Mashimo, S., Iwasa, Y., Chiba, A., & Shinohara, K. (1975) *J. Phys. Soc. Jpn. 39*, 1529.
Maurel, P., Hui Bon Hoa, G., & Douzou, P. (1975) *J. Biol. Chem. 250*, 1376.
Milstein, S., & Cohen, L. A. (1970) *Proc. Natl. Acad. Sci. U.S.A. 67*, 1143.
Page, M. I., & Jencks, W. P. (1970) *Proc. Natl. Acad. Sci. U.S.A. 68*, 1678.
Richards, F. M. (1977) *Annu. Rev. Biophys. Bioeng. 6*, 151.
Simpson, R. T., Riordan, J. F., & Vallee, B. L. (1963) *Biochemistry 2*, 616.
Storm, D. R., & Koshland, D. E., Jr. (1970) *Proc. Natl. Acad. Sci. U.S.A. 66*, 445.
Sturtevant, J. M. (1955) *Discuss. Faraday Soc. No. 20*, 254.
Tanford, C. (1968) *Adv. Protein Chem. 23*, 122.
Warshel, A., & Levitt, M. (1976) *J. Mol. Biol. 103*, 227.
Washburn, E. W., Ed. (1929) *International Critical Tables*, Vol. V, p 12, McGraw-Hill, New York.

Analysis of Protein–Protein Relationships in 30S Ribosome Assembly Intermediates Using Protection from Proteolytic Digestion†

Li-Ming Changchien and Gary R. Craven*

ABSTRACT: Treatment of the intact bacterial ribosome with proteolytic enzymes results in little or no digestion of many of the component proteins [Craven, G. R., & Gupta, V. (1970) *Proc. Natl. Acad. Sci. U.S.A. 67*, 1329]. In contrast, when the proteins are released from the constraints of ribosome structure, they become completely susceptible to proteolytic attack. We have attempted to exploit these observations in an effort to determine the precise steps in ribosome assembly which result in a conversion of the structures of the various proteins from a proteolysis sensitive to a resistant form. Thus, a total of 11 30S ribosome assembly intermediate complexes of proteins and 16S RNA were prepared and digested with trypsin or chymotrypsin. The kinetics of digestion of each protein in the complex were followed by polyacrylamide gel electrophoresis. By a comparison of the digestion pattern of two complexes differing only by the presence of a single protein, it was possible to deduce several specific protective effects of one protein on its neighbor in the complex. On the basis of these studies, we propose nine protein–protein protective effects. The possible relevance of these interrelationships to other well-established proximity relationships is discussed.

The architectural organization of the proteins in the ribosome remains as one of the most difficult unsolved problems in molecular biology. This is not, however, due to lack of effort. Numerous investigators have constructed sophisticated, imaginative, and potentially powerful approaches toward the elucidation of protein–protein distance relationships in the bacterial ribosome. The approaches include the use of bifunctional reagents to covalently cross-link neighboring proteins (e.g., Lutter et al., 1972; Shih & Craven, 1973; Sommer & Traut, 1976; Peretz et al., 1976; Expert-Bezancon et al., 1977), electron microscopy of ribosomes labeled with specific antibodies (Lake, 1977; Tischendorf et al., 1975), energy transfer between pairs of proteins labeled with different fluorescent dyes (Huang et al., 1975), neutron scattering of deuterated protein pairs (Langer et al., 1978), and protection from chemical iodination (Changchien & Craven, 1977). In summary, the combination of these techniques, and others not mentioned here, has produced information about over 80 protein pairs in the 30S ribosome (for reviews, see Changchien & Craven, 1977; Gaffney & Craven, 1978). Unfortunately, despite this impressive catalogue of data, no definitive model of the 30S particle has been presented. Apparently more information is required before a consistent model can be deduced. With this goal in view, we have developed another approach to the determination of protein–protein proximity relationships.

Our approach is to compare the proteolytic sensitivity of various specific ribosomal protein–16S RNA complexes. We have constructed a series of complexes intermediate in the pathway of 30S ribosome assembly. The protein composition of the complexes was selected to allow the direct comparison of two complexes which differed only by the presence of a single protein. This has made it possible to investigate the relative alterations in sensitivity to mild proteolytic digestion induced by the presence of a given protein in a ribonucleoprotein particle. Using either trypsin or chymotrypsin under controlled conditions, we have found that the addition of a new protein to a complex sometimes results in a dramatic change in the susceptibility of one or more other proteins. A number of these effects involve a pair of proteins which have been implicated as close neighbors in the intact 30S ribosome by a variety of independent experiments. We propose that the protection of one protein from enzymatic attack by the presence of a second protein may be due to the physical proximity of the two proteins. We also present arguments defending the hypothesis that proteins situated close together at early stages in ribosome assembly remain in proximity throughout assembly and are found as near neighbors in the final ribosome structure.

Experimental Procedures

Preparation of 30S Ribosomal Subunits and Purification of Ribosomal Proteins. Ribosomes were isolated from *E. coli* MRE 600, and 30S ribosomal subunits were purified from 70S ribosomes by zonal centrifugation as described by Craven & Gupta (1970). Ribosomal proteins were extracted from the purified 30S subunits with 67% acetic acid and further

†From the Laboratory of Molecular Biology and Department of Genetics, University of Wisconsin, Madison, Wisconsin 53706. *Received September 11, 1978; revised manuscript received December 13, 1978.* This work was supported by the Graduate School and the College of Agriculture and Life Sciences, University of Wisconsin—Madison, and by research Grants GM15422 and GM 24109 from the National Institutes of Health.

