those components that were visibly detected are shown in bold, whereas those deduced are shown in regular type. Where two polypeptides or a question mark are listed in parentheses, a specific assignment could not be made from these data.

Two of the protein pairs (D-RS:K-RS and E/P-RS:I-RS) were observed directly with two reagents. Five pairs (K-RS:p38, R-RS:R-RS, K-RS:K-RS, K-RS:Q-RS, E/P-RS:K-RS) that were proposed with one reagent were confirmed with a second reagent. Eight pairs (D-RS:D-RS, D-RS:Q-RS, D-RS:M-RS, K-RS:L-RS, I-RS:R-RS, D-RS:E/P-RS, I-RS:Q-RS, I-RS:L-RS) and a trimer (E/P-RS:I-RS:L-RS) were observed directly, but only with one type of crosslinker. One pair is proposed in which the aminoacyl-tRNA synthetase component was observed directly, but the partner was not (R-RS:p43). However, this association was demonstrated previously using one-dimensional analysis of aminoacyl-tRNA synthetase complex crosslinked with disuccinimidyl tartarate (Filonenko & Deutscher, 1994).

The only synthetase protein observed in off-diagonal spots that could not be paired accurately was Q-RS. It is likely that one or more of the small components of the complex (p43, p38, or p18) are the appropriate partner. However, the analysis system used here does not visualize these small proteins adequately. In this regard, although this study is extensive, it by no means represents a comprehensive listing of all possible protein pairs within the complex. Other crosslinking reagents with different biochemical reactivities or spacer lengths are likely to detect additional associations. Likewise, use of an alternative method of protein identification, such as immunoblotting, coupled with an expanded range of separation, should provide information on the distribution of p43, p38, and p18 within the particle. Nonetheless, the data presented here are sufficient to form an initial model of the protein topography of the multienzyme aminoacyl-tRNA synthetase complex.

Domain structure of the aminoacyl-tRNA synthetase complex

As shown in Figure 7, it is possible to arrange the components of the aminoacyl-tRNA synthetase complex in three dimensions in a manner that is consistent with all the crosslinked pairs observed in this study. Each component is represented by a box roughly proportional to its mass and in the generally accepted stoichiometry (Yang, 1996). In order to visualize all of the components, three views are shown. These are related by 90° rotations about a horizontal axis in the plane of the paper. In each view, polypeptides in the foreground are indicated by filled boxes, and those behind are indicated by open boxes enclosed with dotted lines. Crosslinks are marked by short lines of different patterns. For clarity, each identified pair is labeled in only one view.

Fig. 5. Visualization of protein pairs crosslinked within the aminoacyl-tRNA synthetase complex by EGS. **A:** Digitized image of the second-dimension electrophoretic pattern after cleavage of crosslinks. A lane of noncrosslinked complex serves as mass standard. **B:** Same image overlaid with vertical analysis lines drawn through the standard (lane 1) and off-diagonal spots (2-11). **C:** Densitometric tracing of standard lane with positions of each aminoacyl-tRNA synthetase polypeptide identified by the corresponding one-letter amino acid abbreviation.

This arrangement of the components of the aminoacyl-tRNA synthetase complex gives an overall shape to the particle of two "arms" extending from a central "base." Within this model, three domains are readily apparent. As arbitrarily numbered, domains I and II each form one of the arms of the structure and domain III is the base. Based on the crosslinking data reported here, domain I contains the D-RS dimer, M-RS, and Q-RS, whereas the second is composed of the K-RS and R-RS dimers. Both domains contain four synthetase polypeptides and are of similar mass (ca. 300 kDa). The base of the molecule (domain III) contains the three largest polypeptides (E/P-RS, I-RS, and L-RS), for a combined mass of

Fig. 6. Densitometric analysis of crosslinked pairs formed by EGS. Individual panels contain densitometric tracings along the corresponding line shown in Figure 5B. Only those peaks representing proteins in identified pairs are labeled.

430 kDa. The small proteins, p38 and p43, cannot be placed precisely, but symmetry considerations suggest they may bridge the domains. Throughout this study, no specific information regarding the presence of p18 in any of the crosslinks was obtained, so this component is omitted from the diagram.

