Mechanism of Dissociation of S-Peptide from Ribonuclease S†

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ABSTRACT: Equilibrium constants have been measured for two steps in the dissociation reaction of ^{3}H-labeled S-peptide from ribonuclease S in the low-temperature range, 0–20 °C. The method, which has been published previously, makes use of the large difference between the tritium exchange kinetics of S-peptide when free and when bound to S-protein. This method measures independently: (a) the overall dissociation reaction, and (b) the first step in dissociation, which is unimolecular and has the properties of a partial unfolding reaction. Evidence that the unimolecular process is part of the overall dissociation reaction is provided by a comparison of the dependences of the two equilibrium constants on pH, ionic strength, and temperature. The two steps in dissociation are termed partial unfolding and separation. The partial unfolding reaction is enhanced both at low ionic strength and at low pH, suggesting that unfolding is induced by electrostatic repulsion from a cluster of positive charges. This reaction involves a set of about six highly protected protons in the S-peptide which exchange as a group with fairly uniform exchange rates in all of the conditions studied here. Less protected protons are allowed to exchange out in the initial conditions before beginning these experiments. As suggested previously, these six protons are probably among the H-bonded amide protons of the α helix (residues 7–13). The enthalpy change of partial unfolding is 14 kcal/mol, both at pH 4.25 and pH 6.9. The separation reaction is strongly inhibited by 2′-CMP binding, which implicates the region around His-12 in the S-peptide in this reaction. The separation reaction is also favored at low pH but is not affected by ionic strength. It shows an enthalpy change of 15 kcal/mol. The enthalpy of the overall dissociation process, 29 kcal/mol, is in good agreement with the calorimetric data of Hearn et al. (Hearn, R. P., Richards, F. M., Sturtevant, J. M., and Watt, G. D. (1971), *Biochemistry 10,* 806–817). A basic conclusion from this work is that partial unfolding reactions do occur in native ribonuclease S at low temperatures and are detected by tritium exchange measurements. The dissociation of the S-peptide from ribonuclease S may itself be viewed as a partial unfolding reaction. A second conclusion is that an apparent intermediate in the dissociation process can be studied by ^{3}H exchange; the concentration of this intermediate is too low to be detected by most other methods.

The dissociation reaction of the S-peptide from ribonuclease S (RNase S) is a good system in which to study the mechanism of tertiary folding in proteins. It contains many features of protein folding reactions. The S-peptide has a predominantly random-coil conformation when free in solution at 26 °C (Brown and Klee, 1971; Rocchi et al., 1972). When bound to the S-protein, the S-peptide has a stable secondary structure, an α helix 11 residues in length, and bonding interactions with the S-protein, both H bonds, and hydrophobic interactions. Since the S-protein remains folded as the S-peptide dissociates, this reaction is also an example of a partial unfolding reaction of a specific region in a protein. Transient partial unfolding of the S-peptide region probably occurs in RNase A but cannot be detected due to the short lifetime and small concentration of the partly unfolded species. The cleavage of the polypeptide chain in RNase A which produces RNase S allows the unfolded S-peptide to dissociate from the protein; this increases its lifetime in the unfolded state and allows the reaction to be detected.

The bimolecular nature of this reaction transforms the unimolecular folding problem to the study of a dissociation equilibrium. If the reaction involves partially unfolded intermediates, their presence can be detected by finding initial unimolecular steps in the overall dissociation reaction. For detection both of dissociation and of partially unfolded intermediates, we use the rate of ^{3}H exchange between solvent and S-peptide whose amide protons are ^{3}H labeled. In some conditions, this rate is more than 10^5-fold faster in free S-peptide than in S-peptide bound to RNase S; see Table 1 of Schreier and Baldwin (1976). The kinetic exchange curve for ^{3}H-labeled S-peptide bound to RNase S, measured at high RNase S concentrations, is complex, indicating that different protons exchange at different rates from ^{3}H-labeled S-peptide. We measure here only the slowest exchange reaction of the bound S-peptide. A set of about six protons exchange at a fairly uniform rate in this reaction.

We can write the following two-step reaction mechanism for dissociation of the S-peptide from RNase S, based on the average rate of exchange of these six protons:

$$\text{RNase S} \overset{K_1}{\rightleftharpoons} \text{[partially unfolded intermediate]} \overset{K_2}{\rightleftharpoons} \text{S-peptide} + \text{S-protein} \quad (1)$$

where $K_1K_2 = K_D$, the overall dissociation constant. The evidence that a partially unfolded intermediate in dissociation can be detected by ^{3}H exchange measurements is a principal subject of this paper. It rests on a comparison between the properties of K_1 and of K_D, the overall dissociation constant, which are measured independently of each other. The equilibrium constant K_2 for the second step in dissociation is obtained from the ratio K_D/K_1. The fact that other protons of the S-peptide exchange at faster rates may indicate the existence of other partially unfolded intermediates, or these protons may exchange by some mechanism other than partial unfolding.

Our previous study (Schreier and Baldwin, 1976) led to the following working model. (1) The most slowly exchanging six protons appear to exchange as a group; this probably means

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that they belong to a common structural unit. (2) This is likely to be the α helix of residues 3–13, which contains seven H-bonded amide protons (residues 7–13). (3) The unimolecular reaction in RNase S which allows exchange of these protons is probably unfolding or unzippering of the α helix. (4) This reaction has a pH dependence resembling the overall dissociation process; therefore, it probably is one step in dissociation. (5) If the partial unfolding reaction is unzippering of the α helix, then its enhancement at low pH probably results from electrostatic repulsion between three positive charges in the α helix (Lys-7, Arg-10, and His-12) which are partly compensated within the helix only by Glu-9.