0006-2960/79/0418-1275$01.00/0

fractionated by phosphocellulose column chromatography (Mannex-P_1, high capacity) following the procedure described by Hardy et al. (1969). The concentration of individual proteins was estimated spectrophotometrically on the assumption that 5 absorbance units at 230 nm correspond to 1 mg of protein per mL. The molecular weights of proteins used for the caculation of molar ratios for the reconstitution of complexes were those determined by sequence analysis and $NaDodSO_4$ gel electrophoresis as summarized in Gaffney & Craven (1978). The purified proteins are designated according to the nomenclature of Wittman et al. (1971).

Preparation of 16S RNA. 16S RNA precipitated by acetic acid during the protein extraction was washed several times with 0.03 M Tris-HCl (pH 8.0), 0.02 M magnesium acetate, until the pH of the wash approached 7.0. The RNA was dissolved in 0.03 M Tris-HCl (pH 7.8) and further deproteinized with an equal volume of redistilled phenol, saturated with 0.015 M sodium citrate (pH 7.0), 0.15 M NaCl. After centrifugation at 10000 rpm for 20 min in a Sorvall SS34 rotor, the aqueous phase was recovered and 16S RNA was precipitated with 2 vol of absolute ethanol (precooled to −20 °C). After 12 h at −20 °C, the precipitated 16S RNA was collected by centrifugation at 10000 rpm for 20 min in a Sorvall SS34 rotor and dissolved in 0.02 M Tris-HCl (pH 7.4). The RNA solution was finally dialyzed against the same Tris-HCl buffer overnight at 4 °C and stored in small portions at −70 °C. The concentration of 16S RNA was estimated spectrophotometrically on the assumption that 1 absorbance unit per mL at 260 nm corresponds to 75 pmol.

Reconstitution Complexes. Reconstitution of 16S RNA–protein complexes was performed by a modification of the method of Held et al. (1973). The purified 30S ribosomal proteins were dialyzed overnight against 0.03 M Tris-HCl (pH 8.0), 8 M urea, 1 mM dithiothreitol and then for 12–16 h against 0.03 M Tris-HCl (pH 7.4), 0.02 M magnesium acetate, 0.5 M KCl, 1 mM dithiothreitol. Routinely 25 A_{260} units of 16S RNA in 2 mL of 0.03 M Tris-HCl (pH 7.4), 0.02 M magnesium acetate, 1 mM dithiothreitol were preincubated at 42 °C for 10 min and then mixed with a twofold molar excess of protein(s) in 4 mL of 0.03 M Tris-HCl (pH 7.4), 0.5 M KCl, 0.02 M magnesium acetate, 1 mM dithiothreitol. The incubation of the reconstitution mixture was continued for 30 min at 42 °C. After cooling the sample to 4 °C, the mixture was centrifuged at 15000 rpm for 10 min to remove any precipitated material. The reconstituted particles were isolated by pelleting through 10 mL of 10% sucrose in reconstitution buffer (0.03 M Tris-HCl (pH 7.4), 0.02 M magnesium acetate, 0.33 M KCl, 1 mM dithiothreitol) for 16 h at 30000 rpm in a Beckman 30 rotor. The reconstituted particles were dissolved in 0.01 M Tris-HCl (pH 7.4), 0.06 M KCl, 0.3 mM magnesium acetate and dialyzed overnight against the appropriate buffer. The presence and amount of protein(s) in the sedimented complex were routinely determined by polyacrylamide gel electrophoresis. The relative amounts of each protein were quantitated as previously described (Changchien & Craven 1976). Only complexes containing roughly stoichiometric amounts of each protein were employed.

Polyacrylamide Gel Electrophoresis. The protein composition of the reconstituted complexes was identified by unidimensional polyacrylamide gel electrophoresis as described by Voynow & Kurland (1971). The samples (2.5 A_{260} units of complexes) in urea were treated with RNase A (Calbiochem Corp.) and electrophoresis was carried out on 6-cm 10% polyacrylamide gels for 6 h at 3 mA/gel. Following electrophoresis, gels were stained for 12 h with 0.02% Coomassie blue in 12.5% trichloroacetic acid and destained in 10% trichloroacetic acid.

Proteolytic Digestion of Protein–RNA Complexes. For kinetic studies of trypsin (essentially free of chymotrypsin, purchased from Miles Laboratories) or chymotrypsin (Worthington Biochemical) digestion, an enzyme to complex molar ratio of 1:10 was used. When complexes containing more than 1 protein were digested, the concentration of enzyme was increased by 5% for each additional protein in the complex. The digestion was conducted at room temperature in reconstitution buffer (see above). At various time intervals, samples of 0.1 mL containing 4 A_{260} units of the 16S RNA–protein complex were withdrawn from the reaction mixture and the digestion was terminated by the addition of an excess of either soybean trypsin inhibitor (Worthington Biochemical) or 2-nitro-4-carboxyphenyl-*N*,*N*-diphenylcarbamate (Sigma), a specific inhibitor of chymotrypsin (Erlanger & Edel, 1964). The terminated reaction mixture was cooled to 4 °C and the complexes were isolated by pelleting the particles through 7 mL of 10% (w/v) sucrose in reconstitution buffer.