The reader should note that the model presented in Figure 7 shows only one arrangement of domains and of proteins within the domains that is consistent with the crosslinking data. Other arrangements are possible and much more data from a variety of methods is needed before all components of the complex are definitively located. However, the model presented here not only conforms to the constraints of the crosslinks observed, but is also consistent with previous data concerning the overall shape of the particle and the distribution of proteins within it. Images of both murine and rabbit aminoacyl-tRNA synthetase complex in negatively stained electron micrographs (Norcum, 1989, 1991) are of a "cup" or elongated "U" with a cleft between arms which extend from a base section near one end. With regard to placement of individual synthetases within the complex, a close association of E/P-RS, I-RS, L-RS was indicated by dissociation experiments (Norcum, 1991) in which these three proteins were isolated as a subcomplex after incubation with LiCl, whereas E/P-RS and I-RS were released together by NaSCN. Moreover, interaction of I-RS with E/P-RS has been documented using two-hybrid analysis (Rho

Table 2. Neighboring protein pairs identified from 2D analysis of crosslinked aminoacyl-tRNA synthetase complex

Estimated M_r (kDa)	Crosslinker			Calculated M_r (kDa)
	<i>cis</i> -DDP	SMPT	EGS	
110–115	D-RS:D-RS K-RS:p38	K-RS:p38	K-RS:p38 R-RS:p43	110 110 113
120–130	D-RS:K-RS	D-RS:K-RS		127
135–145	Q-RS:(?) R-RS:R-RS K-RS:K-RS D-RS:Q-RS		Q-RS:(?) R-RS:(R-RS,K-RS) K-RS:(R-RS,K-RS)	140, 142 142, 144 149
145–155			D-RS:M-RS	159
160–170	K-RS:Q-RS		(R-RS,K-RS):Q-RS K-RS:L-RS	164, 166 197
190–210			I-RS:R-RS D-RS:E/P-RS	205 215
215–250	I-RS:Q-RS E/P-RS:K-RS		E/P-RS:(R-RS,K-RS) I-RS:L-RS E/P-RS:I-RS	229 230, 232 260 295
260–300			E/P-RS:I-RS	420
300–450			E/P-RS:I-RS:L-RS	

et al., 1996). This information, coupled with the crosslinks observed here, strongly supports the composition of domain III in the model. In the same dissociation study (Norcum, 1991), D-RS, M-RS, and Q-RS, as well as K-RS, were reported to be tightly associated based on their isolation as a subcomplex of the particle. Interaction of R-RS and K-RS has been observed previously in a variety of studies (reviewed in Yang, 1996). The compositions of

domains I and II are again supported by these data. However, alternative arrangements of these sections of the molecule are clearly possible.

Overall, this study provides additional evidence for the presence of distinct domains within the aminoacyl-tRNA synthetase complex and extends the structural analysis of this particle into three dimensions. That is, the spatial relationships of three structural

Fig. 7. Model of the aminoacyl-tRNA synthetase complex indicating crosslinks observed in this study and the proposed domains. For clarity, each crosslink is indicated in only one view. Complex constituents are drawn with the generally accepted stoichiometry (Yang, 1996) and with box sizes proportional to their masses. In each view, proteins in the foreground have filled boxes, those in the background have open boxes enclosed with dotted lines. Aminoacyl-tRNA synthetases are labeled with the corresponding one-letter amino acid abbreviation. Domains are indicated by boxed areas on the side view.

domains is suggested by interdomain crosslinks. In addition, intradomain crosslinks predict the arrangement of polypeptides within each domain. These data give us the clearest model to date of the protein arrangement within the aminoacyl-tRNA synthetase complex and provide a basis for additional investigations.

Materials and methods

Reagents

Aminoacyl-tRNA synthetase complex was isolated from rabbit reticulocyte lysate (Green Hectares) as described previously (Norcum, 1989), but with two additional steps. Briefly, high molecular mass material in the 30–50% saturated ammonium sulfate precipitate was collected by centrifugation into a sucrose cushion. In order to remove contaminating chaperonin (Norcum, 1996), this was applied to a 2.5×20 cm column of S-Sepharose (Sigma, S1264) equilibrated with 50 mM KCl in column buffer [50 mM Hepes, pH 7.2, 5 mM magnesium acetate, 0.5 mM EDTA, 1 mM dithiothreitol, 10% (v/v) glycerol, 1 mM phenylmethylsulfonyl fluoride, 1 mM *N*- α -tosyl-L-arginine methyl ester]. After washing with one column volume of the same buffer, bound protein was eluted with a linear gradient (300 mL) of 50–500 mM KCl. The fractions eluted from approximately 175–300 mM KCl were pooled for subsequent successive affinity chromatography steps (Norcum, 1991) on heparin (Econo-Pac cartridges, BioRad) and immobilized yeast tRNA (Affi-gel Hz, BioRad). To completely separate copurifying valyl-tRNA synthetase complex, a final ion exchange was performed using HiTrap Q cartridges (Pharmacia) equilibrated with the above buffer containing 75 mM KCl. During a gradient of 75–500 mM, the multienzyme aminoacyl-tRNA synthetase complex eluted as a sharp peak centered at 225 mM KCl. Purified complex was collected by centrifugation and stored in 50 mM Hepes, pH 7.5, 1 mM dithiothreitol, 50% glycerol. If needed, complex was further concentrated by adsorption to 1-mL HiTrap heparin cartridges and elution with 25 mM Hepes, pH 7.2, 300 mM KCl. The integrity of each preparation was confirmed by SDS-PAGE and aminoacylation activities (Norcum, 1991).