To test this working model, we have now performed the following experiments. (1) Allow the other, less protected, protons to exchange out in fixed conditions. Then determine whether the set of about six remaining protons still exchanges as a group when the conditions are varied widely. (2) Determine the temperature dependences of the two reactions. If the unimolecular reaction is a partial unfolding reaction, it is likely to show a significant enthalpy change. Also, the enthalpy of the overall dissociation process should agree with the calorimetric study by Hearn et al. (1971). (3) If the partial unfolding reaction is enhanced at low pH because of charge repulsion within the α helix, it should also be enhanced at low ionic strengths. Moreover, if this reaction is one step in dissociation, then the expected dependence on ionic strength should also appear in the overall dissociation constant. (4) When the inhibitor 2′-CMP is bound to RNase S, it bridges the S-peptide and S-protein (cf. Richards and Wyckoff, 1970) and the phosphate is close enough to His-12 to change its chemical shift substantially (Markley, 1975). By measuring the effects of 2′-CMP binding on the partial unfolding and separation reactions, we should learn more about the nature of these two steps.

Experimental Section

Materials. Ribonuclease S is the product of Sigma Chemical Co., St. Louis, Mo. Lots 32C8140 and 52C-8180 were used. The RNase S is rechromatographed on CM-Sephadex C-25 in order to remove contaminants such as phosphate. The RNase S is absorbed onto a 2 × 50 cm column (maximum capacity ~500 mg) in 0.1 M NaCl and 10 mM Tris (pH 8) and it is eluted with 0.1 to 1.0 M NaCl salt gradient in 10 mM Tris (pH 8). The protein elutes as a single peak at a concentration of 0.5 M NaCl. The contaminants elute at or near the run-through volume. The RNase S is then desalted by either ultrafiltration or Sephadex gel chromatography and then freeze-dried.

The preparation and characterization of pure S-peptide and S-protein from RNase S and the preparation of ^{14}C-labeled S-peptide have been described previously (Schreier and Baldwin, 1976). 2′-Cytidine monophosphate is made by P-L Labs., Milwaukee, Wis. Tritiated H_2O and [^{14}C]formaldehyde are the products of New England Nuclear Co., Boston, Mass. Phosphocellulose paper, No. P 81, is made by the Whatman Paper Co.

Methods. (a) Experimental Procedure. The preparation of ^{3}H-labeled S-peptide and S-protein and initiation of the HX[1] experiments follow essentially the method used in our previous paper (Schreier and Baldwin, 1976). However, two improvements have been made. pH jumps are performed by adding 0.5 vol of a concentrated buffer solution at a pH close to the final desired pH. This procedure prevents possible loss of exchangeable protons due to a transient exposure of the ^{3}H-labeled peptides to high pH. Also, the final concentration, ionic strength, and/or temperature is adjusted 24 h after initiating the tritium exchange reaction. During the 24-h incubation period, the less protected protons in ^{3}H-labeled S-peptide exchange out. The subsequent change in concentration, ionic strength, and/or temperature affects only the highly protected protons. The amount of tritium remaining in ^{3}H-labeled S-peptide is assayed by both the modified freeze-dry method (Schreier and Baldwin, 1976) and a new, convenient filtration developed in our laboratory (Schreier, 1977). Briefly, this method separates tritiated proteins and peptides from tritiated solvent by adsorption onto phosphocellulose filter paper at pH 3.0 and 0 °C. The filters disks can be immediately placed in scintillation vials and counted. The use of an internal ^{14}C concentration standard allows accurate measurement of ^{3}H exchange rates despite some losses on filtering.

(b) Data Analysis. The data have been analyzed by the method described by Schreier and Baldwin (1976). The following simple relation exists between the concentration of free S-protein and the rate of ^{3}H exchange from S-peptide, k_{HX}, provided that two conditions are satisfied: (1) the fractional concentration of free S-peptide is much less than 1 (i.e., the extent of dissociation is small), and (2) the kinetics of ^{3}H exchange from free S-peptide are slow compared both to the kinetics of association of S-peptide and S-protein and to the rate of refolding in the partial unfolding reaction (see eq 1):

$$k_{HX} = \frac{k_c K_D}{[\text{S-protein}]} + k_c K_1 \qquad (2)$$

Here k_c is the rate constant for "chemical" exchange from the free S-peptide, and K_D and K_1 are equilibrium constants defined in eq 1. When k_{HX} in eq 2 is plotted vs. $1/[\text{S-protein}]$, a straight line is obtained whose slope gives K_D and intercept gives K_1, provided k_c is known. The standard procedure is to vary the S-protein concentration by changing the total concentration, keeping a constant 30% excess of S-protein over S-peptide. It is also assumed that partial unfolding gives an exposed ^{3}H-labeled proton whose rate of exchange with solvent is k_c, i.e. the same as in free S-peptide. If this assumption is incorrect (cf. Leichtling and Klotz, 1966), it affects the interpretation of K_1 which may be regarded as a defined, or apparent, equilibrium constant for partial unfolding.

Equation 2 is written for a single amide proton, but we are studying a set of about six protons. Thus, k_c and k_{HX} are average values. The justification for using eq 2 in this case, when more than one proton is being studied, is that this set of six protons apparently exchanges as a group with fairly uniform rate constants. A detailed analysis of the assumption has to wait until exchange rates of the individual protons can be measured by nuclear magnetic resonance (NMR). Our measurements of k_c for the S-peptide are restricted to the range pH 3–4.5; the standard relationship describing the pH and temperature dependences for exposed amide protons has been used to extrapolate these values to higher pHs and temperatures (Englander et al., 1972). The ^{3}H exchange kinetics of free S-peptide do not follow a single first-order curve, and an average value of k_c has been taken from the terminal exchange rate of the six most slowly exchanging protons. These six protons in the free S-peptide may not be the same as the six highly protected protons in bound S-peptide. This possibility introduces a systematic uncertainty corresponding to a factor of about 2 in the estimate of k_c (see Molday et al., 1972).