Results

Ribosomal proteins detached from the organized network of the 30S ribosome are completely susceptible to digestion by the proteolytic enzymes, trypsin and chymotrypsin. This is illustrated by the experiments shown in Figure 1. In separate experiments, intact 30S ribosomes and extracted total protein (TP_{30}) were subjected to proteolytic digestion for different periods of time. Following termination of the enzyme reactions with the appropriate inhibitor, the undigested protein was extracted and analyzed by polyacrylamide gel electrophoresis. As can be seen, the proteins of the 30S ribosome are remarkably resistant to either trypsin or chymotrypsin attack under the same conditions that TP_{30} is completely digested. Some proteins in the 30S particle are sensitive as has been previously detailed (Craven & Gupta, 1970; Chang & Flaks, 1970; Spitnik-Elson & Breiman, 1971; Crichton & Whittmann, 1971; Rummel & Noller, 1973). We interpret these results to mean that the macromolecular interactions inherent in the structure of the ribosome effectively protect the resistant proteins from proteolytic cleavage. Since the assembly of the 30S ribosome, and hence the generation of these macromolecular interactions, is a stepwise process (Mizushima & Nomura, 1970), it should be possible to identify the specific step in assembly during which an individual protein is converted from trypsin or chymotrypsin sensitive to resistant.

Protein S4 Protects Protein S11 from Chymotrypsin Digestion. The preparation of 16S RNA used in our laboratory is capable of independently and specifically binding a total of 13 30S proteins (Hochkeppel et al., 1976). One of these proteins, not previously observed to bind RNA, is S11. This protein remains completely sensitive to chymotrypsin attack when bound to 16S RNA but becomes resistant by the addition of protein S4 to the complex as shown in Figure 2. In the presence of S4, which itself is partially cleaved under these conditions to yield a slightly smaller fragment (Changchien & Craven, 1976; Newberry et al., 1977), protein S11 is converted into two smaller fragments. The gels in Figure 2 are of protein extracted from the digested complexes, which were purified from digestion products by centrifugation through a layer of 10% sucrose in reconstitution buffer (see Experimental Procedures). Since the two fragments of S11 remain bound to the 16S RNA–S4 complex, they must retain critical portions of the binding site. However, the binding sites

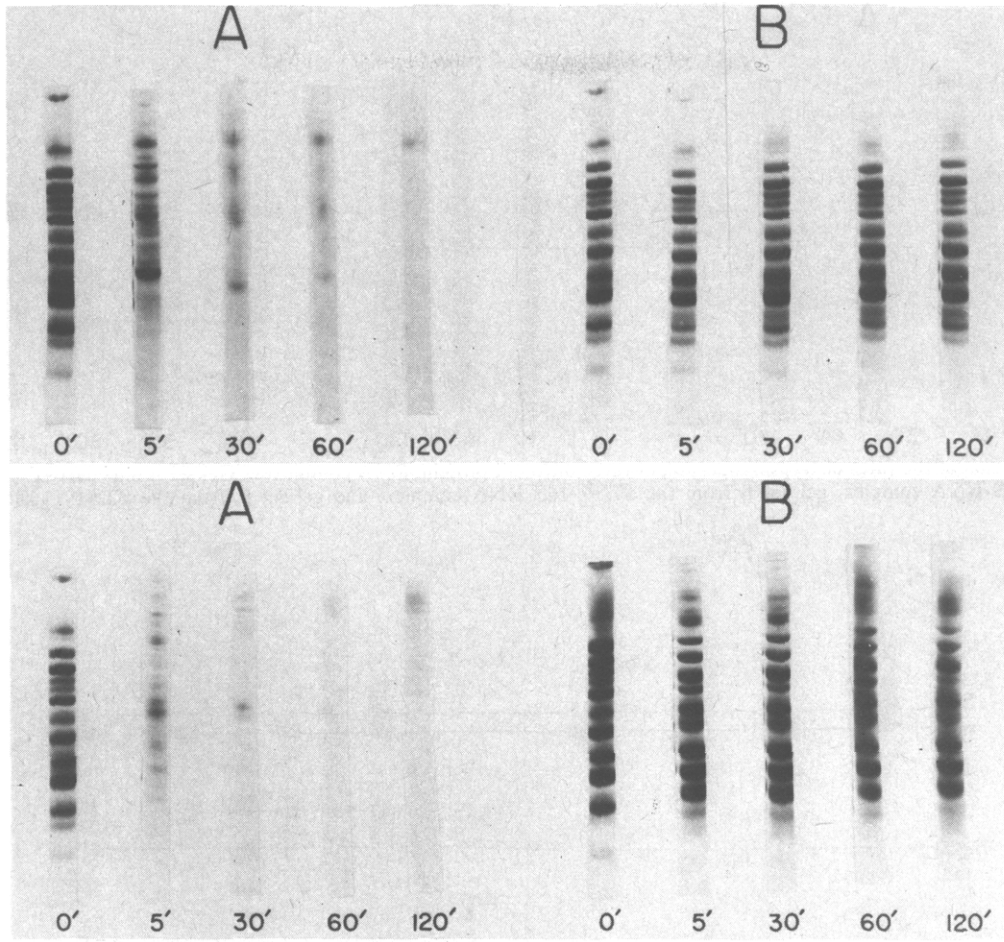

FIGURE 1: Comparison of the rate of digestion of 30S protein in free solution with the rate of digestion of the intact 30S ribosome. Top figure is two sets of polyacrylamide gel electrophoresis patterns obtained after trypsin digestion and the bottom figure is of two similar sets of experiments obtained from chymotrypsin digestion. Gel sets labeled A are of total 30S protein in reconstitution buffer, and the sets labeled B were obtained from intact 30S ribosomes. After the indicated time of digestion, the reactions were terminated by the addition of the appropriate inhibitor.