Crosslinking reagents were purchased from Pierce Chemical Company (EGS, SMPT) and Sigma Chemical Company (*cis*-DDP). Stock solutions at 100 mM in DMSO were stored at -20°C .

Reversible crosslinking

Aminoacyl-tRNA synthetase complex was typically diluted approximately threefold with 25 mM Hepes, pH 7.4, to give protein concentrations of 0.15–0.3 mg/mL (0.15–0.3 μM). Stock solutions of crosslinking reagents were diluted to 1 mM in DMSO, then further diluted 20-fold into the protein solution. For control reactions, DMSO was substituted for crosslinking reagent. Time courses of crosslinking with each reagent (data not shown) were used to select reaction conditions that maximized generation of distinct crosslinked products while avoiding over-crosslinking of the complex into a nonseparable high molecular mass "blob." All reactions were performed at these low protein concentrations to avoid intermolecular crosslinking (Capaldi et al., 1979). Optimized incubation times and temperatures were: 18 min at 30°C for *cis*-DDP, 60 min on ice for SMPT, 15 min at 30°C for EGS. For *cis*-DDP, crosslinking was stopped by addition of one-fifth volume of denaturing electrophoresis loading buffer (60 mM Tris-HCl,

pH 8, 1% SDS, 250 mM DTT, 10% glycerol, 0.1% bromophenol blue). For SMPT, the same denaturant with only 100 mM DTT was used. For EGS, the reaction was quenched for 15 min on ice in the presence of 2 mM each glycine and cysteine and then samples were denatured as for *cis*-DDP. After electrophoresis in the first dimension, gel slices containing both control and crosslinked samples were treated with cleavage reagents. Specifically, crosslinks formed by *cis*-DDP and SMPT were reversed by reduction with 100 mM diethyldithiocarbamate for 15 min at room temperature and 100 mM DTT for 30 min at room temperature, respectively. Cleavage of EGS was by incubation with 1 M hydroxylamine, pH 8.5, for 2–3 h at 37°C .

Electrophoretic methods

Analysis of crosslinked proteins was by diagonal gel electrophoresis using SDS-PAGE in both dimensions. All gels were run using the mini-PROTEAN II slab format from BioRad. First-dimension electrophoretic separation of crosslinked proteins was on 0.75-mm gels using one of two buffer systems: Tris-acetate (Fairbanks et al., 1971) or Tris-glycine (Laemmli, 1970). Gels with the Fairbanks buffer were 5% acrylamide throughout. Those with the Laemmli buffer contained either a gradient of 3–7% acrylamide or were layered 3% acrylamide stacking and 7.5% acrylamide resolving gels. The various types of gels allowed resolution of crosslinked products of similar sizes as well as providing mass estimates up to about 450 kDa (Hillel & Wu, 1977). Two lanes were loaded with each sample: one-third in a lane to be stained for visualization of crosslinked products, two-thirds in a lane to be subjected to crosslink cleavage reagents as described above. For second-dimension electrophoresis, gel slices were incubated with the appropriate cleavage reagents, washed in stacking buffer (60 mM Tris-HCl, pH 6.8, 0.05% SDS) containing 50 mM DTT, then sealed to 1-mm thick 7.5% Laemmli gels with 1.5% agarose in stacking buffer. Gel slices were placed with the top (i.e., highest mass bands) at the far right of the second-dimension gel, so that apparent masses of crosslinked pairs could be estimated by alignment with the stained first-dimension lanes. Visualization of protein was with alkaline silver stain (Giulian et al., 1983). Each pair of gels was stained simultaneously in the same container to ensure equal color development in both control and crosslinked samples.

Data analysis

Digitized images were obtained by direct scanning of gels with a Hewlett Packard ScanJet 4C flatbed scanner at 300 or 600 dpi. The apparent mass of crosslinked products was determined from first-dimension gels using SigmaGel™ analysis software from Jandel, which calculates the size of proteins in specified bands from a plot of migration distance versus mass. The standards used for curve calibration were crosslinked phosphorylase *b* kinase (Sigma) and the individual aminoacyl-tRNA synthetases in the control samples. Previously crosslinked protein pairs were identified on second-dimension gels using the "line" function of the SigmaGel™ software to draw an exact vertical line through spots below the diagonal. That all analysis lines were drawn precisely vertical and began at the same height was confirmed from pixel location of the cursor at each end. Estimated mass of proposed protein pairs was determined from position of noncrosslinked proteins on the diagonal and by alignment with mass standards on the stained first-dimension gel. Components of protein pairs were named by comparison of

peak positions in the densitometric tracing with those in the line through the standard lane that contained noncrosslinked aminoacyl-tRNA synthetase complex. In all cases, the migration of corresponding polypeptides in the noncrosslinked standard lane, the diagonal of the control gel, and the diagonal of the gel after cross-link cleavage were the same. This controls for any effect on electrophoretic behavior of derivitization with the crosslinkers (Wong, 1991) and indicates that protein pairs can be assigned correctly based on migration. In addition, identification of a protein pair was considered valid only when its calculated mass correlated with the apparent mass and the crosslink was confirmed with a second preparation of complex. In several cases, confirmation was also obtained with a separate crosslinking reagent.