[1] Abbreviations used are: HX, hydrogen exchange; RNase, ribonuclease; 2′-CMP, 2′-cytidine monophosphate; Tris, tris(hydroxymethyl)aminomethane.

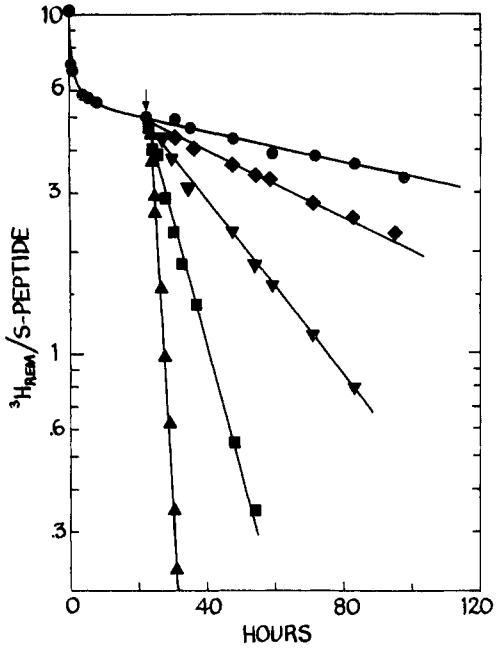

FIGURE 1: The temperature dependence of tritium exchange kinetics of ^{3}H-labeled S-peptide bound to RNase S. ^{3}H-Labeled S-peptide (0.12 mM) is mixed with 0.156 mM S-protein as described under Methods. The sample is left at 0 °C in 50 mM NaOAc (pH 4.25) for 1 day. Aliquots are then brought to the final temperature at the time noted by the arrow: (●) 0 °C; (♦) 4 °C; (▼) 8 °C; (■) 12 °C; (▲) 16 °C.

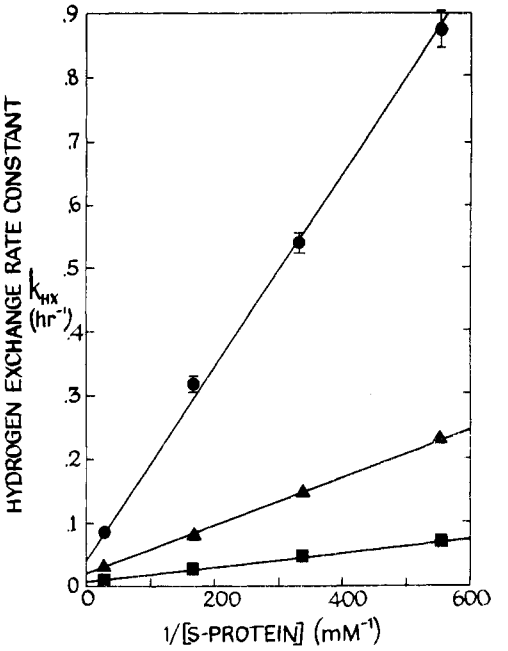

FIGURE 2: The concentration dependence of tritium exchange kinetics of ^{3}H-labeled S-peptide bound to RNase S at three temperatures. The conditions are identical with those in Figure 2. The samples are diluted to their final concentrations when the temperature jump is made: (■) 4 °C; (▲) 7.9 °C; (●) 12.5 °C.

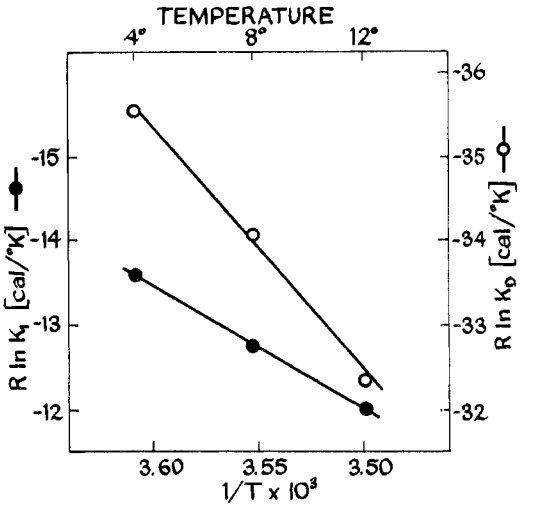

FIGURE 3: The temperature dependence of the equilibrium constant for overall dissociation, K_D (○), and for the partial unfolding reaction, K_I (●).

Results

(a) *Temperature Dependence of K_I and K_D.* The temperature dependences of K_I and K_D have been measured at pH 4.25 where k_c can be measured directly. The technique is illustrated in Figure 1. All but about six ^{3}H-labeled protons are allowed to exchange out at 0 °C at a high protein concentration (0.12 mM), and the temperature is then raised to initiate more rapid exchange. First-order exchange curves are found. When the experiment is repeated at different concentrations, the dilution is made at the same time that the temperature is raised. The results yield linear plots of k_{HX} vs. $1/[\text{S-protein}]$ (Figure 2). Figure 3 shows van't Hoff plots of ln K_D and ln K_I vs. $(1/T)$. A substantial contribution to the temperature dependence of k_{HX} in Figure 1 comes from the temperature dependence of k_c, which is large: the activation enthalpy of k_c is 17 kcal/mol (Englander and Poulsen, 1969). This contribution has been eliminated from the temperature dependences of K_I and K_D shown in Figure 3. The apparent enthalpy changes for the partial unfolding and overall dissociation reactions, calculated from the slopes of the two lines in Figure 3, are 14 and 29 kcal/mol, respectively.

In order to compare our van't Hoff ΔH values with the calorimetric data of Hearn et al. (1971), a more limited study of the temperature dependence of K_D and K_I has been made at pH 6.9. The results are given in Table I. Within experimental error, they yield the same values of ΔH as at pH 4.25 (see Table II).

