Assessment by Sedimentation Equilibrium Analysis of a Heterologous Macromolecular Interaction in the Presence of Self-Association: Interaction of S5 with S8†

Stephen H. Tindall and Kirk C. Aune*

ABSTRACT: The proteins S5 and S8 from the *Escherichia coli* 30S ribosomal subunit have been examined by sedimentation equilibrium methods for behavior in solution as isolated components and in mixtures. The means of resolving two simultaneous associations in this system is discussed, and the energy of association of S5 and S8 is reported. It was found that protein S5 from the MRE 600 strain tends to self-associate weakly at 4 °C in a manner that can be described as an isodesmic self-association with an association constant and corresponding standard Gibbs free energy equal to (7.7 ± 0.7) × 10^3 M^{-1} and −4.9 ± 0.1 kcal/mol, respectively. Protein S8 was found to have a molecular weight of 15 800 and was monomeric in a pure state. Mixtures of S5 and S8 clearly demonstrated the presence of an S5–S8 complex in addition to the self-association of S5. The equilibrium constant of association for the formation of a simple S5–S8 complex at 4 °C and the corresponding standard Gibbs free energy were found to be (5.5 ± 1.0) × 10^4 M^{-1} and −6.0 ± 0.1 kcal/mol, respectively.

In connection with a study of isolated ribosomal protein–protein interactions separate from the intact ribosome, an interesting complication was encountered. During the examination of solutions of S5 and S8 from *Escherichia coli* strain MRE 600, it was observed that protein S5 undergoes a self-association which confuses the definition of the system by ordinary sedimentation analysis. Such a self-association would have, in the past, precluded the study of a heterogeneous association of these proteins because of the number of variables. A novel method for analyzing this type of association using the analytical ultracentrifuge is employed which circumvents this problem, thereby allowing the association of S5 with S8 to be studied and the energy of association to be determined.

The characterization of these two proteins in solution is of some interest. Protein S8 forms a specific complex with the 16S ribosomal ribonucleic acid (rRNA)[1] (Mizushima & Nomura, 1970; Ungewickell et al., 1975; Zimmermann et al., 1972; Muto & Zimmermann, 1978) and plays a vital role in the assembly of the 30S particle in vitro as represented in the assembly map of Mizushima & Nomura (1970). S5 plays roles in the binding of fMet-tRNA and in large subunit–small subunit association (Traut et al., 1974).

Materials and Methods

All chemicals used in this study were of reagent grade or better. Ultrapure Gdn·HCl and ultrapure sucrose were purchased from Bethesda Research Laboratories, Inc. Equipment and amino acid analysis were as discussed previously (Rohde et al., 1975) except that (a) spectral determinations were performed with a Beckman ACTA CIII spectrophotometer and a Varian 635 spectrophotometer, (b) zonal centrifugation was accomplished with a Ti-15 rotor instead of a Ti-14 rotor, and (c) dialyses of samples for chromatography were carried out by employing Spectrapor 3 tubing to improve recovery of the smaller proteins.

Mid-log *Escherichia coli* MRE 600 in the form of frozen cell paste was purchased from Grain Processing Corp., Muscatine, IA. Ribosome preparation, separation of subunits, protein extraction, and initial chromatography using phosphocellulose were as described previously (Rohde et al., 1975) except that sucrose was untreated for RNase, urea solutions were treated with 10 g of Norit and 10 g of mixed-bed ion-exchange resin AG 501-X8(D) per L to remove color and ionic contaminants prior to preparing urea buffers, and PMSF was added prior to the disruption of the cells. Urea solutions were stored frozen until needed, and all urea buffers contained 12 mM methylamine as described (Rohde et al., 1975). All chromatography was performed at 4 °C.

Proteins from the initial phosphocellulose chromatography were identified as discussed previously (Rohde et al., 1975) except that the urea gel system used consisted of 8 M urea, 9% acrylamide, 0.4% methylenebis(acrylamide), and 0.3 M acetate at pH 4.5. The appropriate pooled fractions were applied to a Sephacryl S-300 (Pharmacia Fine Chemicals) column in 7.5 M urea at pH 5.8. Final fractionation was accomplished by carboxymethylcellulose chromatography in 7.5 M urea at pH 6.5. Immediately after each rechromatography step, samples were dialyzed into 15% acetic acid to remove urea and stored frozen. Small portions of the pooled fractions were subjected to 8 M urea–polyacrylamide gel electrophoresis, $NaDodSO_4$–, and 8 M urea IEF polyacrylamide gel electrophoresis as described below.

Comparisons of the amino acid analyses, according to the method described by Rohde et al. (1975), of proteins S5 and S8 relative to the purified proteins isolated by Craven et al. (1969) and Kaltschmidt et al. (1970a) provided unique identification of both proteins (correlation coefficients of 0.99). By this method, a correlation coefficient of greater than 0.98 is considered to be excellent. The purity of these proteins was judged to be greater than 98%. Under conditions in which

† From the Marrs McLean Department of Biochemistry, Baylor College of Medicine, Houston, Texas 77030. *Received September 4, 1980.* This work was supported in part by the National Institutes of Health (GM22244 and GM00071) and the Robert A. Welch Foundation (Q-592). A preliminary report of this work was presented (Tindall & Aune, 1980).

