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Measurement of the Refolding Combination Reaction between S-Peptide and S-Protein†

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ABSTRACT: S-Peptide combines with S-protein during the refolding of ribonuclease S. The kinetics of combination have now been measured by a specific probe, the absorbance (492 nm) of a fluoresceinthiocarbamyl (FTC) group on lysine-7 of S-peptide. pK changes of the FTC group detect both initial combination and later, first-order, stages in folding. Combination with the slow-folding species of S-protein occurs with a half-time of 0.4 s at 50 μM, whereas complete folding takes 50 s (pH 6.8, 31 °C). Thus combination takes place at an early stage in folding. The second-order rate constant of the refolding combination reaction (5×10^4 M^{-1} s^{-1}) is 100-fold smaller than that for combination with folded S-protein, which probably reflects the lower affinity of S-protein for S-peptide in the initial complex. Inhibition by S-peptide of combination between FTC-S-peptide and S-protein shows that the refolding combination reaction is specific and reversible. Both the fast-folding and slow-folding species of unfolded S-protein participate in the refolding combination reaction.

Ribonuclease S (RNase S)[1] dissociates into S-protein plus S-peptide upon unfolding, and these reassociate during refolding (Richards & Logue, 1962; Labhardt & Baldwin, 1979a). This property can, in principle, be used to characterize the site at which combination occurs during folding and to determine the interactions responsible for combination. This may be an effective way of studying how recognition takes place between different parts of a protein during folding. The first step in this work is to find a probe that detects combination during folding and then to measure the kinetics of combination. Initial combination is not detected by changes in tyrosine absorbance or fluorescence in the refolding of unlabeled RNase S (Labhardt & Baldwin, 1979a), and the refolding combination reaction has also not yet been observed by circular dichroism (A. M. Labhardt, unpublished results). We report here that an FTC group covalently attached to the ϵ-NH_2 group of Lys-7 by the Edman reaction provides a probe that detects the refolding combination reaction.

Unfolded RNase S contains both fast-folding (U_F) and slow-folding (U_S) species, as does RNase A. The U_F and U_S species have different refolding pathways that have been

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[1] Abbreviations: RNase A, bovine pancreatic ribonuclease A; RNase S, a derivative of RNase A in which the peptide bond between residues 20 and 21 has been cleaved; S-peptide, residues 1-20 of RNase S; S-protein, residues 21-124 of RNase S; FTC, fluoresceinthiocarbamyl; FTC-S-peptide, des-1,7^t-FTC-S-peptide; FTC-RNase S, FTC-S-peptide combined with S-protein; U_F and U_S, fast-folding and slow-folding forms of an unfolded protein; p, S-peptide (or FTC-S-peptide); pN, native RNase S (or FTC-RNase S); A, absorbance; GdmCl, guanidinium chloride; t_m, temperature midpoint of a thermal unfolding transition; τ, time constant of a reaction (reciprocal of the apparent rate constant); C>p, cytidine 2',3'-phosphate; 2'-CMP, cytidine 2'-phosphate; k_{cat}, turnover number; K_m, Michaelis-Menten constant; Tris, tris(hydroxymethyl)aminomethane; $NaDodSO_4$, sodium dodecyl sulfate; CD, circular dichroism; CM, carboxymethyl.

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studied previously (Labhardt & Baldwin, 1979a; Labhardt, 1980, 1982b). The U_S:U_F ratio has been measured accurately for RNase A as 82:18 (F. X. Schmid, unpublished results) and approximately for RNase S as 75:25 (Labhardt, 1982b). Available evidence for RNase A indicates that the U_S species are produced after unfolding by the slow cis–trans isomerization of essential proline residues (Schmid & Baldwin, 1978; L.-N. Lin and J. F. Brandts, unpublished results). S-Protein has all four proline residues of RNase A and probably has the same U_S species. The major unfolded species of RNase A is a slow-folding species that is denoted as U_S^{II} (Schmid & Blaschek, 1981). If U_S^{II} is produced by proline isomerization after unfolding, then proline isomerization must occur as the last step in the folding of U_S^{II} in strongly native folding conditions where structural folding intermediates are stable. U_S^{II} folds at 0–10 °C to give a well-populated, nativelike intermediate I_N before proline isomerization occurs (Cook et al., 1979; Schmid, 1981; Schmid & Blaschek, 1981). At least one intermediate preceding I_N in the folding of RNase A has been detected by measurements of protection of peptide NH protons against exchange with water (Schmid & Baldwin, 1979; Kim & Baldwin, 1980).

The experiments reported here do not distinguish between different U_S species, and we refer to them collectively as U_S. We assume here that proline isomerization gives rise to the U_S species. Fast CD measurements of RNase S refolding (Labhardt, 1980, 1982a,b; A. M. Labhardt, unpublished results) show that structural intermediates with distinctive CD spectra are observed in the refolding of U_S. Both because U_S is the major unfolded form and because there is other evidence for well-populated structural intermediates in the folding of U_S, we ask here whether FTC-S-peptide can combine with an early folding intermediate of U_S.

The pathway of folding of U_S is written as

$$U_S \rightleftharpoons I_1 \quad (1)$$

$$p + I_1 \rightleftharpoons pI_1 \quad (2)$$

$$pI_1 \rightleftharpoons \ldots pN \quad (3)$$

Here I_1 is the first folding intermediate formed by U_S that has a binding site for S-peptide (p), N stands for folded S-protein, and pN stands for native RNase S. The experiments described here indicate that proline isomerization occurs after pI_1 is formed, so that I_1 contains a wrong proline isomer, as does U_S.