(b) *Ionic Strength Dependence.* The dependences on ionic strength of K_I and K_D have been measured by a technique similar to the one used to study temperature dependence. A concentrated sample is allowed to release all but about five slowly exchanging protons in a preincubation, and more rapid exchange is then started by simultaneously diluting the protein and adjusting the salt concentration. Exchange curves for 0.5, 0.05, and 0.0007 M NaCl are shown in Figure 4 at pH 4.3, 0 °C, 10^{-5} M of bound ^{3}H-labeled S-peptide. Values of K_I and K_D at each salt concentration are shown in the inset (solid lines). There is about a sevenfold increase both in K_I and in K_D in going from 0.5 to 0.0007 M NaCl. This dependence of K_D on ionic strength has not been noted in previous work, which covered a smaller range of ionic strength.

An interesting feature of the ionic strength study is that the exchange rate of the free S-peptide also increases markedly at low ionic strength, at 0 °C, pH 4.3 (Figure 5). This behavior, which is not observed in unstructured peptides, suggests that some structure in the free S-peptide is influencing its exchange kinetics (see Discussion).

(c) *2′-CMP Binding.* To study the effects on K_I and K_D of the binding of specific ligands, 2′-CMP was chosen because it has the smallest dissociation constant of the mononucleotide inhibitors of RNase A (Anderson et al., 1968). At 0 °C, pH 6.4, the dissociation constant is in the micromolar range, and

TABLE I: Temperature Dependence of the Equilibrium Constants for Dissociation and Partial Unfolding of S-Peptide Bound to RNase S.

pH	Temp (°C)	k_c[a] (s^{-1})	K_D[b] (M)	K_1[b,c]
4.25	4.0	2.1×10^{-3}	1.6×10^{-8}	1.0×10^{-3}
4.25	7.9	3.2×10^{-3}	3.4×10^{-8}	1.6×10^{-3}
4.25	12.5	5.3×10^{-3}	8.0×10^{-8}	2.3×10^{-3}
6.9	0	0.66	8.8×10^{-11}	5.9×10^{-6}
6.9	3.6	0.99	2.3×10^{-10}	7.0×10^{-6}
6.9	8.4	1.7	3.7×10^{-10}	1.3×10^{-5}

[a] See text for definition of k_c. [b] Calculated using eq 2 in text. [c] In Schreier and Baldwin (1976) the symbol K_B was used instead of K_1.

TABLE II: van't Hoff Enthalpy and Entropy of Dissociation and of Partial Unfolding for S-Peptide Bound to RNase S.[a]

pH	Temp. range (°C)	Reaction[b]	ΔH (kcal/mol)	ΔS (eu/mol)
4.25	4–12.5	Dissociation (K_D)	29	71
4.25	4–12.5	Partial unfolding (K_1)	14	39
6.9	0–8.4	Dissociation (K_D)	28	57
6.9	0–8.4	Partial unfolding (K_1)	13	24

[a] Based on the data in Figure 3 and Table I. [b] The concentration range for K_D measurements is 120 to 1.8 μM in RNase S. See legends of Figures 2 and 3 for more details.

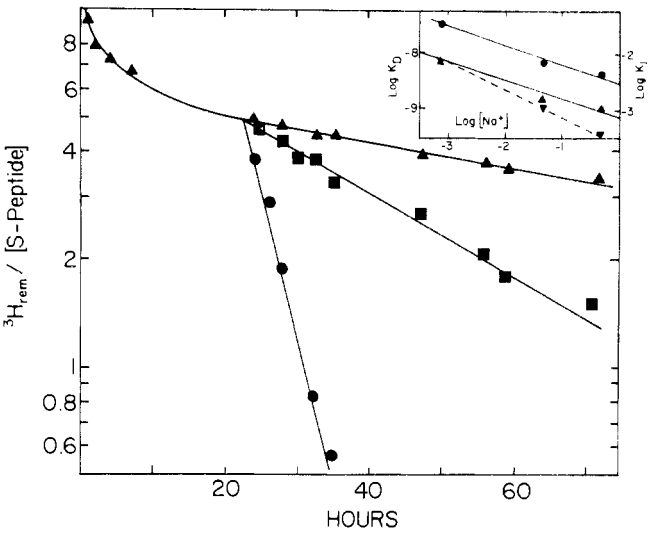

FIGURE 4: Dependence on ionic strength of the tritium exchange kinetics of bound ^{3}H-labeled S-peptide at 0 °C, pH 4.3. Tritium exchange is initiated with 0.12 mM ^{3}H-labeled S-peptide in the presence of 0.156 mM S-protein as described in Methods. After 24 h aliquots are diluted to the final concentration of 10 μM ^{3}H-labeled S-peptide as the salt concentration is simultaneously adjusted to: 0.500 M NaCl, 0.025 M sodium acetate, 0.25 sodium formate (▲); 0.025 M NaCl, 0.025 mM sodium acetate, 0.025 mM sodium formate (■); and 0.001 M sodium acetate and 0.001 M sodium formate (●). Inset figure: dependence of K_D and K_1 on ionic strength: (●) K_D; (▲, ▼) K_1 calculated with different values of k_c. See Discussion for details.

is strongly pH dependent. Figure 6 shows that the binding of 2′-CMP has a large effect on the separation step of the dissociation reaction. In the absence of 2′-CMP, there is the usual large difference between the exchange curves measured at 6 and at 120 μM RNase S. In the presence of 3 mM 2′-CMP, both exchange curves are identical and are shifted to the limiting curve for exchange in the absence of dissociation of S-peptide. The value of K_D becomes too small to measure, whereas the apparent decrease in K_1 is only fivefold (Table III). Thus, the binding of 2′-CMP strongly inhibits the separation step but only mildly inhibits the partial unfolding step of the dissociation reaction.