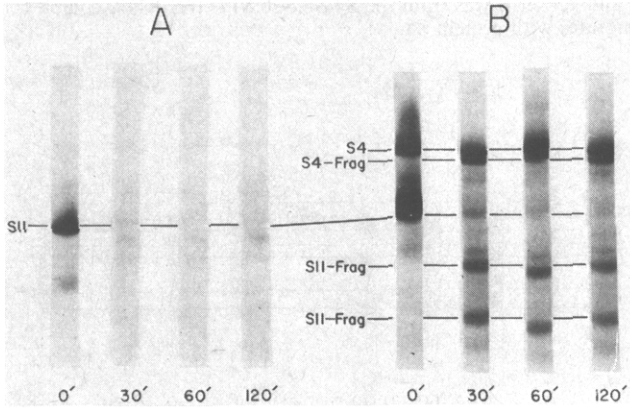

FIGURE 2: Comparison of the rate of chymotrypsin digestion of the protein S11–16S RNA complex with the rate of digestion of the S4,S11–16S RNA complex. Gel set A is that obtained with S11–16S RNA and set B is the product of the S4,S11–16S RNA complex.

of the S11 fragments are apparently rendered unstable by the cleavage as they fail to rebind 16S RNA after dissociation from the complex. This is in contrast to the fragment of S4 which retains the full capacity to associate with RNA (Changchien & Craven, 1976; Newberry et al., 1977). We conclude from these experiments that protein S4 has the capacity to specifically protect protein S11 for chymotrypsin digestion. This is in contrast to trypsin digestion where we find no change in S11 susceptibility in the presence of S4.

Proteins S9 and S19 Independently Protect S7 from Trypsin Digestion. Protein S7 remains totally sensitive to proteolytic attack when specifically bound to 16S RNA (Changchien & Craven, 1976). Both proteins S9 and S19 independently can become associated with the S7–16S RNA complex (Mizushima & Nomura, 1970). When the S7,S9–16S RNA complex was treated with trypsin, the S7 showed a reduced susceptibility to trypsin (gels B, Figure 3). A more dramatic alteration in trypsin sensitivity for protein S7 was seen in the case of the S7,S19–16S RNA complex (gels C, Figure 3). Similar results with these complexes were obtained with chymotrypsin. We suggest that protein S19 completely protects protein S7 from proteolytic digestion and protein S9 affords a partial protection of S7 under these conditions.

Protein S17 Protects Protein S20 from Trypsin Digestion. A complex of 16S RNA and proteins S4, S8, and S20 was prepared and digested with trypsin under standard conditions. In addition a complex of 16S RNA and the same three proteins along with a fourth protein, S17, was made and treated at the same time. The results of the digestion of these two complexes are shown by the gels in Figure 4. The presence of protein S17 in the complex protects protein S20 from trypsin cleavage.

Protein S3 Protects Protein S14 from Trypsin Digestion. Several other complexes were prepared and studied for their sensitivity to trypsin digestion. The polyacrylamide gel patterns reproduced in Figure 5 can be interpreted to mean that protein S3 has a clear capacity to protect protein S14 from trypsin degradation. In this figure a complex of 16S RNA and proteins S4, S7, S10, S9, S14, and S19 is compared for its susceptibility to trypsin with a complex containing proteins

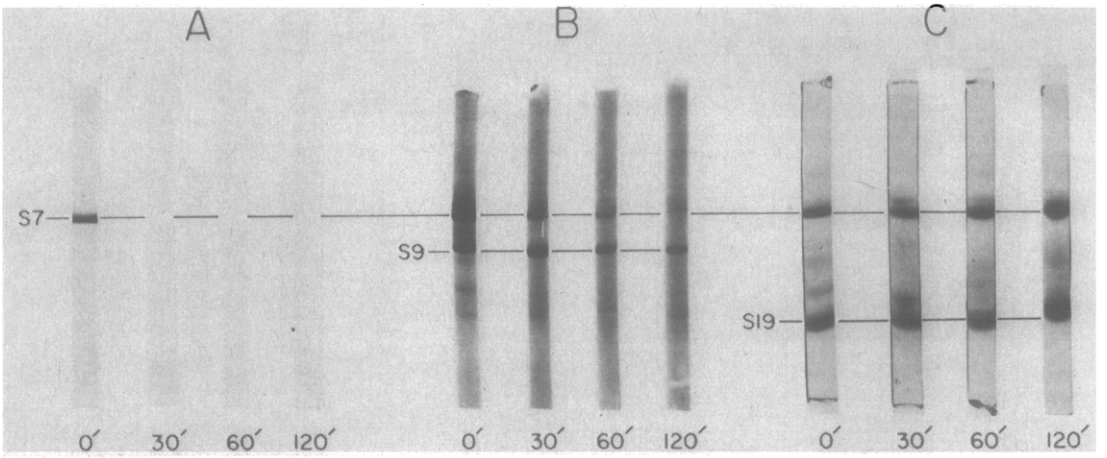

FIGURE 3: Comparison of the rates of trypsin digestion of three different protein–RNA complexes. Gel set A was derived from digestion of the protein S7–16S RNA complex, gel set B from the S7,S9–16S RNA complex, and gel set C from the S7,S19–16S RNA complex.