[1] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; TMK reconstitution buffer, 0.03 M Tris, 0.02 M $MgCl_2$, 0.35 M KCl, 0.003 M NaN_3, and 0.01 M 2-mercaptoethanol, pH 7.4; Gdn·HCl, guanidine hydrochloride; IEF, isoelectric focusing; RNA, ribonucleic acid; rRNA, ribosomal RNA; fMet-tRNA, formylmethionyl transfer RNA; MSC, molecular sieve chromatography; PMSF, phenylmethanesulfonyl fluoride.

0006-2960/81/0420-4861$01.25/0

2% contaminants could be detected by direct gel scanning, the proteins migrated as single bands on the 8 M urea gels, on $NaDodSO_4$ gels, and on 8 M urea IEF gels.

$NaDodSO_4$–polyacrylamide gel electrophoresis was accomplished as described previously (Rohde et al., 1975). The 8 M urea IEF polyacrylamide gel electrophoresis system was a modification of methods found elsewhere (Righetti, 1979). The 8 M urea IEF gel consisted of 8 M urea, 4.85% acrylamide, 0.15% methylenebis(acrylamide), and 2% ampholytes with the upper and lower buffers predicated by the range of ampholytes used. In all cases, protein bands were detected by a stain consisting of 27% ethanol, 10% glacial acetic acid, 0.04% Coomassie Brilliant Blue R-250, and 0.05% Crocein Scarlet. In the case of staining IEF gels, 0.5% copper(II) sulfate was added to both stain and destain.

Protein solutions for ultracentrifuge studies were prepared from samples that had been exposed to denaturing conditions during purification. Solutions of these proteins were prepared by dissolution of lyophilized protein in 6 M Gdn·HCl followed by rapid dilution into TMK in a manner similar to that described previously (Rohde et al., 1975). Amounts on the order of 1–3 mg of lyophilized protein were dissolved in 50 μL of 6 M Gdn·HCl, pH 8.4, containing 0.04 M Tris and 1% 2-mercaptoethanol. This solution was incubated at 40 °C for 30 min, diluted with 0.95 mL of TMK + 1% 2-mercaptoethanol, incubated at 40 °C for 30 min, placed in Spectrapor 6 dialysis tubing, and dialyzed versus TMK + 1% 2-mercaptoethanol at approximately 30 °C for 30 min. Overnight dialysis was then carried out at 4 °C. Both samples were centrifuged at 48000g for 30 min at 4 °C and then chromatographed using a 1.5 × 30 cm column of G-100 in TMK to remove the small amount of aggregation. The peak fractions pooled represented at least 90% of the mass applied to the column. These solutions were either dialyzed versus TMK for analytical ultracentrifugation or stored frozen. Samples stored frozen for periods of several months exhibited the same behavior upon analytical ultracentrifugation as did freshly prepared samples.

Analytical ultracentrifugation in TMK and in 6 M Gdn·HCl, plate reading and associated error, and calculation of apparent partial specific volumes were as described elsewhere (Rohde et al., 1975). Point-average molecular weight analyses were determined by methods discussed previously (Kar & Aune, 1974). The apparent molecular weights by $NaDodSO_4$–polyacrylamide gel electrophoresis (Weber et al., 1972) and urea molecular sieve chromatography (Ackers, 1975) have been discussed.

Analysis of data from the analytical ultracentrifuge was accomplished with a Cromemco System Three computer using modified procedures and Fortran programs derived from earlier discussed methods (Aune & Rohde, 1977). The complication of the system studied here required some modification of those techniques as described below.

The analysis of data must accommodate an indefinite (isodesmic) self-association. Historically, analyses of isodesmic self-associations for homogeneous macromolecular systems have been based on the use of molecular weight averages as functions of concentration (Adams, 1967; Teller, 1973). These methods cannot be applied to a heterogeneous system. An isodesmic self-association is a self-association in which a protein can form all possible n-mers with the concentration of any given polymer state, C_i, described by

$$C_i = (1/K)(C_1K)^n \quad (1)$$

where K, C_1, and n are the isodesmic association constant, the monomer concentration, and the number of monomer subunits involved in the structure under consideration, respectively.

Table I[a]

mol wt method	proteins S5	proteins S8
sequence (strain K)	17 506[b]	12 272[c] 12 215[d]
$NaDodSO_4$–polyacrylamide gel electrophoresis[f]	21 000 ± 300	16 000 ± 300
urea MSC[g]	20 200 ± 300	14 600 ± 300
sedimentation equilibrium[h]		
6 M Gdn·HCl	16 200 ± 400	14 600 ± 600
TMK	*i*	15 800 ± 100

[a] ± reflects the precision of the measurement. [b] Wittmann-Liebold & Greuer (1978). [c] Stadler (1974). [d] Stadler & Wittmann-Liebold (1976). [e] Allen & Wittmann-Liebold (1978). [f] The accuracies of the $NaDodSO_4$ molecular weights are ±10%. [g] The accuracies of the urea molecular sieve chromatography molecular weights are ±7–10%. [h] The accuracies of the sedimentation equilibrium molecular weights are on the order of their precisions. [i] Self-associating.