There should be a corresponding intermediate I_2 on the folding pathway of U_F. I_2 differs from I_1 in having correct

$$U_F \rightleftharpoons I_2 \quad (4)$$

$$p + I_2 \rightleftharpoons pI_2 \quad (5)$$

$$pI_2 \rightleftharpoons \ldots pN \quad (6)$$

proline isomers and possibly also in other respects. Previous evidence for the existence of pI_2 is based on the facts that RNase S refolds more rapidly than S-protein alone except at very low concentrations, when the folding reactions of the U_F forms are compared, and that the RNase S folding rate increases strongly with concentration (Labhardt & Baldwin, 1979a). This means that a complex between S-protein and S-peptide, pI_2, is formed early in folding and that the complex folds more rapidly than S-protein itself. At low concentrations, around 1 μM, reaction 5 is slow enough that folding can also occur by the following pathway:

$$U_F \rightleftharpoons \ldots N \quad (7)$$

$$p + N \rightleftharpoons pN \quad (8)$$

A change in pathway from reactions 7 and 8 at low concentration to reactions 4–6 at higher concentrations can explain the apparent 1.6-order kinetics of the refolding of the U_F species of RNase S (Labhardt & Baldwin, 1979a).

A folded species of S-protein is also present at pH 1.7, below 40 °C (Labhardt & Baldwin, 1979b). It has been denoted as I_3 because S-protein undergoes a pH-induced unfolding transition below pH 7 that appears to be complete near pH 2.2, 22 °C, when measured by tyrosine absorbance [Richards & Logue, 1962; cf. also Shindo & Cohen (1976)], and I_3 appears to be a loosely folded species. I_3 is measured by the property of combining rapidly with S-peptide at pH 6.8. In this respect, it is no different from the neutral-pH form of folded S-protein (see Table I). Two steps have been resolved in the formation of native RNase S from $p + I_3$:

$$p + I_3 \rightleftharpoons pI_3 \quad (9)$$

$$pI_3 \rightleftharpoons pN \quad (10)$$

Reaction 10, which is first order, can be measured at concentrations above 32 μM by using 2′-CMP binding as a fast probe for the formation of the substrate binding site. pI_3 has a half-life of 68 ms at 20 °C, pH 6.8 (Labhardt & Baldwin, 1979b). It is not known whether the formation of native RNase S from p + N (the neutral pH form of folded S-protein) also involves a measurable first-order step after combination. I_3 shows a broad thermal unfolding transition at pH 1.7 with a t_m of 23 °C, as measured by combination with S-peptide at pH 6.8; I_3 is in slow equilibrium with U_F and U_S (Labhardt & Baldwin, 1979b). In order to eliminate I_3 from the species present before refolding, we added 1 M GdmCl (31 °C, pH 1.7) in some experiments, which is diluted to 0.13 M GdmCl in the pH 1.7 → pH 6.8 jump that initiates refolding.

Materials and Methods

Materials. 2′-CMP, C>p, S-peptide, and RNase S were purchased from Sigma Chemical Co. The RNase S was purified by chromatography on CM-Sephadex (Pharmacia) according to Garel (1976), and some S-peptide was also separated from the S-protein according to Doscher & Hirs (1967). The concentrations of stock samples (which were stored frozen) were determined by quantitative amino acid analysis: the standards were run concurrently. Pyridine, triethylamine, and ethyl acetate were purchased from J. T. Baker while all other materials were reagent grade. Solutions were prepared with quartz-distilled water.

Preparation of FTC-S-peptide. Des-1,7$^{\epsilon}$-FTC-S-peptide was prepared by subtractive Edman degradation of S-peptide. Twenty milligrams of S-peptide (Sigma) was dissolved in 3 mL of H_2O and combined with a solution of 145 mg of 5-fluorescein isothiocyanate (Eastman) in 2.5 mL of triethylamine and 7 mL of pyridine. This mixture was incubated at 37 °C in the dark, under argon, in a glass-stoppered test tube for 3 h. After the incubation, 4 mL of ethyl ether and 2 mL of H_2O were added, and the mixture was extracted 3 times with 4 mL of ethyl acetate (saturated with H_2O). The aqueous layer was dried at 60 °C in a vacuum drying oven. Six milliliters of anhydrous trifluoroacetic acid was added to the residue, and it was incubated at 37 °C for 1 h. The excess trifluoroacetic acid was evaporated under a stream of argon.

The residue was dissolved in 0.1 M KCl, centrifuged to clear the insoluble material, and chromatographed on a Sephadex G-25 column (1.5 × 30 cm). The major peak fractions (A 490 nm) were pooled, lyophilized, and desalted on a Sephadex G-10 column (1.8 × 27 cm) to give 20 mg of FTC-S-peptide.