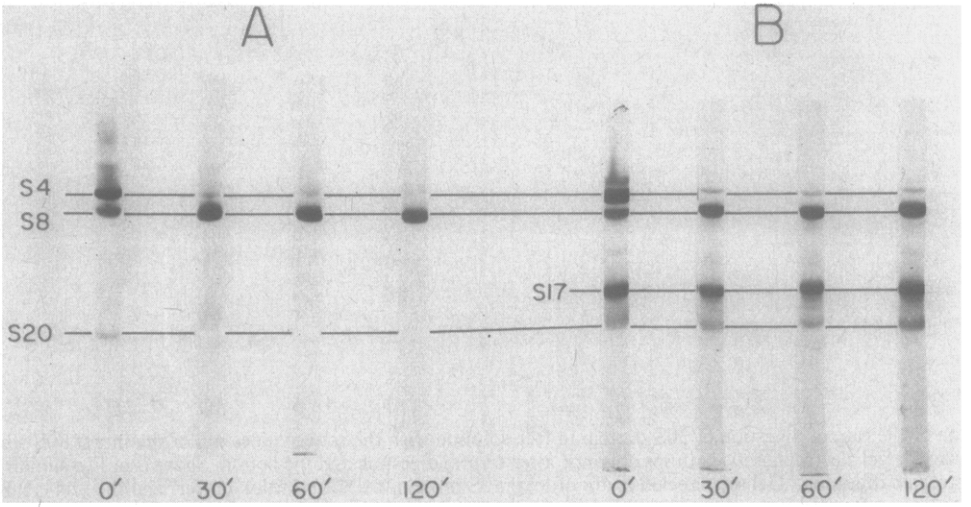

FIGURE 4: Comparison of the rates of trypsin digestion of two protein–RNA complexes differing only in the presence of protein S17. Gel set A was produced by the digestion of the S4,S8,S20–16S RNA complex, and gel set B was from the S4,S8,S20,S17–16S RNA complex. Note that the digestion of S4 yields a slightly smaller fragment which comigrates with protein S8.

S3, S4, S7, S10, S9, S14, and S19. The presence of S3 in the second particle shows essentially complete protection of S14. This effect is seen, although protein S3 itself undergoes a partial cleavage to yield a fragment with an electrophoretic mobility coincident with S4 fragment.

Some Other Possible Protective Effects. We have surveyed a number of other complexes for trypsin sensitivity involving between 7 and 17 component proteins. Due to the number of proteins in the complex, it was generally difficult to identify with certainty any protective effects. However, several tentative alterations in trypsin sensitivity were found and one is illustrated by the polyacrylamide gel analyses reproduced in Figure 6. In this set of experiments, a complex containing proteins S4, S5, S6, S8, S11, S12, S13, S15, S16, S17, S18, and S20 was treated with trypsin and compared with a second particle containing the same series of proteins with the addition of S7. There are several noticeable differences observed in the digestion pattern of this complex when protein S7 is present. Perhaps the most convincing change in trypsin sensitivity is seen in S12/S13.

The presence of protein S7 in the particle appears to have several other protective effects. Protein S11 changes from a conformation in which it is totally susceptible to trypsin attack to one in which it is only very slowly degraded. In addition, as previously observed (Changchien & Craven, 1976), protein S4 is appreciably protected from degradation into the S4 fragment migrating coincident with protein S8. Finally, it is

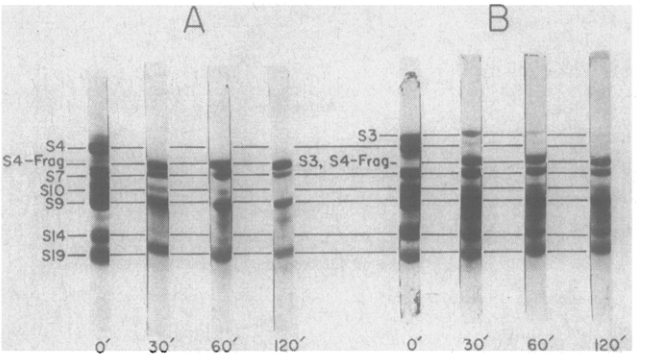

FIGURE 5: Comparison of the rates of trypsin digestion of two protein–RNA complexes differing only in the presence of protein S3. Gel set A was obtained by the digestion of an S4,S7,S9,S10,S14,S19 complex, and gel set B was derived from a complex with the same protein composition with the addition of protein S3.

also possible to observe in Figure 6 that either protein S5 or S6 has become less susceptible to trypsin digestion in the presence of protein S7. This can be seen by the disappearance of one of the two bands denoted as S5/S6 fragments seen in gel A of Figure 6. Unfortunately resolution of the S5/S6 region is poor due to the presence of the S4 fragment which migrates immediately below. However, despite all the complications of these experiments involving so many proteins, we would like to suggest that protein S7 in the particle

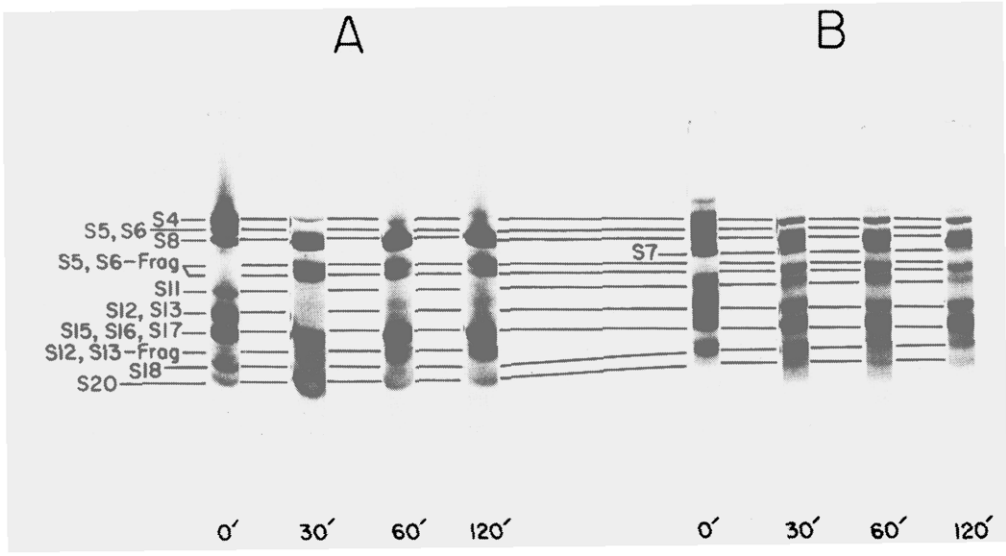

FIGURE 6: Comparison of the rates of trypsin digestion of two protein–RNA complexes differing only in the presence of protein S7. Gel set A involved a 16S RNA complex containing proteins S4, S5, S6, S8, S11, S12, S13, S15, S16, S17, S18, and S20. The gel set labeled B was from a complex containing the same proteins except for the addition of protein S7.

produces a marked change in the trypsin sensitivity of proteins S4, S11, S12, S13, and either protein S5 or S6.