The concentration fitting procedures (Aune & Rohde, 1977) can be modified to accommodate this type of an association. At sedimentation equilibrium, the protein concentration at any radial position in the ultracentrifuge, $C(r)$, is given by

$$C(r) = C_1(a)\frac{\exp[\sigma_1(r^2 - r_a^2)/2]}{\{1 - KC_1(a)\exp[\sigma_1(r^2 - r_a^2)/2]\}^2} \quad (2)$$

where

$$\sigma_1 = M_1(1 - \bar{v}\rho)\omega^2/(RT) \quad (3)$$

and where $C_1(a)$, M_1, $\bar{v}$, ρ, ω, r, r_a, R, and T are the meniscus concentration of monomer, the molecular weight, the partial specific volume, the solution density, the angular velocity, and radial position, the radial position of the meniscus, the gas constant, and the absolute temperature, respectively.

The other modification involves the constraining of the predicted composition of the system to within experimental error of the known input composition. The details of these methods will be reported elsewhere.

Results

Before the interaction of S5 with S8 was studied, S5 and S8 were characterized with respect to their molecular weight. Their individual behaviors in TMK reconstitution buffer as assessed by sedimentation equilibrium were studied since it could not be assumed a priori that these proteins do not undergo self-association which would confuse the analysis of mixtures of S5 and S8.

S5 from *Escherichia coli* strain K has a sequence molecular weight of 17 506 (Wittmann-Liebold & Greuer, 1978). Table I contains the $NaDodSO_4$–polyacrylamide gel electrophoresis and the 7.5 M urea molecular sieve chromatography apparent molecular weights obtained for S5. Since the $NaDodSO_4$–polyacrylamide gel electrophoresis and molecular sieve chromatography molecular weights each have accuracies of approximately ±10% (Weber & Osborn, 1975; Fish et al., 1969), these values are in agreement with the sequence value. Also listed in Table I and in agreement with the sequence value is the molecular weight obtained from sedimentation equilibrium in 6 M Gdn·HCl.

S5 in TMK reconstitution buffer at 4 °C studied by sedimentation equilibrium shows a tendency to self-associate as indicated in Figure 1. This figure is a plot of interpolated point-average molecular weights from two experiments as a

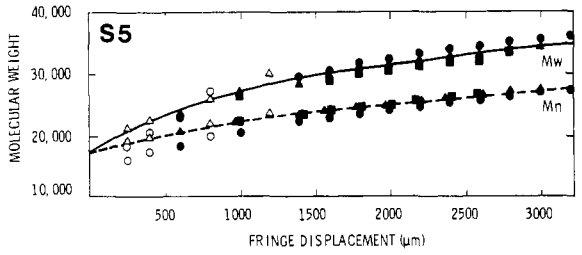

FIGURE 1: Purified S5 self-association at sedimentation equilibrium. Representative number- and weight-average molecular weights in TMK buffer versus protein concentration expressed as fringe displacement where displacements of 1140 μm = 1 mg/mL. Smooth curves are means of all data points available for the two experiments with extrapolations to 17 500. Experimental conditions: 0.22 mg/mL initial concentration of S5 at 22 000 (○) and 26 000 (△) rpm; 0.36 mg/mL initial concentration of S5 at 28 000 (●), 32 000 (▲), and 36 000 (■) rpm.

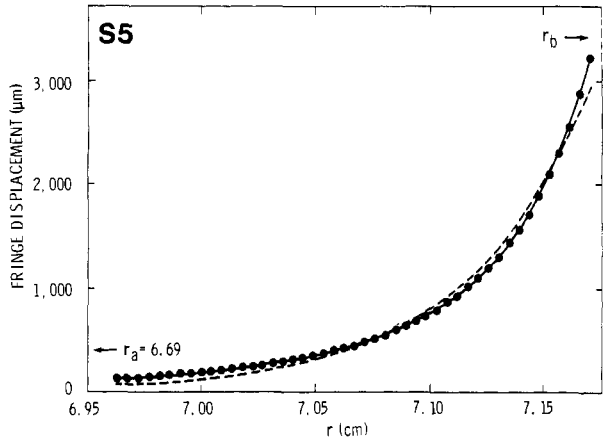

FIGURE 2: Protein S5 at sedimentation equilibrium. Fringe displacement versus radial position. Solid line is best fit as an indefinite (isodesmic) self-association with monomer molecular weight of 17 500. Dashed line is best fit as a single molecular species. Experimental conditions: 0.36 mg/mL initial concentration of S5 at 28 000 rpm and 4 °C.

function of fringe displacement, a quantity related to the protein concentration.