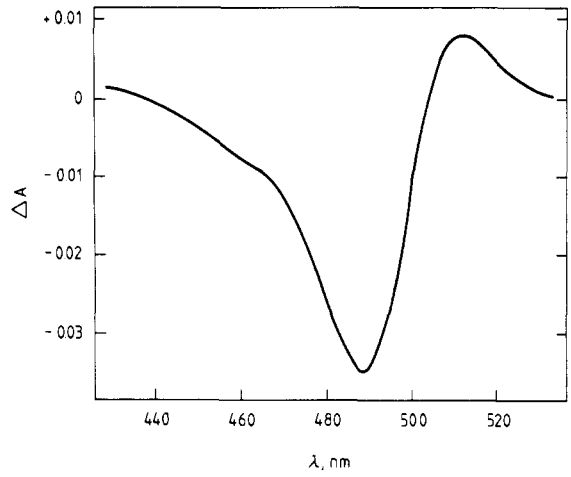

FIGURE 1: Equilibrium difference spectrum between FTC-RNase S and FTC-S-peptide (reference cell) at pH 6.8, 22 °C, in 0.1 M sodium cacodylate, 8.8 μM, with 1-cm cells; A_{490} = 0.34 for FTC-S-peptide alone.

The product exhibited a single fluorescent spot on urea–Na-DodSO$_4$ slab gel electrophoresis (Swank & Munkres, 1971). The extinction coefficient of FTC-S-peptide is 77 000 ± 4000 M^{-1} cm^{-1} at 488 nm, pH 11, as determined by quantitative amino acid analysis with standards run concurrently (Ridge, 1978).

Stopped-Flow Measurements. These were made as described by Labhardt & Baldwin (1979a).

Results

(A) Properties of FTC-RNase S. (I) Difference Spectrum. Figure 1 shows the equilibrium difference spectrum between FTC-S-peptide and FTC-RNase S at 22 °C, pH 6.8. There is a 10% decrease in absorbance at λ_{max} (488 nm) upon combination with S-protein. Difference spectra of the type shown in Figure 1, taken at several pHs near 6.8, can be represented by synthetic spectra resulting from a pK shift of +0.20 and a red shift of $\Delta\lambda_{max}$ = +1.5 nm on combination.

(II) Stoichiometry. Figure 2a shows the reaction half-time as a function of concentration for combination between folded S-protein (pH 6.8) and FTC-S-peptide, as measured by FTC fluorescence. The concentration of FTC-S-peptide is held fixed at 0.1 μM, and the ratio r of S-protein to FTC-S-peptide is varied (r = 0, 1, 2, ...). In the pseudo-first-order range ($r \geq 4$), the slope of ln τ vs. ln r is −1, and the second-order rate constant is 4.8 × 10^6 M^{-1} s^{-1}. The reaction is complete within 1 s at all concentrations shown. When S-protein is omitted, no kinetics are seen.

The amount of complex formed, measured by the change in fluorescence intensity, is plotted against r in Figure 2b. Within experimental error, the graph is a titration curve with a sharp end point at a 1:1 mole stoichiometry. Similar experiments have been made with RNase A in place of S-protein. No systematic kinetics are seen in this case.

(III) Enzymatic Activity. With C>p as the substrate, the enzymatic activities of FTC-RNase S and RNase S are nearly the same when assayed at 20 °C, pH 7.15, in 0.1 M Tris-HCl buffer–0.1 M NaCl, by the method of Crook et al. (1960). The value found for k_{cat} is 4 s^{-1} for both RNase S and FTC-RNase S, and the values found for K_m are 37 mM and 32 mM, respectively.

(IV) Unfolding Transition. Figure 3 shows the thermal unfolding transition of FTC-RNase S at pH 6.8, as measured by A_{492}. The FTC chromophore monitors overall unfolding in a cooperative transition like that of RNase S monitored by tyrosine absorbance. The t_m is 46 °C at 8.8 μM. At 8.8 μM, the t_m of RNase S measured by CD is some 4 °C lower (Labhardt, 1981), suggesting that the association constant of FTC-S-peptide with S-protein is larger than that of unlabeled S-peptide [cf. Labhardt (1981)].

(V) Reversibility. Figure 4b shows the displacement of FTC-S-peptide from FTC-RNase S by excess S-peptide. The reaction follows apparent first-order kinetics with τ = 72 s at 31 °C, pH 6.8. The change in fluorescence intensity upon displacement is equal and opposite to the intensity change on forming FTC-RNase S from S-protein and FTC-S-peptide (Figure 4a). Thus formation of FTC-RNase S is a reversible reaction. In a similar experiment, Richards & Logue (1962) found that S-peptide displaces tetramethyl-S-peptide from tetramethyl-RNase S with a half-time of 60 s at pH 4.5, 22 °C, and Niu et al. (1980) found that S-peptide very slowly (in hours) displaces ^{13}C-labeled peptide residues 1–15 from combination with S-protein at 4 °C, pH 5.4.

(B) Rapid Combination between FTC-S-peptide and Folded S-Protein. Figure 5 shows the reaction half-time as a function of concentration for combination between I_3 and FTC-S-peptide at 31 °C, pH 6.8. The second-order rate constant is similar (4-fold higher) to that observed previously for combination with S-peptide (Labhardt & Baldwin, 1979b; see