Discussion

A number of independent investigators have attempted to explore the structural intricacies of the bacterial ribosome using proteolytic enzymes as a probe (Craven & Gupta, 1970; Chang & Flaks, 1970; Spitnik-Elson & Breiman, 1971; Crichton & Wittmann, 1971; Rummel & Noller, 1973). The most striking observation in all these experiments is that when members of the compact ribonucleoprotein particle, the ribosomal proteins show differential sensitivities to proteolysis. These distinguishable degrees in accessibility to trypsin were generally interpreted as a reflection of the macromolecular interactions inherent in the organization of the ribosome. More specifically, those proteins which were observed to be least sensitive when exposed to trypsin were thought to be involved in the greatest number of interactions with other proteins and the RNA. The intermolecular associations surrounding a protein were imagined to effectively protect that protein from digestion until a stage in the trypsin attack had been reached which removed those component proteins and/or RNA at which point the protein would become susceptible to the proteolysis. If this simple model accurately describes the effect of proteolytic enzymes on ribosome structure, it should be possible to determine the precise stage during the assembly of the particle at which a given protein is transformed from a proteolytic enzyme-sensitive form to a configuration relatively resistant to proteolytic dergradation.

The results detailed in this communication show that it is indeed feasible to determine precise steps in the pathway of ribosome self-assembly responsible for the conversion of an individual protein from an environment which leaves it accessible to proteolytic attack into one which effectively resists enzymatic cleavage. Thus we have found that the first stage in 30S ribosome assembly, the formation of a specific complex with 16S RNA, inhibits proteolysis of proteins S8, S15, S17, and most of S4. Of the other proteins capable of individually binding 16S RNA, proteins S7, S13, S20, and S11 were observed to be completely exposed to the action of trypsin or chymotrypsin despite being tightly associated with the RNA (Changchien & Craven, 1976). However, we then discovered that three of these proteins, S7, S11, and S20, become proteolysis resistant at early phases in ribosome assembly. Protein S7 is protected from trypsin after the addition of protein S19 or S9. Similarly protein S11 becomes partially resistant when protein S4 is added to the complex. Finally protein S20 is converted to a resistant form after the binding of S17. Protein S13 apparently is not changed into a trypsin resistant configuration until somewhat later in the assembly process. All of the protection effects we have observed are summarized in Table I.

Table I: Summary of Proposed Proteolysis Protection Relationships

	assembly[a]	cross-link[b]	other[c]	EM model[d]
S4→S11	no	no	no	near
S9→S7	yes	yes	yes	near
S19→S7	yes	no	yes	near
S17→S20	no	no	yes	near
S3→S14	no	no	no	near
S7→S12 and/or S13	no/no	no/yes	no/yes	near/near
S7→S11	no	no	no	near
S7→S4	yes	no	yes	near
S7→S5 or S6	no/no	no/no	no/no	near/far

[a] Mizushima & Nomura (1970). [b] See Expert-Bezancon et al. (1977) for summary. [c] Langer et al. (1978); Huang et al. (1975); Changchien & Craven (1977). [d] Stöffler & Wittmann (1977); Gaffney & Craven (1978).

Table I also summarizes protein–protein relationships observed by other authors between the various pairs we have found to be involved in protection from proteolysis. As can be seen, all of our protection effects involve proteins which have been implicated to be situated in close proximity within the intact 30S particle by at least one other method. Most notable is the pair S7 and S9. These two proteins are interdependent in assembly (Mizushima & Nomura, 1970), have been shown to be very close by fluorescence transfer measurements (Huang et al., 1975), have centers of mass approximately 34 Å apart (Langer et al., 1978), reside together on a 3′-proximal fragment of 16S RNA (Morgan & Brimacombe, 1973), are cross-linked by the bifunctional reagent bis(dimethyl suberimidate) (Lutter et al., 1972), and are involved in chemical protection effects (Changchien & Craven, 1977). Furthermore, in both immunoelectron microscopic models of the 30S

particle, sections of S7 and S9 have been found to have antigenic sites very near one another (Lake, 1977; Tischendorf et al., 1975; Gaffney & Craven, 1978). In this case the protection of S7 from trypsin digestion by the presence of S9 most likely is due to the close physical proximity of the two proteins.

Since our investigations involved the simple complex between 16S RNA and the two proteins, we suggest that proteins S7 and S9 are situated near one another from early stages in assembly throughout the complete process of assembly. Indeed, we have found that these two proteins can be cross-linked together with bis(dimethyl suberimidate) present in the two protein RNA complex as well as the final assembled particle (Shih & Craven, manuscript in preparation).

Several other protein pairs showing definitive protection from proteolytic digestion have been found to be close neighbors in the ribosome. Thus the protective relationship between S17 and S20 may well reflect close physical proximity, as fluorescence transfer measurements indicate that these two proteins are extremely close (Huang et al., 1975). Furthermore, the S4–S7, the S9–S7, and the S19–S7 relationships involve proteins which have been found to be interdependent for assembly (Mizushima & Nomura, 1970) and to afford protection for iodination (Changchien & Craven, 1977).