Discussion

(a) *Evidence That the First Step in Dissociation Is a Partial Unfolding Reaction.* A basic question in this work is whether the unimolecular reaction is part of the dissociation process, or whether ^{3}H exchange from the bound S-peptide occurs by some unrelated mechanism such as diffusion of solvent into the protein. To answer this question we note first that the overall dissociation reaction, as it occurs at low temperatures where S-protein remains folded, may itself be viewed as a partial unfolding reaction. Then, by determining the properties of the overall dissociation reaction, we have a model system for estimating the behavior of a more localized unfolding reaction. In particular, the enthalpy change is of interest because of the large enthalpy changes that occur in the overall unfolding transitions of proteins (cf. Privalov and Khechinashvili, 1974) and because diffusional mechanisms for exchange are expected to have relatively small activation enthalpies (Woodward and Rosenberg, 1971a,b). Moreover, the properties of the overall dissociation reaction are required to be directly related to those of the unimolecular process if the latter is one step in dissociation, specifically, $K_D = K_1K_2$. Thus, we can look for any unusual changes in K_1 with pH, ionic strength, or temperature and ask if these are reflected in K_D.

The suggestion was made previously (Schreier and Baldwin, 1976) that the unimolecular process is part of the dissociation reaction for the following reasons. (1) A set of about six protons appears to exchange as a group; these are the most slowly exchanging protons when S-peptide is bound to RNase S. If they do exchange as a group, they presumably belong to a common structural unit. This is likely to be the α helix formed by residues 3–13, which has seven H-bonded amide protons (residues 7–13). (2) Both K_1 and K_D increase strongly as the pH is lowered below 7. In regard to (1) we now add to the evidence that these six protons exchange as a group by allowing the other, more rapidly exchanging, protons to be released in a preincubation and then studying the exchange behavior of these six protons alone in a variety of conditions. Linear first-order exchange curves are formed in all cases, indicating that the six protons do exchange at a fairly uniform rate.

The substantial enthalpy change associated with K_1, 14 kcal/mol, is consistent with the postulate that exchange occurs via a partial unfolding reaction. The enthalpy change in the overall dissociation reaction is about twice as large or 29 kcal/mol, which is consistent with the unimolecular reaction being one step in dissociation.

Our previous interpretation of the pH dependence of K_1 as arising from charge repulsion between three positive charges in the α helix (Schreier and Baldwin, 1976) leads to the prediction that K_1 should increase at low ionic strength. A sevenfold increase is observed here (Figure 5) which is qualitatively consistent with this prediction. A larger effect might be expected from the fact that there is a 1000-fold increase in K_1 as the pH is lowered from 7 to 3 (Schreier and Baldwin, 1976). This may be explained in part by a salt bridge between the carboxylate anion of Glu-2 and the guanidinium cation of Arg-10 (Finn et al., 1972). If the partial unfolding reaction is helix unzippering, then titration of Glu-2 will contribute to the

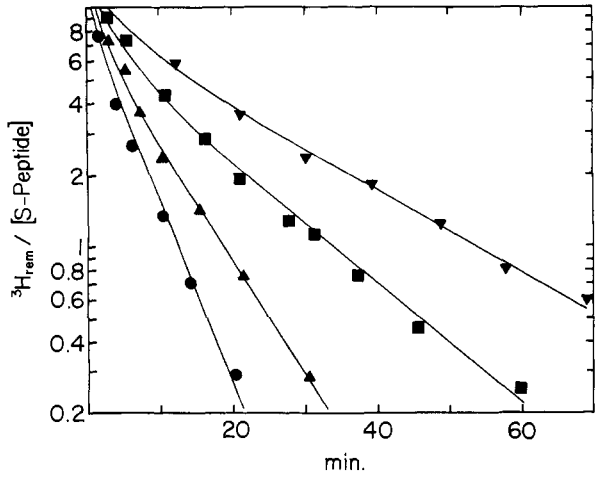

FIGURE 5: Dependence on ionic strength of the exchange kinetics of free ^{3}H-labeled S-peptide at 0 °C, pH 4.3. Exchange is initiated by dissolving freeze-dried ^{3}H-labeled S-peptide into: 1.5 M NaCl, 0.025 M sodium acetate, 0.025 M sodium formate (▼); 0.5 M NaCl, 0.025 M sodium acetate, 0.025 M sodium formate (■); 0.025 M NaCl, 0.025 M sodium acetate, 0.025 M sodium formate (▲); and 0.001 M sodium acetate, 0.001 M sodium formate (●). Conductivity measurements are used to determine ionic strength. In addition to the exchange of the protons shown here, one very slowly exchanging proton was found (half-time of exchange about 20 min) (see Discussion). Its exchange kinetics have been subtracted from the data shown here.

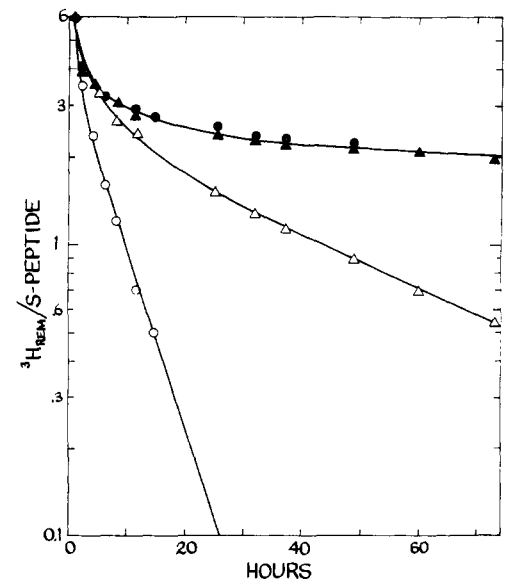

FIGURE 6: Effect of 3 mM 2′-CMP on the exchange behavior of bound ^{3}H-labeled S-peptide at two concentrations with 30% excess S-protein present at pH 6.4, 0 °C. See Table III for measurements of K_1 and K_D. Tritium exchange is initiated as described in Methods. The two concentrations of bound ^{3}H-labeled S-peptide are: 0.120 mM (△) and 0.006 mM (○); filled symbols show the effect of adding 3 mM 2′-CMP.

increase in K_1 at low pH but the salt bridge will oppose any increase in K_1 at low ionic strength.