Curve-fitting S5 data from several experiments in terms of monomer, monomer–dimer, and monomer–dimer–trimer associations failed to give consistent association constants for these models. A better model for the S5 self-association was the indefinite (isodesmic) self-association model. In this model, monomer S5 forms all possible n-mers with an intrinsic association constant for each step. This mode of self-association is treated in depth elsewhere (Adams, 1967; Teller, 1973). It can be shown that the monomer molecular weight, M_1, can be obtained from Figure 1 according to the expression $M_1 = 2M_n - M_w$ where M_n and M_w are the number- and weight-average molecular weights, respectively, at any protein concentration (Teller, 1973). The monomer molecular weight was computed to be 17 900 ± 500 from S5 data in Figure 1. Hence, all S5 data were fit, incorporating eq 2 into the methods and employing a monomer molecular weight equal to its sequence value of 17 500 and a partial specific volume of 0.740 mL/mg. Such a fit is illustrated by the solid line in Figure 2. The best fit of these data as a single molecular weight species, indicated by the dashed line in Figure 2, would predict a molecular weight of 27 300 in this experiment. The isodesmic association constant and corresponding standard Gibbs free energy obtained from five experiments having different initial concentrations of S5 with each experiment performed at two or three different speeds were $(7.7 \pm 0.7) \times 10^3$ M^{-1} and −4.9 ± 0.1 kcal/mol, respectively. The fitting error for these experiments varied from 4 to 10 μm, which is within plate reading error.

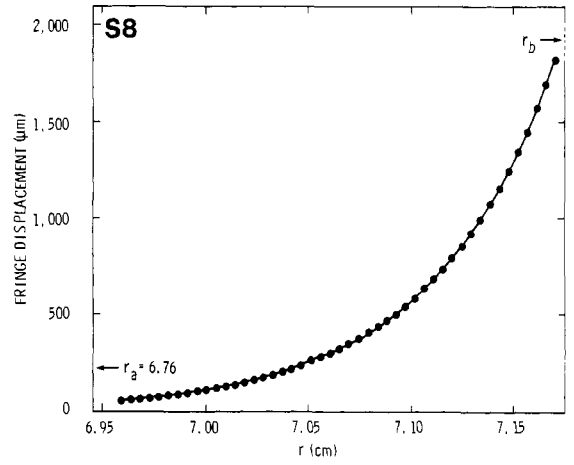

FIGURE 3: Protein S8 at sedimentation equilibrium. Fringe displacement versus radial position. Solid line is best fit as a single molecular species with molecular weight 15 800. Experimental conditions: 0.29 mg/mL initial concentration of S8 at 36 000 rpm and 4 °C.

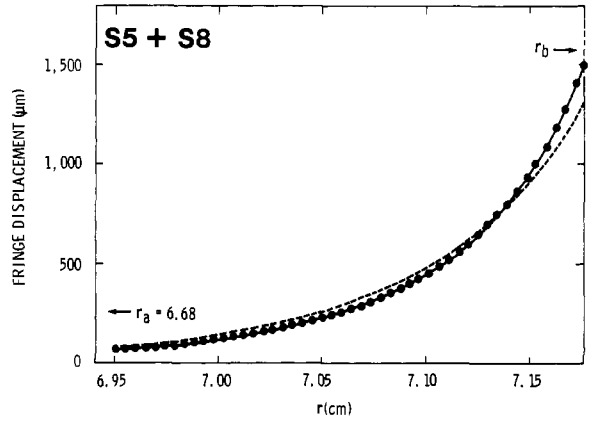

FIGURE 4: Proteins S5 and S8 as a mixture at sedimentation equilibrium. Fringe displacement versus radial position. Solid line is best fit for the presence of a 1:1 molar S5–S8 complex. Dashed line is best fit in the absence of complex. Experimental conditions: 0.070 mg/mL initial concentration of S5 and 0.13 mg/mL initial concentration of S8 at 30 000 rpm and 4 °C.

S8 from *Escherichia coli* strain K is reported to have sequence molecular weights of 12 272 (Stadler, 1974), 12 215 (Stadler & Wittman-Liebold, 1976), and 13 996 (Allen & Wittman-Liebold, 1978). Table I contains the $NaDodSO_4$–polyacrylamide gel electrophoresis and the 7.5 M urea molecular sieve chromatography apparent molecular weights obtained for S8. Also listed in Table I is the molecular weight obtained from sedimentation equilibrium in 6 M Gdn·HCl.

Analysis of sedimentation equilibrium data from five separate experiments shows that S8 in TMK reconstitution buffer at 4 °C has no tendency to self-associate and, as indicated in Table I, has a molecular weight of 15 800 ± 100 as calculated by using a partial specific volume of 0.746 mL/mg. The solid line of Figure 3 represents such a fit.