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References

- Abdella PM, Smith PK, Royer GP. 1979. A new cleavable reagent for cross-linking and reversible immobilization of proteins. *Biochem Biophys Res Comm* 87:734-742.
- Capaldi RA, Briggs MM, Smith RJ. 1979. Cleavable bifunctional reagents for studying near neighbor relationships among mitochondrial inner membrane complexes. *Methods Enzymol* 56:630-642.
- Cusack S. 1995. Eleven down and nine to go. *Nature Struct Biol* 2:824-831.
- Dang CV, Yang DCH. 1979. Disassembly and gross structure of particulate aminoacyl-tRNA synthetases from rat liver. *J Biol Chem* 254:5350-5356.
- Fairbanks G, Steck TL, Wallach DF. 1971. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. *Biochemistry* 10:2606-2617.
- Filonenko VV, Deutscher MP. 1994. Evidence for similar structural organization of the multienzyme aminoacyl-tRNA synthetase complex in vivo and in vitro. *J Biol Chem* 269:17375-17378.
- Giulian GG, Moss RL, Greaser M. 1983. Improved methodology for analysis and quantitation of proteins on one-dimensional silver-stained slab gels. *Anal Biochem* 129:277-287.
- Gonias SL, Oakley AC, Walther PJ, Pizzo SV. 1984. Effects of diethyldithiocarbamate and nine other nucleophiles on the intersubunit protein cross-linking and inactivation of purified human α_2 -macroglobulin by cis-diaminedichloroplatinum(II). *Cancer Res* 44:5764-5770.
- Hillel Z, Wu CW. 1977. Subunit topography of RNA polymerase from *Escherichia coli*. A cross-linking study with bifunctional reagents. *Biochemistry* 16:3334-3342.
- Kisselev LL, Wolfson AD. 1994. Aminoacyl-tRNA synthetases from higher eukaryotes. *Prog Nucleic Acids Res Mol Biol* 48:83-142.
- Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680-685.
- Mirande M. 1991. Aminoacyl-tRNA synthetase family from prokaryotes and eukaryotes: Structural domains and their implications. *Prog Nucleic Acids Res Mol Biol* 40:95-142.
- Norcum MT. 1989. Isolation and electron microscopic characterization of the high molecular mass aminoacyl-tRNA synthetase complex from murine erythroleukemia cells. *J Biol Chem* 264:15043-15051.
- Norcum MT. 1991. Structural analysis of the high molecular mass aminoacyl-tRNA synthetase complex—Effect of neutral salts and detergents. *J Biol Chem* 266:15398-15405.
- Norcum MT. 1996. Novel isolation method and structural stability of a eukaryotic chaperonin: The TCP-1 ring complex from rabbit reticulocytes. *Protein Sci* 5:1366-1375.
- Quevillon S, Mirande M. 1996. The p18 component of the multisynthetase complex shares a protein motif with the β and γ subunits of eukaryotic elongation factor 1. *FEBS Lett* 395:63-67.
- Rho SB, Lee KH, Kim JW, Shiba K, Jo YJ, Kim S. 1996. Interaction between human tRNA synthetases involves repeated sequence elements. *Proc Natl Acad Sci USA* 93:10128-10133.
- Schulman LH. 1991. Recognition of tRNAs by aminoacyl-tRNA synthetases. *Prog Nucleic Acids Res Mol Biol* 41:23-87.
- Thorpe PE, Wallace PM, Knowles PP, Relf MG, Brown ANF, Watson GJ, Knyba RE, Wawrzynczak EJ, Blakey DC. 1987. New coupling agents for the synthesis of immunotoxins containing a hindered disulfide bond with improved stability in vivo. *Cancer Res* 47:5924-5931.
- Traut RR, Casiano C, Zecherle N. 1989. Crosslinking of protein subunits and ligands by the introduction of disulphide bonds. In: Creighton TE, ed. *Protein function—A practical approach*. Oxford: IRL Press. pp 101-133.
- Wong SS. 1991. Application of chemical cross-linking to soluble proteins. In: *Chemistry of protein conjugation and cross-linking*. Boca Raton, Florida: CRC Press. pp 221-231.
- Yang DCH. 1996. Mammalian aminoacyl-tRNA synthetases. *Curr Topics Cell Reg* 34:101-136.