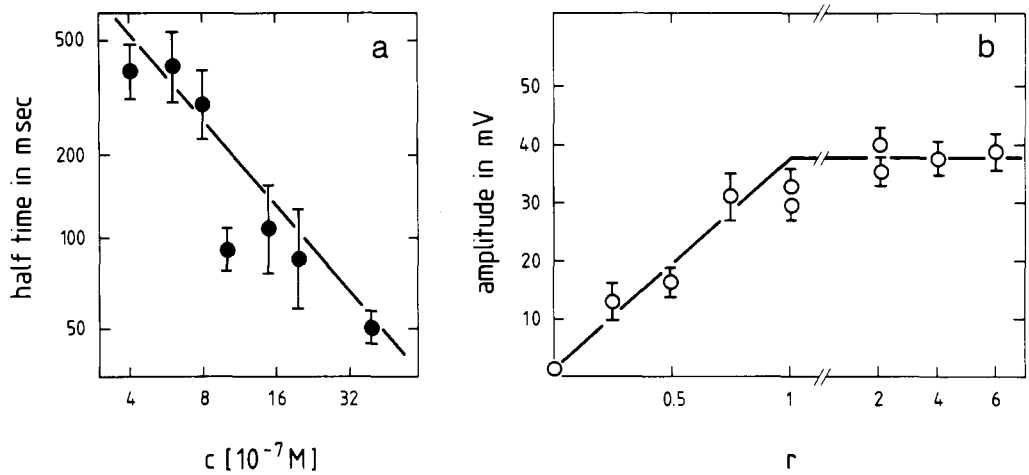

FIGURE 2: (a) Kinetics of combination between 0.1 μM FTC-S-peptide and folded S-protein (pH 6.8, 31 °C), measured by FTC fluorescence (see text for details). The reaction half-time is shown as a function of concentration of S-protein. (b) Titration of FTC-S-peptide against folded S-protein (pH 6.8, 31 °C). The amplitude of the combination reaction (a) is shown as a function of the ratio (r) of S-protein to FTC-S-peptide. Fluorescence intensity was measured with excitation at 494 nm, observation at λ ≥ 515 nm.

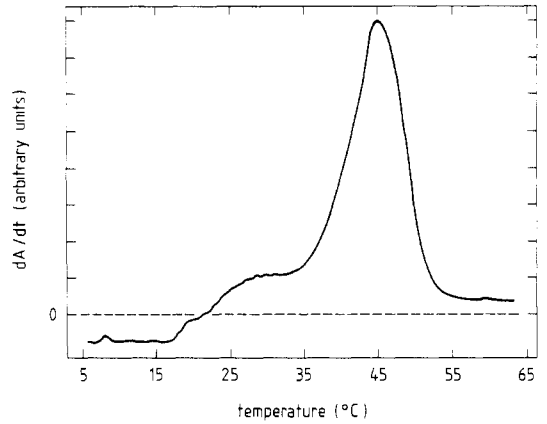

FIGURE 3: Thermal unfolding transition of 8.8 μM FTC-RNase S at pH 6.8; the derivative curve of A_{492} vs. temperature is shown (see Figure 1 for conditions).

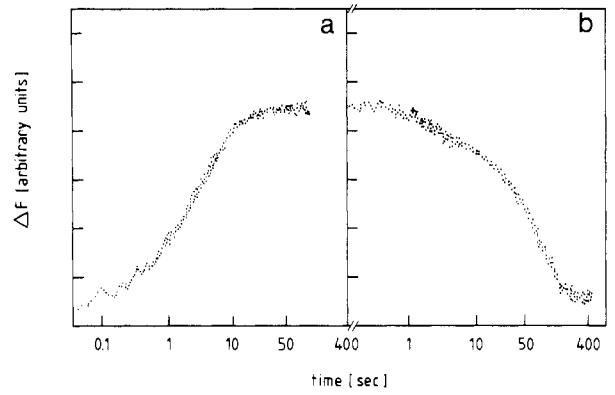

FIGURE 4: (a) Kinetic changes in fluorescence intensity measured during combination of 0.1 μM FTC-S-peptide with 0.1 μM folded S-protein at 31 °C, pH 6.8, in 0.1 M sodium cacodylate buffer; excitation was at 494 nm, observation was at wavelengths larger than 515 nm. (b) Displacement kinetics: 500-fold excess unlabeled S-peptide displaces FTC-S-peptide from FTC-RNase S in the same conditions as in (a).

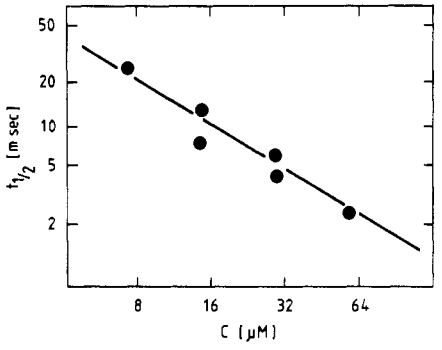

FIGURE 5: Second-order kinetics of combination between FTC-S-peptide and I_3 (pH 1.7 folded form of S-protein), measured at 31 °C, pH 6.8, in 0.05 M sodium cacodylate–0.05 M $NaClO_4$, by FTC absorbance at 492 nm.

Table I). Combination is measured by FTC absorbance after a pH jump (pH 1.7 → pH 6.8). The second-order rate constant is 6.7×10^6 M^{-1} s^{-1}, which agrees with the less precise value given above for combination of FTC-S-peptide with N.