Figure 4 illustrates typical sedimentation equilibrium data for a mixture of S5 and S8 in TMK buffer at 4 °C. The solid curve represents the theoretical fit for a system containing self-associating S5, monomer S8, and a complex of S5 and S8 which has a 1:1 stoichiometry. The dashed line represents the best fit for self-associating S5 and monomer S8 in the absence of a 1:1 complex. In both cases, S5 self-association is represented by incorporating the determined self-association constant and eq 2 into the fitting methods.

Table II: Parameters Determined from Mixtures of S5 and S8 by Using Sedimentation Equilibrium Curve Fitting

rpm	$\bar{R}$ (μm)[a]	S5/S8 mass ratio	complex (%)	equilibrium $K \times 10^4$ M^{-1}	ΔG° (kcal)
22 000	6.4	0.77[b]	27	6.1	−6.1
28 000	6.0	0.77	39	8.7	−6.3
32 000	7.2	0.77	41	7.8	−6.2
26 000	6.0	0.56[c]	24	4.3	−5.9
30 000	6.0	0.56	23	3.2	−5.7
34 000	8.0	0.56	21	2.2	−5.5

[a] Average residual fitting error. [b] S5 (0.08 mg/mL) and S8 (0.10 mg/mL) in TMK buffer. [c] S5 (0.07 mg/mL) and S8 (0.13 mg/mL) in TMK buffer.

Indicated in Table II is a summary of fitting results obtained from two experiments using the model for 1:1 complex formation stated above. Included in Table II are the association constants and the corresponding standard Gibbs free energies at each angular velocity employed in each experiment. The average weighted association constant and corresponding standard Gibbs free energy for these two experiments are (5.5 ± 1.0) × 10^4 M^{-1} and −6.0 ± 0.1 kcal/mol, respectively. These constants were calculated according to a weighting procedure based on the residual and not determined by merely averaging those values found in Table II. The method used for determining the error of these values is discussed elsewhere (Aune & Rohde, 1977).

Discussion

The structural integrity of the ribosomal subunits from *Escherichia coli* is determined by three major types of interactions. These are the interactions of RNA with RNA, RNA with protein, and protein with protein. Of interest here is the possible interaction of protein S5 with protein S8. Several lines of evidence *suggest* a possible interaction between these proteins. The binding of S5 to the 30S subunit in vitro is greatly stimulated by the prior binding of S8 to the 16S rRNA (Nomura & Held, 1974). These two proteins have been chemically cross-linked in the ribosome (Lutter et al., 1972), subsequently isolated as a dimer by denaturing methods such as those employed here, and reconstituted to form an active ribosome (Lutter & Kurland, 1973). Their centroid to centroid separation distance in the 30S subunit has been determined by neutron scattering to be 35 Å (Engelman et al., 1975). However, it is clear that *none* of the above addresses interaction directly.

Since sedimentation equilibrium methods can only deal with reasonably simple systems, the objective here has been limited to an assessment of what physical attraction exists between S5 and S8 free from the other subunit components. To define mixtures of these proteins, it is necessary to understand quantitatively the behavior of the individual purified proteins.

It has been shown here that in TMK reconstitution buffer at 4 °C, S5 undergoes a self-association which can be described as an indefinite (isodesmic) self-association with a monomer molecular weight of 17 500, an isodesmic association constant of (7.7 ± 0.7) × 10^3 M^{-1} at 4 °C, and a corresponding standard Gibbs free energy of −4.9 ± 0.1 kcal/mol. This low energy self-association probably plays little or no role in the intact 30S ribosomal subunit since S5 is thought to occur in at most one copy per subunit (Hardy, 1975). This protein had not been previously observed to undergo self-association in this laboratory using the *Escherichia coli* strain B protein (Rohde et al., 1975). Such a difference is not unexpected since S5's from *Escherichia coli* strains B and MRE 600 have been shown to be electrophoretically different (Kaltschmidt et al., 1970b). Moreover, this study involved the use of concentrations much higher than those previously used (Rohde et al., 1975) such that the interaction would demonstrate itself.

S8 from *Escherichia coli* strain K is reported to have sequence molecular weights of 12 272 (Stadler, 1974), 12 215 (Stadler & Wittmann-Liebold, 1976), and 13 996 (Allen & Wittmann-Liebold, 1978). In TMK buffer at 4 °C, S8 does not undergoe self-association and can best be described as having a molecular weight of 15 000 ± 100. This molecular weight differs significantly from that of the first published *Escherichia coli* strain K S8 sequence (Stadler, 1974). It differs less significantly from the revision reported later (Allen & Wittmann-Liebold, 1978). The latest version is only 1800 less than the physicochemical molecular weight reported here. Parenthetically, the last 23 C-terminal residues of the shorter version bear no relation to the latest version. The difference in molecular weight between the data here and the latest sequence value may reflect an actual difference in the molecular weight of S8 from *Escherichia coli* strain MRE 600 used in this laboratory and S8 from *Escherichia coli* strain K used in the sequencing study. However, studies which compared S8 from these two strains using two-dimensional electrophoresis concluded that S8 was electrophoretically identical in the two strains (Kaltschmidt et al., 1970b). Also, S8 from *Escherichia coli* B has been reported to have a molecular weight of 16 600 ± 1200 (Rohde et al., 1975). It is interesting to note that physical studies on S8 elsewhere (Dzionara et al., 1970) gave a molecular weight of 15 500 by $NaDodSO_4$–polyacrylamide gel electrophoresis and sedimentation equilibrium. Purity and identification criteria suggest that it is not likely that the protein used in this study was mistakenly identified. It is possible that a proteolytic inhibitor used in preparations here can account for the difference. In any event, since the molecular weight of S8 reported from sedimentation equilibrium studies has had a more stable history, the value reported in Table I is used for the interpretation of the data presented here.