We are tempted to conclude from the summary of results listed in Table I that proteins which protect one another from proteolytic digestion do so because they are located very near one another in the ribonucleoprotein particle. If this hypothesis is correct, then this method of analysis has led to several new protein-protein proximity relationships not found by cross-linking, fluorescence, or neutron scattering.

A remarkable feature of the particles studied in our experiments is that, in general, the proteins are extremely resistant to proteolysis. Even those proteins, which at certain stages in assembly show some sensitivity, often are cleaved into discrete fragments which remain associated with the complex. This observation is in marked contrast to those reported by other workers (Spitnik-Elson & Breiman, 1971; Crichton & Wittmann, 1971). These investigators found that the 30S ribosome can be digested essentially to completion if the trypsin concentration is increased. However, the concentration of trypsin used in our experiments is roughly the same as used by these other workers. This discrepancy could be due to a number of factors. We have found that commencial preparations of trypsin are often substantially contaminated with RNase activity which could cause degradation of the particle. This in turn would increase the accessibility of the proteins to enzymatic digestion. We have taken great care to use trypsin preparations completely free of contaminating RNase.

Secondly, the ionic conditions employed for the enzyme digestion probably play an important role in the stability of the particle. We have used a high ionic strength buffer (reconstitution buffer) throughout our studies which most likely keeps the particles in a highly compact form. These other workers conducted their experiments in somewhat lower ionic strength buffers making it more likely that any destabilization of the particle caused by the enzymatic removal of accessible proteins would result in an "opening up" of the structure, thereby increasing the exposure of many previously resistant proteins.

The fact that we do observe a high degree of resistance to proteolytic attack in all particles we have examined is surprising in light of the known chemical and physical structure of the ribosomal proteins. Most of the proteins contain a substantial number of lysine and arginine residues (Craven et al., 1969) and several have been implicated by physical measurements and immunoelectron microscopy to assume an unusually extended conformation (Lake et al., 1974; Tischendorf & Stöffler, 1975; Gulik et al., 1978; Moore et al., 1977). In addition, chemical modification experiments have shown that a very large number of the lysine ϵ-amino groups are exposed and accessible to reaction with a variety of reagents (Cantrell & Craven, 1977; Chang & Craven, 1977, 1978). All of these considerations taken together would seem to make it highly probable that the ribosome would be extremely susceptible to trypsin digestion. One possible rationalization of these observations is that the trypsin-sensitive groups (i.e., peptide bonds) must be involved in protein–protein and protein–RNA interactions making them in general inaccessible within the compact structure of the ribosome. Since we find a similar resistance to the proteolytic digestion by chymotrypsin, which selects aromatic amino acid side groups, a similar conclusion can be drawn about these amino acids. It would be of interest to examine the sensitivity of the ribosome to a variety of proteases. This might reveal whether this resistance to proteolysis is a characteristic of all regions of the ribosomal proteins or is a special consequence of particular amino acid functional groups.

References

Cantrell, M., & Craven, G. R. (1977) *J. Mol. Biol. 115*, 389.

Chang, F. N., & Flaks, J. G. (1970) *Proc. Natl. Acad. Sci. U.S.A. 67*, 1321.

Chang, C., & Craven, G. R. (1977) *J. Mol. Biol. 117*, 401.

Chang, C., & Craven, G. R. (1978) *Eur. J. Biochem. 88*, 165.

Changchien, L.-M., and Craven, G. R. (1976) *J. Mol. Biol. 108*, 381.

Changchien, L.-M., & Craven, G. R. (1977) *J. Mol. Biol. 113*, 103.

Craven, G. R., & Gupta, V. (1970) *Proc. Natl. Acad. Sci. U.S.A. 63*, 1329.

Craven, G. R., Voynow, P., Hardy, S. J. S., & Kurland, C. G. (1969) *Biochemistry 8*, 2906.

Crichton, R. R., & Wittmann, H. G. (1971) *Mol. Gen. Genet. 114*, 95.

Erlanger, B. F., & Edel, F. (1964) *Biochemistry 3*, 346.

Expert-Bezancon, A., Barritault, D., Milet, M., Guerin, M.-F., & Hayes, D. H. (1977) *J. Mol. Biol. 112*, 603.

Gaffney, P. T., & Craven, G. R. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 3128.

Gulik, A., Freund, A. M., & Vachette, P. (1978) *J. Mol. Biol. 119*, 391.

Hardy, S. J. S., Kurland, C. G., Voynow, P., & Mora, G. (1969) *Biochemistry 8*, 2897.

Held, W. A., Mizushima, S., & Nomura, M. (1973) *J. Biol. Chem. 248*, 5720.

Hochkeppel, H.-K., Spicer, E., & Craven, G. R. (1976) *J. Mol. Biol. 101*, 155.

Huang, K., Fairclough, R. H., & Cantor, C. R. (1975) *J. Mol. Biol. 97*, 443.

Lake, J. A. (1977) *11th FEBS Symp. 43*, 121.

Langer, J. A., Engelman, D. M., & Moore, P. B. (1978) *J. Mol. Biol. 119*, 463.

Lutter, L. C., Zeichardt, H., Kurland, C. G., & Stöffler, G. (1972) *Mol. Gen. Genet. 119*, 357.

Mizushima, S., & Nomura, M. (1970) *Nature (London) 226*, 1214.

Moore, P. B., Langer, J. A., Schoenborn, B. P., & Engelman, D. M. (1977) *J. Mol. Biol. 112*, 199.

Morgan, J., & Brimacombe, R. (1973) *Eur. J. Biochem. 37*, 472.