Interpretation of the ionic strength effect is complex because of the strong dependence on ionic strength of the exchange rate of the free S-peptide (Figure 5). This probably reflects α-helix formation by the free S-peptide itself at low temperatures. A circular dichroism study of peptide 1–13 by Brown and Klee (1971) indicates that partial helix formation does occur in the temperature range 0–20 °C at pH 5–6. They found that helix formation increases strongly with salt concentration, in agreement with our ^{3}H exchange data. If α-helix formation is responsible for this salt dependence, then calculating K_1 by the use of k_c values measured for the free S-peptide underestimates the change in K_1 with ionic strength, since the product of the partial unfolding reaction is presumed to be the completely unfolded α helix. Figure 4 (inset) also shows the K_1 values (dashed line) calculated on the assumption that the S-peptide is completely unfolded at the lowest ionic strength shown in Figure 4.

(b) *Properties of the Second Step in Dissociation: the Separation Reaction.* K_2 is independent of pH, within experimental error (a factor of 2), between pH 4.3 and 8.3 and then increases below 4.3 by one order of magnitude at pH 2.7 (Schreier and Baldwin, 1976). This suggests that titration of one carboxyl group, with a rather low pK, results in dissociation of the S-peptide. This group might be Asp-14, which is H-bonded to Tyr-25; replacement of the carboxyl group of Asp-14 by a carboxamide group drastically weakens the binding of S-peptide to RNase S (Filippi et al., 1975). K_2 is independent of ionic strength (Figure 5), suggesting that nonionic interactions predominate in the separation step. The enthalpy change associated with K_2 is 15 kcal/mol, which accounts for half of the total enthalpy change on dissociation.

If the partial unfolding step is correctly identified as helix unzippering, then the separation reaction must occur after the α helix unfolds. Conversely, in the combination of S-peptide with S-protein, combination should take place before the α helix forms. A study of the bonding interactions between S-peptide and S-protein focuses attention on residues His-12, Asp-14, and Ser-16. There are main-chain H bonds between His-12 and Val-47, Asp-14 and Val-47, Ser-16 and His-48 (Wyckoff et al., 1970), and between His-12 and Thr-45 (Patel et al., 1975). Moreover, there is the tyrosine–carboxylate H bond of Tyr-25–Asp-14 mentioned above. The H bond between Ser-16 and His-48 is not necessary for binding: peptide 1–14 binds to S-protein with an affinity equal to native S-peptide (Finn et al., 1972). Chemical substitution experiments indicate that Phe-8 and Met-13 are also important in binding: cf. Richards and Wyckoff, 1970. Our 2′-CMP binding results, taken together with these structural results, suggest that residues 12–14 may serve as an "anchor" in the initial combination of S-peptide with S-protein. 2′-CMP is known to bind close to His-12 in RNase S (cf. Richards and Wyckoff, 1970) and to have a large effect on the chemical shift of the C-2 proton of His-12 (Markley, 1975). The binding of 2′-CMP causes K_D to become too small to measure at pH 6.4, 0 °C, but has only a small effect on K_1 (Table III), indicating that residues close to His-12 are strongly involved in the separation reaction.

TABLE III: Effect of 2′-CMP on the Equilibrium Constants for Dissociation and Partial Unfolding of S-Peptide Bound to RNase S.[a]

[2′-CMP] (mM)	K_D[b] (M)	K_1[b]
0	2.2×10^{-10}	2.4×10^{-5}
3	$\leq 10^{-12}$	4.4×10^{-6}

[a] 0.12 M sodium cacodylate, pH 6.4, 0 °C. See legend of Figure 6 for more details. [b] Calculated from tritium exchange data using eq 2 in the text with a value of $k_c = 0.21$ s^{-1}.

(c) *Comparison between Our Data for K_D and Results in the Literature.* We have already compared our previous data for K_D with values in the literature (Schreier and Baldwin, 1976). There is substantial disagreement in the literature between values obtained by spectrophotometric techniques (cf. Woodfin and Massey, 1968; Rocchi et al., 1972; Dunn and Chaiken, 1975), or by a column binding technique (Gawronski

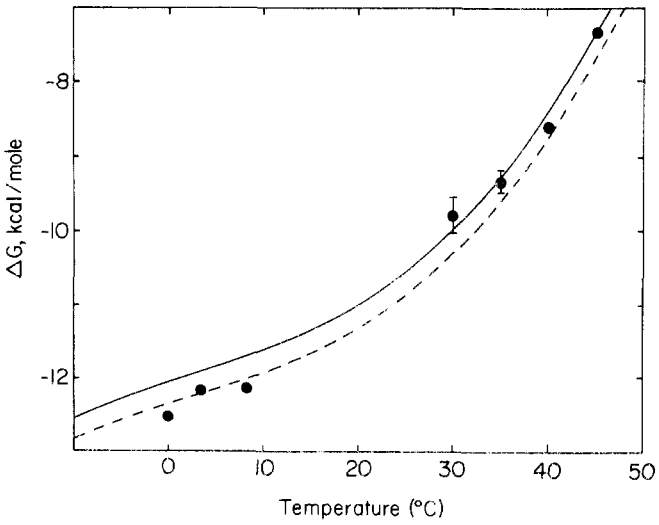

FIGURE 7: Comparison of K_D values measured at 0–8.4 °C, pH 6.9, by ^3H exchange kinetics, with the 25–40 °C, pH 7.0, values of K_D based on enzyme dilution experiments (Hearn et al., 1971). The data are given as the standard free-energy change for the reaction, ΔG. The line through the two sets of data is calculated from the calorimetric results of Hearn et al. (1971) and the integrated Gibbs–Helmholz equation, $\Delta G = A - BT \ln T - CT^2 + DT$, where the values of A, B, and C are determined from the calorimetric data and D is obtained by fitting these data to the K_D determinations. Hearn et al. (1971) calculated $D = 98.4053$ with 4 points and we use here $D = 98.4042$ in order to fit better both sets of K_D values (7 points): (—), $D = 98.4053$; (- - -) $D = 98.4042$.