Mixtures of S5 and S8 in TMK at 4 °C cannot be analyzed in terms of self-associating S5 and monomer S8 only. When the interaction of two proteins is studied by the sedimentation equilibrium data analysis method used here, three criteria must be satisfied before a model is deemed acceptable. First, the error of the fit must be in the range of errors expected for plate reading. Second, the composition of the mixture being studied must be properly predicted by the fit for the model of interest. Third, the results must be consistent from one experimental condition to another.

Fits of the data in terms of the noninteraction model which disregard the known starting concentrations provide an error on the order of the plate reading error, but greatly overestimate the amount of S5 present in the mixtures, thereby violating criterion two. This problem is circumvented by the method employed here. Information concerning the starting concentrations of the components is injected into the analysis, thus eliminating such false solutions. The only model which meets the criteria of composition balance, residual error, and experimental consistency is the model in which monomer S5 and monomer S8 combine to form a complex having 1:1 stoichiometry with an association constant and standard Gibbs free energy of (5.5 ± 1.0) × 10^4 M^{-1} at 4 °C and −6.0 ± 0.1 kcal/mol, respectively. Any additional S5 not directly involved in the 1:1 complex undergoes a less significant self-association as described above. Fits of the data in terms of this model have an error on the order of the plate reading error, properly

predict the composition of the mixture of S5 and S8, and provide a consistent association constant under different experimental conditions.

Although the interaction of S8 with larger than monomer forms of S5 cannot be excluded, this interaction would not affect the association constant determined for 1:1 complex formation because of the small amount of higher aggregates of S5.

Littlechild & Malcolm (1978) have developed a method for purifying ribosomal proteins which is based on LiCl extraction of intact subunits. Claims have been made (Giri et al., 1977; Morrison et al., 1977a,b; Littlechild & Malcolm, 1978; Littlechild et al., 1979; Littlechild, 1980) that the proteins so produced have conformations more like those found in the intact ribosome than those obtained by urea purification which has been employed in this work. We feel that both methods produce proteins that approximate the native conformations of ribosomal proteins in the intact subunits.

The urea-purified proteins used in these comparative studies (Giri et al., 1977; Morrison et al., 1977a,b; Littlechild & Malcolm, 1978; Littlechild et al., 1979; Littlechild, 1980) were not subjected to reconstitution conditions (buffer composition, temperature, etc.) which are necessary to form active, reconstituted subunits from urea-purified proteins. These comparisons of physical properties are not completely valid since the urea-purified proteins which were used had not been "renatured". Proteins S5 and S8 used in this paper were subjected to renaturation conditions that we believe produce proteins with native conformations. Some or all of the differences that were observed in the comparative studies may have been due to the presence of aggregated protein in solutions of urea-treated proteins which is not removed by centrifugation. We have frequently observed the presence of such aggregated material in our samples and have subjected our samples to gel filtration chromatography to remove it prior to use.

It should be noted that workers using the LiCl extraction method have often referred to the method as a nondenaturing method (Giri et al., 1977; Morrison et al., 1977a,b; Littlechild & Malcolm, 1978; Littlechild et al., 1979; Littlechild, 1980). Denaturation chemists have long recognized LiCl as a protein denaturant (Lapanje, 1978). For example, careful studies by Dreizen & Gershman (1970) showed that treatment of myosin with 1 M LiCl causes light-chain dissociation and, concomitantly, produces an irreversible inactivation of the CaATPase activity.

Three important points should be noted concerning the interaction of S5 with S8 observed in this study. First, the interaction between S5 and S8 is specific. The energy of interaction of S5 with S8 is on the order of the energy difference between native and denatured proteins (Brandts, 1964; Aune & Tanford, 1969; Salahuddin & Tanford, 1970). The energy difference between native and denatured proteins reflects the energy involved in forming specific, intramolecular contacts which should be similar to intermolecular contacts. Second, the strength and specificity with which S5 and S8 interact show that S5 and S8 can chemically recognize each other free in solution in addition to being in close proximity in the 30S subunit as suggested by cross-linking (Lutter et al., 1972) and neutron-scattering studies (Engelman et al., 1975). A chemical recognition is also suggested, but not proven, by assembly studies which demonstrate that the presence of S8 greatly stimulates the binding of S5 to the subunit (Nomura & Held, 1974). Third, the interaction of S5 with S8 occurs in vitro in the absence of other ribosomal components. These three points taken together suggest that the interaction proven to exist in solution by the study presented here provides part of the energy required to account for the observed stability of the ribosome.