Newberry, V., Yazuchi, M., & Garrett, R. A. (1977) *Eur. J. Biochem. 76*, 51.
Peretz, H., Towbin, H., & Elson, D. (1976) *Eur. J. Biochem. 63*, 83.
Rummel, D. P., & Noller, H. F. (1973) *Nature (London) 245*, 72.
Shih, C. Y., & Craven, G. R. (1973) *J. Mol. Biol. 78*, 651.
Sommer, A., & Traut, R. R. (1976) *J. Mol. Biol. 106*, 995.
Spitnik-Elson, P., & Breiman, A. (1971) *Biochim. Biophys. Acta 254*, 457.
Stöffler, G., & Wittmann, H. G. (1977) *Molecular Mechanisms of Protein Biosynthesis*, pp 117–202, Academic Press, New York.
Tischendorf, G. W., Zeichardt, H., & Stöffler, G. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 4820.
Voynow, P., & Kurland, C. G. (1971) *Biochemistry 10*, 517.
Wittmann, H. G., Stöffler, G., Hindennach, I., Kurland, C. G., Randall-Haxelbauer, L., Birge, E. A., Nomura, M., Kaltschmidt, E., Mizushima, S., Traut, R. R., & Bickle, T. A. (1971) *Mol. Gen. Genet. 11*, 327.

Accumulation of Polyadenylated mRNA during Liver Regeneration†

Vladimir Atryzek and Nelson Fausto*

ABSTRACT: Cytoplasmic and polysomal polyadenylated mRNA [poly(A)$^+$-mRNA] increased by 120% prior to the onset of DNA synthesis during the regeneration of rat liver following partial hepatectomy. Despite this large change in cytoplasmic mRNA and an approximately 50% increase in total nuclear RNA, the amount of polyadenylated nuclear RNA increased by only 15–20% during this time. Neither the average size of nuclear or of cytoplasmic polyadenylated mRNA nor the length of their poly(adenylic acid) [poly(A)] tracts changed during liver regeneration. Polysomal poly(A)$^+$-mRNA increased proportionately more and at a faster rate than rRNA during the first day following partial hepatectomy. Normal livers contained a substantial proportion of cytoplasmic poly(A)$^+$-mRNA not associated with polysomes but this proportion was not altered in 3-h regenerating liver. Thus, in regenerating liver, most preexisting cytoplasmic mRNA does not appear to be recruited into polysomes prior to the substantial increase in the amount of cytoplasmic poly(A)$^+$-mRNA.

The regenerative response of the liver following removal of two-thirds of its mass provides a suitable in vivo system for studying the stimulation of cell replication. The early stages of this response in rats consist of a hypertrophic phase, lasting approximately 12–16 h, during which the rate of protein synthesis rises, and a subsequent hyperplastic phase which is characterized by a peak in DNA synthesis at about 24 h followed 6–8 h later by mitosis (reviewed by Bucher & Malt, 1971).

Qualitative and quantitative alterations in mRNA populations may be expected to be important determinants of the type and amounts of protein synthesized during liver regeneration. The complexity of nuclear and cytoplasmic mRNA at the early stages of liver regeneration as well as the homology between RNA populations from normal and regenerating liver have been determined by molecular hybridization methods (Colbert et al., 1977; Tedeschi et al., 1978). However, these methods do not provide a direct measurement of the total amounts of liver mRNA. Although the rate of synthesis of mRNA is probably increased very shortly after partial hepatectomy (Glazer, 1976, 1977), little is known about the actual amounts of mRNA present in regenerating livers, though previous studies suggest that they may be increased (Greene & Fausto, 1974). Therefore, we determined the absolute amounts of poly(A)$^+$-mRNA[1] present in various subcellular fractions of the liver at different stages of hepatic regeneration following partial hepatectomy. For these estimations it was necessary to determine the poly(A) content, the size of the poly(adenylic acid) tracts present in RNA preparations, as well as the average length of poly(A)$^+$-mRNA molecules in normal and regenerating liver.

Materials and Methods

Materials. Ribonuclease-free deoxyribonuclease was treated with iodoacetate (Zimmerman & Sandeen, 1966) prior to use. Bentonite was prepared according to the method of Brownhill et al. (1959). Solutions and plastic ware were sterilized by autoclaving. All glassware was siliconized (1% dichlorodimethylsilane in toluene) and kept at 300 °C for at least 6 h.

Animals. Three to eight male rats (Holtzmann; Charles River Breeding Labs.), weighing 120–160 g, were used for each RNA preparation. The animals were kept in a temperature-controlled room under alternating 12-h light–dark cycles.

Surgical procedures were performed under continuous ether–oxygen anesthesia, which is known not to affect hepatic levels of nucleoside triphosphates or of cyclic nucleotides (Bucher & Swaffield, 1966; Fausto & Butcher, 1976). Control animals were sham-operated; i.e., they were laparotomized and their livers manipulated but not removed. Partial hepatectomy, resulting in the removal of 70% of the liver mass, was done according to the method of Higgins & Anderson (1931). All

† From the Division of Biology and Medicine, Brown University, Providence, Rhode Island 02912. *Received August 22, 1978; revised manuscript received December 18, 1978.* This work was supported by U.S. Public Health Service Grant CA-23226 from the National Cancer Institute.

[1] Abbreviations used: poly(A), poly(adenylic acid); poly(A)$^+$-mRNA, polyadenylated mRNA; poly(U), poly(uridylic acid); rRNA, ribosomal RNA; HnRNA, heterodisperse, high molecular weight nuclear RNA; SET, 10% sodium dodecyl sulfate, 50 mM EDTA, 250 mM Tris-HCl, pH 7.4; $NaDodSO_4$, sodium dodecyl sulfate; TNMDH, 50 mM Tris-HCl, pH 7.4, 25 mM NaCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 100 μg/mL sodium heparin.

0006-2960/79/0418-1281$01.00/0