Figure 6 shows the thermal unfolding transition of I_3 at pH 1.7, as measured by combination with FTC-S-peptide after a stopped-flow pH jump to pH 6.8. It agrees with the transition curve measured previously by combination with S-peptide (Labhardt & Baldwin, 1979b) and allows this reaction to be identified as the combination of p with I_3. When 1 M GdmCl is added in the initial conditions (pH 1.7, 31 °C), this

Table I: Association–Dissociation Reactions of S-Peptide with S-Protein[a]

	RNase S	FTC-RNase S[a]
	(A) $p + N \rightleftharpoons pN$	
K_A	10^7 M^{-1} (30 °C)[b]	(3×10^8 M^{-1}) (31 °C)
k_{on}	(1.2×10^6 M^{-1} s^{-1})[c]	4.8×10^6 M^{-1} s^{-1}
k_{off}	(1.2×10^{-1} s^{-1})	1.4×10^{-2} s^{-1}
	(B) $p + I_3 \rightleftharpoons pI_3$	
k_{on}	1.2×10^6 M^{-1} s^{-1} (30 °C)[d]	6.7×10^6 M^{-1} s^{-1} (31 °C)
	7×10^5 M^{-1} s^{-1} (20 °C)[d]	2.3×10^6 M^{-1} s^{-1} (20 °C)
	2×10^5 M^{-1} s^{-1} (20 °C, 0.5 M GdmCl)[e]	
	(C) $p + I_1 \rightleftharpoons pI_1$	
K_A	(6×10^4 M^{-1}) (32–47 °C)[f]	
k_{on}	(10^4 M^{-1} s^{-1}) (31 °C)	5×10^4 M^{-1} s^{-1} (31 °C)
		3×10^4 M^{-1} s^{-1} (31 °C, 0.13 M GdmCl)

[a] All values refer to pH 6.8 and either 0.1 M sodium cacodylate or 0.05 M sodium cacodylate–0.05 M $NaClO_4$; values given in parentheses are calculated from other data (e.g., K_A may be given as the ratio of measured values of k_{on} and k_{off}); all data for FTC-RNase S are from this work. [b] Hearn et al. (1971); Labhardt (1981). [c] k_{on} has been found to be the same for $p + N \rightarrow pN$ as for $p + I_3 \rightarrow pI_3$ (A. M. Labhardt, unpublished results). [d] Labhardt & Baldwin (1979b). [e] Labhardt (1982b). [f] Labhardt (1981).

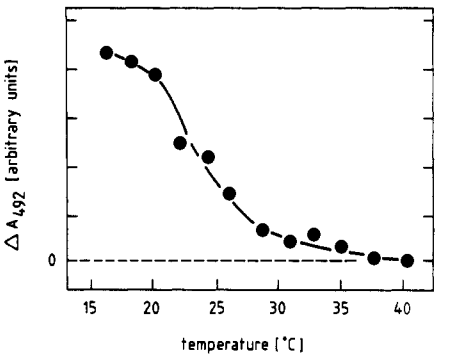

FIGURE 6: Thermal unfolding transition of I_3 at pH 1.7 measured by the extent of combination between FTC-S-peptide and S-protein after a pH jump from pH 1.7 to pH 6.8; 0.05 M sodium cacodylate, 0.05 M $NaClO_4$, 8.8 μM S-protein, and FTC-S-peptide.

reaction disappears completely, which confirms its assignment as the combination of p with I_3.

(C) Detection of the Refolding Combination Reaction by the FTC Probe. When I_3 is eliminated by adding 1 M GdmCl at pH 1.7, only two refolding reactions are observed at pH 6.8, 31 °C, by FTC absorbance. Their time constants as a function of concentration are shown in Figure 7a, and their relative amplitudes are given in Figure 7b. One reaction is second order and is seen over the entire concentration range studied (0.5–50 μM); the other reaction is first order and is not seen below 2 μM. The extent of the first-order reaction increases linearly with concentration above 2 μM. Its time constant (50 s) indicates that it is part of the folding process of the U_S species [cf. Labhardt & Baldwin (1979a)]. The relative amplitudes of the two reactions are shown in Figure 7b.

We identify the second-order reaction as the sum of the two combination reactions 2 and 5, involving complex formation between FTC-S-peptide and intermediates in the folding of both U_F and U_S. The first-order reaction represents a late stage in the $pI_1 \rightleftharpoons$ ...pN conversion; when measured by tyrosine absorbance or 2′-CMP binding, the kinetics of this reaction show some complexity (Labhardt & Baldwin, 1979a). The first-order reaction is not observed at concentrations below 2 μM because then combination is slow enough to be rate lim-