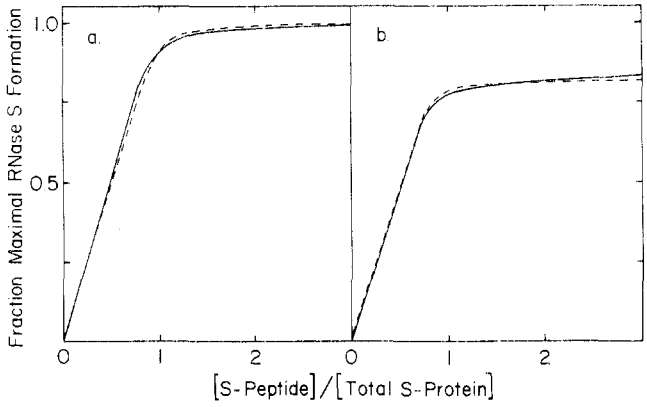

FIGURE 8: Computed titration curves for a hypothetical mixture of two kinds of RNase S molecules with $K_D = 10^{-6}$ M (20%) and with $K_D = 10^{-9}$ M (80%). The S-peptide is assumed to be uniform and the S-protein to be a 20:80 mixture: (a) total S-protein concentration, 10^{-5} M; the dashed line shows the expected curve at 10^{-5} M for a homogeneous sample with $K_D = 10^{-7}$ M; (b) total S-protein concentration, 10^{-7} M, dashed line shows the expected curve for a homogeneous sample with $K_D = 10^{-9}$ at an S-protein concentration of 0.82×10^{-7} M.

and Wold, 1972a,b), or by specific enzyme activity, making use of the loss in activity when RNase S dissociates (Richards and Vithayathil, 1959; Hearn et al., 1971). Our value for K_D at pH 7.0, 0 °C, agrees with the high-temperature enzyme activity measurements of Hearn et al. (1971) when their calorimetric data for the binding of S-peptide to RNase S are used to extrapolate their measurements of K_D from 25 to 40 °C down to 0 °C.

We have now measured K_D as a function of temperature and find a van't Hoff ΔH in good agreement with the calorimetric data of Hearn et al. (1971). Moreover, our measurements of K_D at different low temperatures at pH 6.9 agree satisfactorily with their 25–40 °C data at pH 7.0, using the calorimetric results to connect the two sets of data (Figure 7). The circular dichroism titration studies of Filippi et al. (1975) measure α-helix formation at 222 nm, which should reflect strongly α-helix formation by residues 3–13 of the S-peptide. One can calculate from their data (see their Figure 3) that K_D is not greater than 10^{-7} M at pH 6.8, 25 °C, in agreement with our data and those of Hearn et al. (1971).

The high values of K_D obtained by other spectrophotometric titrations may reflect the presence of some molecules which are not completely native and which dissociate more readily than RNase S. Figure 8 shows computed titration curves at two concentrations for a hypothetical mixture of two kinds of RNase S molecules: 20% nonnative, $K_D = 10^{-6}$ M; 80% native, $K_D = 10^{-9}$ M. The S-peptide is assumed to be uniform and the S-protein to be a 20:80 mixture. When the titration is made at a total S-protein concentration of 10^{-5} M the curve shown in Figure 8a is found. One would calculate an apparent K_D of $\sim 10^{-7}$ M from this curve if the sample is assumed to be homogeneous. The S-protein concentration needed for spectrophotometric titrations is usually $1–2 \times 10^{-5}$ M. When K_D is measured by titration of enzymatic activity, 100-fold lower concentrations can be used. In this concentration range, the less stable molecules (20% of the hypothetical mixture above) will be largely dissociated, and only the titration which produces native RNase S (80% of the hypothetical mixture) will be observed. Figure 8b illustrates this case. An apparent K_D of $\sim 10^{-9}$ M would be computed from this curve. Note that the apparent saturation of S-protein with S-peptide is achieved at a smaller ratio of S-peptide/total S-protein than in Figure 8a.

(d) *α-Helix Formation by Free S-Peptide.* The data shown in Figure 5, for the rate of ^3H exchange from S-peptide at 0 °C, pH 4.3, have some interesting implications. They suggest that kinetic measurements of proton exchange may provide a sensitive probe for the formation of weak secondary structure in unfolded proteins and peptides. In the model compound *N*-methylacetamide, the rate of proton exchange increases only by 15% on decreasing the NaCl concentration from 2.8 to 0 M (Schleich et al., 1971). In Figure 5, the rate of proton exchange in ^3H-labeled S-peptide decreases by a factor of 3 as the NaCl concentration is decreased from 1.5 to 0.001 M, indicating the presence of structure that is destabilized at low ionic strengths causing an increase in the exchange rate. In view of the circular dichroism study by Brown and Klee (1971), this structure is almost certainly α-helix formation. Using our present technique, it would be difficult to measure faster rates of exchange at higher pHs or temperatures. However, other techniques could be used: stopped-flow infrared studies or NMR measurements of proton spectra in H_2O using correlation spectroscopy.

It should be noted that the exchange of one very slow proton, whose exchange half-time is about 20 min, has been subtracted from the data shown in Figure 5. This proton may be the amide proton next to the ionized α-carboxyl group; according to Molday et al. (1972) a 20-fold decrease in exchange rate is expected for this proton.