The interaction of S8 with the 16S rRNA is apparently of sufficient strength to account for its localization in the subunit for it can be isolated bound to the 16S rRNA (Zimmermann et al., 1972). However, the energy of interaction of S5 with S8 is not great enough to explain the immobilization of S5 in the subunit. Several similar interactions between S5 and other proteins in the structure would provide sufficient energy to effectively trap S5 in the structure. Such a matrix of protein–protein and protein–RNA interactions would account for the structural stability of the ribosome.

This paper has presented a novel, if not unique, method for studying multiple associations in solution. This method is not limited to studying a heterogeneous association in the presence of self-associations. Although it may not be possible to determine simultaneously several association constants in a mixture of proteins, it is possible to determine one association constant provided all other associations occurring in the mixture are known and can be accounted for. The maximum number of associations which could be accounted for in this manner is not known, but as shown in this study, it can easily deal with at least two.

Acknowledgments

We thank Dr. William Bradley for his assistance with amino acid analysis, Dr. Harris Busch for the use of his Ti-15 rotor, and Chee Ming Li and Linda Talbert for their expert technical assistance.

References

Ackers, G. K. (1975) *Proteins, 3rd Ed. 1*, 2.
Adams, E. T., Jr. (1967) in *Fractions 3*, pp 1–18, Spinco Division of Beckman Instruments, Inc., Palo Alto, CA.
Allen, G., & Wittmann-Liebold, B. (1978) *Hoppe-Seyler's Z. Physiol. Chem. 359*, 1509.
Aune, K. C., & Tanford, C. (1969) *Biochemistry 8*, 4579.
Aune, K. C., & Rohde, M. F. (1977) *Anal. Biochem. 79*, 110.
Brandts, J. F. (1964) *J. Am. Chem. Soc. 86*, 4291.
Craven, G. R., Voynow, P., Hardy, S. J. S., & Kurland, C. G. (1969) *Biochemistry 8*, 2906.
Dreizen, P., & Gershman, L. C. (1970) *Biochemistry 9*, 1688.
Dzionara, M., Kaltschmidt, E., & Wittmann, H. G. (1970) *Proc. Natl. Acad. Sci. U.S.A. 67*, 1909.
Engelman, D. M., Moore, P. B., & Schoenborn, B. P. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3888.
Fish, W. W., Mann, K. G., & Tanford, C. (1969) *J. Biol. Chem. 244*, 4898.
Giri, L., Littlechild, J., & Dijk, J. (1977) *FEBS Lett. 79*, 238.
Hardy, S. J. S. (1975) *Mol. Gen. Genet. 140*, 253.
Kaltschmidt, E., Dzionara, M., & Wittmann, H. G. (1970a) *Mol. Gen. Genet. 109*, 292.
Kaltschmidt, E., Stöffler, G., Dzionara, M., & Wittmann, H. G. (1970b) *Mol. Gen. Genet. 109*, 303.
Kar, E. G., & Aune, K. C. (1974) *Anal. Biochem. 62*, 1.
Lapanje, S. (1978) in *Physicochemical Aspects of Protein Denaturation*, Wiley, New York.
Littlechild, J. (1980) *FEBS Lett. 111*, 51.
Littlechild, J. A., & Malcolm, A. L. (1978) *Biochemistry 17*, 3363.
Littlechild, J., Morrison, C. A., & Bradbury, E. M. (1979) *FEBS Lett. 104*, 90.
Lutter, L. C., & Kurland, C. G. (1973) *Nature (London), New Biol. 243*, 15.