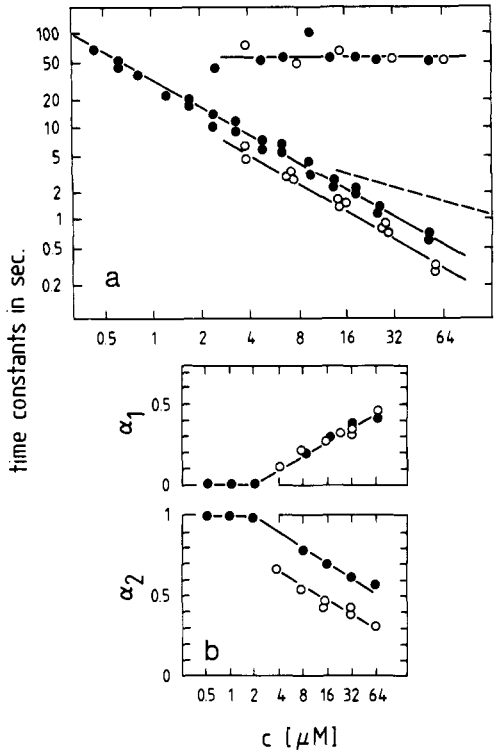

FIGURE 7: (a) Kinetics of combination between FTC-S-peptide and S-protein at equimolar concentrations during folding after a pH jump from pH 1.7 to pH 6.8, 31 °C, in 0.05 M sodium cacodylate–0.05 M $NaClO_4$: (filled circles) 1 M GdmCl present at pH 1.7 and 0.13 M GdmCl present at pH 6.8; (open circles) no GdmCl present. The kinetics of a later first-order folding reaction are also shown. The FTC absorbance at 492 nm is measured. The half-time of the combination reaction and the time constant of the first-order folding reaction are shown as a function of concentration. Previous measurements of the $p + U_F \rightarrow pN$ reaction of RNase S (Labhardt & Baldwin, 1979a) are shown as a dashed line for comparison. (b) The relative amplitudes are given as a function of initial FTC-S-peptide (=S-protein) concentration: (filled circles) 1 M GdmCl present initially at pH 1.7; (open circles) no GdmCl; see (a) for conditions. A third reaction is also present when no GdmCl is present initially: see Figure 5. α_1 and α_2 are the amplitudes of the first- and second-order reactions, divided by the total amplitude, for the two reactions shown in (a).

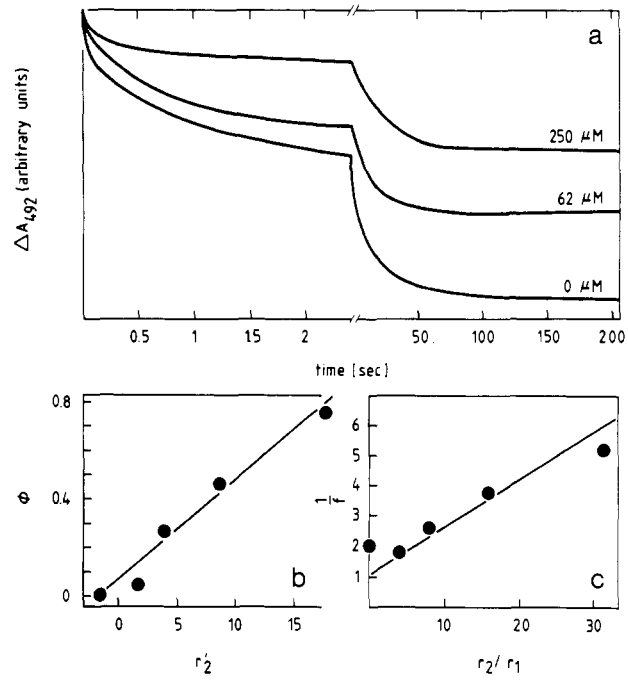

FIGURE 8: (a) Competition between S-peptide and FTC-S-peptide for S-protein during refolding. Formation of a complex between FTC-S-peptide and S-protein is measured by absorbance at 492 nm at fixed concentrations of S-protein (9.6 μM) and of FTC-S-peptide (8 μM) at varying concentrations of added S-peptide (from top to bottom, 250, 62, and 0 μM). Complex formation is measured in two time ranges: (range a) 2.4–200 s; (range b) 0–2.4 s. Conditions: refolding is initiated by a pH jump at 31 °C from pH 1.7 to pH 6.8, in 0.05 M sodium cacodylate–0.05 M $NaClO_4$. (b) Degree of competition after 200 s of refolding and equilibration. The final amount of FTC-RNase S formed is measured from the overall absorbance change in both time ranges, 0–200 s, and is divided by the amount formed in the absence of added S-peptide to give the fraction f from which the ordinate ϕ is computed (see Appendix). The abscissa r_2' depends chiefly on the ratio of total S-peptide to total S-protein concentration. At equilibrium, this plot gives a straight line whose slope measures K_1/K_2, the ratio of the binding constants to S-protein of FTC-S-peptide and of S-peptide. The solid line corresponds to $K_1/K_2 = 20.6$. (c) Degree of competition during the initial 2.4 s of refolding. The data are plotted according to eq a4, which assumes that FTC-S-peptide and S-peptide compete only kinetically for S-protein so that the complexes formed within the first 2.4 s do not equilibrate with each other. The slope of the line gives the ratio of the two on-rates for complex formation in this case (see Appendix). The solid line corresponds to the case in which FTC-S-peptide combines with refolding S-protein 5 times more rapidly than S-peptide combines with refolding S-protein.

iting. The change in relative amplitudes of the two reactions with concentration (Figure 7b) shows that the rate-limiting step in the overall reaction changes with concentration.

Because the folding of the U_F species of RNase S is not observed as a separate reaction, it must be superimposed on the refolding combination reaction (the first-order reaction of Figure 7 is too slow to mask the folding of $p + U_F$). The kinetics of the $p + U_F \rightarrow pN$ reaction, as measured previously for unlabled RNase S (Labhardt & Baldwin, 1979a), are shown by the dashed line in Figure 7a. It can be seen that the second-order combination reaction measured in Figure 7a is always as fast as, or faster than, the $p + U_F \rightarrow pN$ reaction. It is likely that reactions 2 and 5 have similar second-order rate constants for combination of p with I_1 and with I_2. By analogy with the behavior shown in Figure 7a for U_S, one might expect to resolve an additional first-order step in the refolding of U_F at some threshold concentration above 8 μM. Some kinetic complexity has been observed in the refolding combination reaction at high concentrations, but we have not tried to resolve two separate reactions.