References

Anderson, D. G., Hammes, G. G., and Walz, F. G., Jr. (1968), *Biochemistry 7*, 1637–1645.

Brown, J. E., and Klee, W. A. (1971), *Biochemistry 10*, 470–476.

Dunn, B. M., and Chaiken, I. M. (1975), *J. Mol. Biol. 95*, 497–511.

Englander, S. W., Downer, N. W., and Teitelbaum, H. (1972), *Annu. Rev. Biochem. 41*, 903–924.

Englander, S. W., and Poulsen, A. (1969), *Biopolymers 7*, 379–393.

Filippi, B., Moroder, L., Borin, G., Samartsev, M., and Marchiori, F. (1975), *Eur. J. Biochem. 52,* 65–76.
Finn, F. M., Dadok, J., and Bothner-By, A. A. (1972), *Biochemistry 11,* 455–461.
Gawronski, T. H., and Wold, F. (1972a), *Biochemistry 11,* 442–448.
Gawronski, T. H., and Wold, F. (1972b), *Biochemistry 11,* 449–455.
Hearn, R. P., Richards, F. M., Sturtevant, J. M., and Watt, G. D. (1971), *Biochemistry 10,* 806–817.
Leichtling, B. H., and Klotz, I. M. (1966), *Biochemistry 5,* 4026–4037.
Markley, J. L. (1975), *Biochemistry 14,* 3546–3554.
Molday, R. S., Englander, S. W., and Kallen, R. G. (1972), *Biochemistry 11,* 150–158.
Patel, D. J., Canuel, L. L., Woodward, C., and Bovey, F. A. (1975), *Biopolymers 14,* 959–974.
Privalov, P. L., and Khechinashvili, N. N. (1974), *J. Mol. Biol. 86,* 665–684.
Richards, F. M., and Vithayathil, P. J. (1959), *J. Biol. Chem. 234,* 1459–1465.
Richards, F. M., and Wyckoff, H. C. (1970), *Enzymes, 3rd Ed., 3,* 647–806.
Rocchi, R., Borin, G., Marchiori, F., Moroder, L., Peggion, E., Scoffone, E., Crescenzi, V., and Quadrifoglio, F. (1972), *Biochemistry 11,* 50–57.
Schleich, T., Rollefson, B., and von Hippel, P. H. (1971), *J. Am. Chem. Soc. 93,* 7070–7074.
Schreier, A. A. (1977), *Anal. Biochem.* in press.
Schreier, A. A., and Baldwin, R. L. (1976), *J. Mol. Biol. 105,* 409–426.
Woodfin, B. M., and Massey, V. (1968), *J. Biol. Chem. 243,* 889–892.
Woodward, C. K., and Rosenberg, A. (1971a), *J. Biol. Chem. 246,* 4105–4113.
Woodward, C. K., and Rosenberg, A. (1971b), *J. Biol. Chem. 246,* 4114–4121.
Wyckoff, H. W., Tsernoglou, D., Hanson, A. W., Knox, J. R., Lee, B., and Richards, F. M. (1970), *J. Biol. Chem. 245,* 305–328.

Synthesis and Biological Activity of a λ Pseudo Operator†

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ABSTRACT: The chemical and enzymatic syntheses of bacteriophage λ pseudo operator DNA are described. The 17 base-paired duplex contains the DNA which has been proposed as the binding site for cI repressor protein. The synthetic duplex is twofold symmetric and represents the best possible nucleotide summation of the six proposed operator sites in the leftward and rightward operators. However, it does not correspond exactly to any single proposed operator sequence. The chemical synthesis includes the deoxyoligonucleotides d(T-A-T-C-A-C), d(C-G-C-C-G-G-T-G-A-T-A), d(T-A-T-C-A-C-C), and d(G-G-C-G-G-T-G-A-T-A). These deoxyoligonucleotides were joined with T4 DNA ligase to form d(T-A-T-C-A-C-C-G-C-C-G-G-T-G-A-T-A) and d(T-A-T-C-A-C-C-G-G-C-G-G-T-G-A-T-A). The cI repressor protein was found to bind to the duplex formed from these two segments.

An important unsolved problem at the gene level of regulation is how various proteins interact with DNA control regions to regulate transcription. The major leftward and rightward control regions of bacteriophage λ could prove to be very useful for studying this problem. These regions are illustrated in the simplified drawing shown in Figure 1. The cI repressor binds at multiple sites (operators) within these control regions (Maniatis and Ptashne, 1973a,b). *Escherichia coli* RNA polymerase transcribes from the leftward promoter P_L toward gene N (Hershey, 1971) and in opposite directions from the rightward promoters P_R and P_{RM} toward cro and cI, respectively (Meyer et al., 1975). Accumulated evidence also indicates that sequences within the operators are recognized by *E. coli* RNA polymerase as part of the promoter sites (Maniatis et al., 1973; Maurer et al., 1974; Allet et al., 1974). Finally, the cro protein appears to bind to the same or similar sequences as does the cI repressor (Folkmanis et al., 1976). Therefore, within these closely linked control regions can be found three promoters and multiple operator sites. These interact with cI repressor, *E. coli* RNA polymerase, and cro protein to regulate the lysogenic and lytic responses of bacteriophage λ (Ptashne et al., 1976; Folkmanis et al., 1976). We would like to contribute toward an understanding of how these various functional units interact to attenuate and direct transcription. The first steps in this study—the chemical and enzymatic syntheses of a pseudo λ operator DNA and a demonstration of its biological activity—are presented in this paper.

Our initial objective is to define the cI repressor binding sites. The approach involves the chemical and enzymatic syntheses of λ control DNA. Although cI repressor binding sequences remain to be established, considerable evidence suggests that six partially symmetric regions in both leftward and rightward control DNA define the sites (Maniatis et al., 1975). Basic assumptions for research reported in this paper are that these partially symmetric sequences constitute the λ repressor binding sites and that the completely symmetric operator se-

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