Lutter, L. C., Zeichhardt, H., Kurland, C. G., & Stoffler, G. (1972) *Mol. Gen. Genet. 119*, 357.
Mizushima, S., & Nomura, M. (1970) *Nature (London) 226*, 1214.
Morrison, C. A., Bradbury, E. M., & Garrett, R. A. (1977a) *FEBS Lett. 81*, 435.
Morrison, C. A., Bradbury, E. M., Littlechild, J., & Dijk, J. (1977b) *FEBS Lett. 83*, 348.
Muto, A., & Zimmermann, R. A. (1978) *J. Mol. Biol. 121*, 1.
Nomura, M., & Held, W. A. (1974) in *Ribosomes* (*Nomura*, M., Tissieres, A., & Lengyel, P., Eds.) pp 193–223, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Righetti, P. G. (1979) in *Electrokinetic Separation Methods* (Righetti, P. G., Van Oss, C. J., & Vanderhoff, J. W., Eds.) p 476, Elsevier/North-Holland Biomedical Press, Amsterdam and New York.
Rohde, M. F., & Aune, K. C. (1975) *Biochemistry 14*, 4344.
Rohde, M. F., O'Brien, S., Cooper, S., & Aune, K. C. (1975) *Biochemistry 14*, 1079.
Salahuddin, A., & Tanford, C. (1970) *Biochemistry 9*, 1342.
Stadler, H. (1974) *FEBS Lett. 48*, 114.
Stadler, H., & Wittmann-Liebold, B. (1976) *Eur. J. Biochem. 66*, 49.
Teller, D. C. (1973) *Methods Enzymol. 27*, 346.
Tindall, S. H., & Aune, K. C. (1980) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 39*, 1741.
Traut, R. R., Heimark, R. L., Sun, T.-T., Hershey, J. W. B., & Bollen, A. (1974) in *Ribosomes* (Nomura, M., Tissieres, A., & Lengyel, P., Eds.) p 271, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Ungewickell, E., Garrett, R., Ehresmann, C., Stiegler, P., & Fellner, P. (1975) *Eur. J. Biochem. 51*, 165.
Weber, K., & Osborn, M. (1975) *Proteins, 3rd Ed. 1*, 180.
Weber, K., Pringle, J. R., & Osborn, M. (1972) *Methods Enzymol. 26*, 3.
Wittmann-Liebold, B., & Greuer, B. (1978) *FEBS Lett. 95*, 91.
Zimmermann, R. A., Muto, A., Fellner, P., Ehresmann, C., & Branlant, C. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 1282.

Membrane Channel Forming Polypeptides. 270-MHz Hydrogen-1 Nuclear Magnetic Resonance Studies on the Conformation of the 11–21 Fragment of Suzukacillin†

M. Iqbal and P. Balaram*

ABSTRACT: 270-MHz ^{1}H NMR studies on the synthetic suzukacillin fragments Boc-Leu-Aib-Gly-Leu-Aib-OMe (13–17), Boc-Gln-Aib-Leu-Aib-Gly-Leu-Aib-OBz (11–17), Boc-Leu-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib-OMe (13–21), and Boc-Gln-Aib-Leu-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib-OMe (11–21) have been carried out in $CDCl_3$ and $(CD_3)_2SO$. The intramolecularly hydrogen-bonded amide hydrogens in these peptides have been identified by using solvent titration experiments and temperature coefficients of NH chemical shifts in $(CD_3)_2SO$. The peptides are shown to favor conformations stabilized by intramolecular 4→1 hydrogen bonds. The 11–21 fragment adopts a highly folded, largely 3_{10} helical conformation stabilized by seven intramolecular hydrogen bonds. An eighth NH group [Gly(5)] appears to be involved in a weaker interaction. Evidence for the possible participation of the Gln side-chain carboxamide group in hydrogen bonding to the peptide backbone is also presented.

The 24-residue, α-aminoisobutyric acid (Aib)[1] containing polypeptide suzukacillin modifies the permeability properties of lipid bilayers by the formation of transmembrane channels (Jung et al., 1976; Boheim et al., 1976). The presence of a large number of Aib residues in the sequence (Figure 1) greatly restricts conformational freedom of the peptide backbone. The tendency of Aib-containing sequences to adopt 3_{10} helical conformations has been clearly established in studies of alamethicin fragments (Nagaraj et al., 1979; Rao et al., 1979, 1980; Nagaraj & Balaram, 1981a) and model peptides (Prasad et al., 1979, 1980; Shamala et al., 1978; Venkatachalapathi et al., 1981; Venkatachalapathi & Balaram, 1981). As part of a continuing program to elucidate the conformational characteristics of Aib-containing membrane active peptides, we have undertaken a detailed study of suzukacillin. An earlier report described the 3_{10} helical folding of the amino-terminal decapeptide (1–10) segment (Iqbal & Balaram, 1981). In the present paper, we summarize the results of 270-MHz ^{1}H NMR studies on the 11–21 suzukacillin fragment and compare the results obtained with studies on smaller fragments. It is clearly shown that the 11–21 fragment is highly folded in solution, and the NMR evidence strongly favors a conformation in which seven NH groups participate in intramolecular hydrogen bonding.

Materials and Methods

The peptides Boc-Leu-Aib-Gly-Leu-Aib-OMe (**1**), Boc-Gln-Aib-Leu-Aib-Gly-Leu-Aib-OBz (**2**), Boc-Leu-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib-OMe (**3**), and Boc-Gln-Aib-Leu-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib-OMe (**4**) were synthesized

† From the Molecular Biophysics Unit, Indian Institute of Science, Bangalore 560 012, India. *Received January 27, 1981.* This work has been supported by a grant from the Department of Science and Technology, Government of India. M.I. is the recipient of a Teacher-Fellowship of the University Grants Commission. P.B. is the recipient of a UGC Career Award.

[1] Abbreviations used: Aib, α-aminoisobutyric acid; Boc, *tert*-butyloxycarbonyl; OMe, methyl ester; OBz, benzyl ester; TLC, thin-layer chromatography.

0006-2960/81/0420-4866$01.25/0