(D) Inhibition of the Refolding Combination Reaction by Unlabeled S-Peptide. To test whether combination between FTC-S-peptide and refolding S-protein is specific, we have measured competition in this reaction between S-peptide and FTC-S-peptide (Figure 8). The concentrations of FTC-S-peptide (8 μM) and of S-protein (9.6 μM) are held constant, and the concentration of S-peptide is varied. Refolding is measured after a pH jump (pH 1.7 → pH 6.8) at 31 °C, as before. The extent of combination between FTC-S-peptide and S-protein is determined (a) at 200 s after initiating refolding, a time that is long enough to allow for complete refolding and nearly complete equilibration between all components, and (b) at 2.4 s, which is the half-time for combination in the absence of added S-peptide.

Figure 8a shows the progress curves for binding of FTC-S-peptide to refolding S-protein as a function of increasing S-peptide concentration. It is evident that S-peptide does compete with FTC-S-peptide for S-protein in both time ranges. In range a, a steady decrease in the extent of labeled complex formation is observed with increasing amounts of S-peptide. In range b, one observes the expected speeding up of the combination reaction together with a decrease in the amount of the FTC-S-peptide–S-protein complex. The data for range

a have been plotted in Figure 8b according to eq a2 (see Appendix), which assumes that there is a simple, equilibrium binding competition between S-peptide and FTC-S-peptide. The justification is that all reactions, association ($p + I_1 \rightarrow pI_1$), folding ($pI_1 \rightarrow pN$), and dissociation ($pN + p^* \rightarrow p^*{\cdot}N + p$ and vice versa), are faster than 200 s. The slope of Figure 8b measures the ratio of the equilibrium binding constants and is found to be about 20, with FTC-S-peptide forming a tighter complex with S-protein than does S-peptide.

The extent of binding of FTC-S-peptide to S-protein in range b (2.4 s) is plotted according to eq a4 in Figure 8c. This equation assumes that the ratio of the association rates $k_{on}^{S\text{-}pep}$ and k_{on}^{FTC} determines the competition and that dissociation from either pI_1 or pN can be neglected during the initial 2.4 s. This is known to be correct for the displacement of FTC-S-peptide from pN by S-peptide, which has a time constant of 72 s at 31 °C (Figure 4). The slope in Figure 8c indicates that the on-rate for FTC-S-peptide combining with refolding S-protein is about 5 times the on-rate for unlabeled S-peptide.

Discussion

(A) Measurement of the Refolding Combination Reaction. The evidence is as follows that the new second-order reaction detected by the FTC probe represents specific combination between FTC-S-peptide and refolding S-protein. (1) The refolding combination reaction has been observed thus far only by the FTC probe. In the refolding of RNase S, recombination is not detected as a separate step, by monitoring either tyrosine absorbance or fluorescence (Labhardt & Baldwin, 1979a). (2) Nevertheless, combination during refolding is specific, as shown by the inhibition of the reaction with unlabeled S-peptide. Although the FTC group participates in forming pI_1, as judged by the relative on-rates for S-peptide and for FTC-S-peptide, combination does not result only from an interaction between the FTC group and the nonpolar side chains of S-protein. (3) The refolding combination reaction does not arise from a species that is partly folded in the initial conditions. It can be observed when only U_F and U_S are present initially (Figure 7). (4) The refolding combination reaction should be part of the folding pathway of FTC-RNase S: at concentrations low enough that combination is rate limiting for folding, it is the only reaction seen by the FTC probe (Figure 7). At higher concentrations, where combination is no longer rate limiting, the FTC probe also detects a first-order step in the folding of U_S. (5) Both the U_F and U_S species of S-protein participate in refolding combination reactions, as shown by the failure to observe folding of U_F as a separate reaction (see the discussion of Figure 7 under Results).

Combination occurs at an early stage in the folding of U_S, as shown by the half-time for combination (0.4 s at 50 μM in 0 M GdmCl) as compared to the time constant for first-order folding of U_S (50 s). The fact that the on-rate for combination between p and I_1 is 100-fold smaller than for combination between p and N confirms that combination with p occurs at an early stage ($U_S \rightleftharpoons I_1$) in the folding of U_S.

(B) Comparison with Results for Nuclease T′. The folding kinetics of another dissociable small protein, nuclease T′, have been studied (Light et al., 1974). Nuclease T′ is formed by complementation between overlapping fragments of staphylococcal nuclease. Its folding kinetics are independent of concentration down to the lowest concentration studied, 13 μM. Unlike S-protein, neither fragment of nuclease T′ can fold to form a stable structure. Consequently, combination must occur before much folding can take place, and so combination occurs at an early stage in folding as reported here for RNase S. The role of proline isomerization in the refolding kinetics of nuclease has not yet been studied, and no U_S species have been reported although nuclease contains six proline residues.

(C) Rate Constants for Association and Dissociation. Two different combination reactions between S-peptide and S-protein have now been studied: combination during folding and combination after folding of S-protein. The available data for these two reactions, including both studies of S-peptide and FTC-S-peptide, are collected in Table I. Assumed values that are calculated from other data are given in parentheses. The data for combination during folding are consistent with the formation of a less tight initial complex than the one formed after folding of S-protein.

Comparing FTC-S-peptide with S-peptide shows that the FTC group gives more rapid combination and slower dissociation in all cases. This probably reflects a tighter binding to S-protein of FTC-S-peptide than of S-peptide in each case.

Appendix

Competition between S-peptide (p) and FTC-S-peptide (p*) for binding with S-protein (P):

$$p^* + P \overset{K_1}{\rightleftharpoons} p^*P \qquad p + P \overset{K_2}{\rightleftharpoons} pP \tag{a1}$$

is described at equilibrium by

$$K_1/K_2 = \left(r_2 \frac{f}{1-f} - f\right) \Big/ (r_1 - f) \tag{a2}$$

with r_1 = (total FTC-S-peptide concentration)/c_0, r_2 = (total S-peptide concentration)/c_0, and $f = [p^*P]/c_0$, with c_0 being the total S-protein concentration. The derivation of eq a2 is straightforward and assumes that the free S-protein concentration can be neglected ($[P] \ll c_0$) or else that $f = r_1$. Hence, plotting $\phi = (r_1 - f)(1-f)/f$ against $r_2' = r_2 - (1-f)$ should yield a straight line with the slope K_1/K_2. The data plotted in Figure 8b were measured for $r_1 = 0.83$ and r_2 ranging from 0 to 26.

To quantitate competition in the formation of the initial complexes, we assume that only the forward rates are important:

$$p^* + P \xrightarrow{k_1} p^*P \qquad p + P \xrightarrow{k_2} pP \tag{a3}$$

Numerical integration of eq a3, followed by fitting the data to an analytical expression, shows that the fraction f of labeled p*P is given at $t \rightarrow \infty$ by

$$1/f = 1 + (k_2/k_1)(r_2/r_1) \tag{a4}$$

In the absence of added S-peptide ($r_2 = 0$) it follows that $f = 1$ at $t = \infty$; with increasing amounts of S-peptide a straight line is expected when plotting $1/f$ against r_2/r_1, and the slope should measure the ratio of the on-rates. The experimental f values used in Figure 8c were taken from the kinetic progress curves at $t = 2.4$ s (which is $t_{1/2}$ in the absence of added S-peptide, and so $f = 0.5$ at 2.4 s in this case). With increasing amounts of S-peptide, the reaction speeds up. Therefore, the $1/f$ values determined at 2.4 s are expected to start off at 2 (for $r_2 = 0$) and to approach asymptotically the line defined by eq a4 with increasing S-peptide concentration. This behavior is indeed observed.

Registry No. RNase, 9001-99-4; S-peptide, 17205-06-0; FTC-S-peptide, 83968-07-4; C>p, 633-90-9; fluorescein isothiocyanate, 3326-32-7.

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Hemoglobin Function in the Water–Ethylene Glycol Cosolvent System: Linkage between Oxygen Binding and Hydration†

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ABSTRACT: The effects of ethylene glycol (EG) on the oxygen binding properties of human hemoglobin are described in this report. Under the conditions used, the hemoglobin molecule remains in the intact tetrameric form in up to 70% (w/w) EG, corresponding to a mole fraction of EG of 0.4. Interaction between the cosolvent and the hemoglobin is quite weak. Only at high concentrations of EG are the effects on the oxygen binding curve detectable. In the range of mole fraction of EG up to 0.2, oxygen affinity is decreased. In the range of mole fraction of EG between 0.2 and 0.4 (corresponding to molar concentrations of 8–12 M EG), hemoglobin oxygen affinity increases, eventually becoming higher than the value obtained in the absence of EG. Experiments were carried out in the presence of 0.013, 0.10, and 1.0 M NaCl to evaluate the linkage between EG and chloride as allosteric effectors and the possible general effect of ionic strength on oxygen binding properties of hemoglobin in the presence of cosolvent. The effects of EG on hemoglobin ligation are discussed in terms of a model in which EG interacts with hemoglobin in a weak allosteric fashion at the lower concentration range (less than mole fraction of 0.2) while at the higher range (mole fraction of 0.2–0.4) perturbations of protein hydration lead to stabilization of the high-affinity form of hemoglobin.

Hemoglobin is the main protein in the mammalian red cell, present in a concentration of about 30% by weight. The cellular concentration of potassium and chloride ions is maintained within certain levels, and metabolic pathways produce adenosine triphosphate and 2,3-diphosphoglycerate, both of which have well-defined roles in the physiology of the red cell. Thus, 2,3-diphosphoglycerate is an allosteric effector by virtue of its differential interaction with the two conformational forms of the hemoglobin molecule. Such allosteric effects, including the effect of small anions and protons, have received considerable attention both experimentally and theoretically. The theory of linked thermodynamic functions as developed by Wyman (1964, 1965) represents a framework within which allosteric effects can be modeled and described. Structural information about the hemoglobin molecule, including the conformational extremes and the interactions between the protein and its effectors, has been refined to high resolution by means of X-ray crystallography (Fermi, 1975; Baldwin, 1980; Arnone, 1972). Studies of molecular dynamics of proteins in general and heme proteins in particular, using both low-temperature ligation measurements (Austin et al., 1975; Alberding et al., 1978), hydrogen exchange (Englander & Mauel, 1972; Hedlund et al., 1978), and energy minimization calculations (Gelin & Karplus, 1979), have provided another dimension of insight into protein function.

Despite extensive accumulation of experimental data and theory the role of the solvent as a functional component remains poorly understood. From structural, energetic, and dynamic points of view, descriptions of interactions between the protein and surrounding solvent molecules are still primarily qualitative. This is not to say that the general area of protein "hydration" and/or "solvation" has been ignored. Rather, it is the functional aspects of protein–solvent interactions that remain essentially uncharacterized. Thus, in the case of hemoglobin, one may ask if it is possible to define the role protein–water interactions play in the transfer of free energy between the binding sites of allosteric effectors and

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