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The Design and Production of Semisynthetic Ribonucleases With Increased Thermostability by Incorporation of S-Peptide Analogues With Enhanced Helical Stability

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ABSTRACT **Recent work has shown that, with synthetic analogues of C-peptide (residues 1-13 of ribonuclease A), the stability of the peptide helix in H_2O depends strongly on the charge on the N-terminal residue. We have asked whether, in semisynthetic ribonuclease S reconstituted from S-protein plus an analogue of S-peptide (1-15), the stability of the peptide helix is correlated with the T_m of the reconstituted ribonuclease S. Six peptides have been made, which contain Glu9 → Leu, a blocked α-COO^- group ($-CONH_2$), and either Gln11 or Glu11. The N-terminal residue has been varied; its charge varies from +2 (Lys) to −1 (succinyl-Ala). We have measured the stability of the peptide helix, the affinity of the peptide for S-protein (by C.D. titration), and the thermal stability of the reconstituted ribonuclease S.**

All six peptide analogues show strongly enhanced helix formation compared to either S-peptide (1-15) or (1-19), and the helix content increases as the charge on the N-terminal residue changes from +2 to −1. All six peptides show increased affinity for S-protein compared to S-peptide (1-19), and all six reconstituted ribonucleases S show an increase in T_m compared to the protein with S-peptide (1-19). The T_m increases as the charge on residue 1 changes from +2 to −1. The largest increment in T_m is 6°.

The results suggest that the stability of a protein can be increased by enhancing the stability of its secondary structure.

Key words: peptide helix, protein stability, framework model of folding

INTRODUCTION

Increasing attention is being given to producing proteins with increased stability. Apart from their important practical uses, more stable proteins should provide much-needed information on the interrelationships between enzyme structure, stability, and function. The only approach which has been effective (and only in some cases) is the introduction of cross-links: these have been introduced chemically in lysozyme[1] and RNaseA*,[2] producing inactive proteins with greatly increased thermostabilities, and by engineering unique disulphide bonds into dihydrofolate reductase[3] and T4 lysozyme,[4] producing active enzymes with apparently increased stability. Another possible approach is to stabilize individual pieces of secondary structure within the native structure. We ask whether it is possible to stabilize RNAseS by increasing the stability of one of its α-helices.

Bovine pancreatic RNaseA is cleaved specifically by subtilisin at peptide bond 20-21 to give S-protein (21–124) and the N-terminal S-peptide (1–20); the separated fragments can reassociate with high affinity to yield fully active, stable RNaseS.[5] Residues 3–13 of the peptide moiety form an α-helix in RNaseA or RNaseS. The isolated S-peptide[6,7] or C-peptide (1–13)[8,9] also shows partial α-helix formation at low temperatures. Recent work[10] has shown that it is possible to manipulate systematically the helical stability of C-peptide analogues in aqueous solution by varying the charge on the N-terminal residue, which interacts with the helix dipole. In RNaseA or S, residue 1 is isolated on the surface of the protein, so that substitutions ought not to interfere with packing of residues in the interior. Therefore, we set out to use a similar approach here: to vary the charge on the N-terminal residue and then to measure peptide helix stability as well as the affinity of the S-peptide analogue for S-protein (by a C.D. binding assay[11]), and also the thermal stability of the reconstituted, semisynthetic RNaseS. S-peptide itself is a poor helix former[7] and the first stage of this study was the design and synthesis of analogues which would show improved helix formation but still show strong binding to S-protein.

MATERIALS AND METHODS

S-peptide and S-protein were prepared by the procedure of Doscher and Hirs[12], and S-peptide (1–15)

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*Abbreviations: RNaseA, bovine pancreatic ribonuclease A; S-protein, residues 21–124 of RNaseA; S-peptide (1–15), (1–19), 1–20), residues 1–15, 1–19, or 1–20 of RNaseA; RNaseS, 1:1 molar ratio complex formed from S-protein plus S-peptide; T_m, temperature midpoint of a thermal unfolding transition; S.D., standard deviation; K_a, association constant; 2′,3′ cCMP, cytidine 2′:3′-cyclic monophosphoric acid (sodium salt); C.D., circular dichroism; NMR, nuclear magnetic resonance; HPLC, high-pressure liquid chromatography.

was prepared from S-peptide following the procedure of Potts et al[13] as described previously.[14] The concentration of RNase was calculated by using an extinction coefficient at 278 nm of 9,800 $cm^{-1}M^{-1}$.[15]

Hydrolysis of 2′, 3′ cCMP[16] was followed in 2 mM substrate, 100 mM cacodylate buffer, pH 7.1, at 27.5°C. RNA hydrolysis was followed at pH 5.0, 28°C, by the method of Kunitz[17] with bakers' yeast RNA (Sigma type XI) as the substrate.

Peptide Synthesis

Peptides were synthesized by the solid phase method[18] with methylene chloride as the solvent and with 33% trifluoroacetic acid, 1% indole/methylene chloride for deprotection. Coupling reactions were monitored with the Kaiser test and repeated until complete. All peptides were synthesized as the COOH-terminal amides on p-methylbenzhydrylamine poly (styrene/1% divinyl-benzene) resin.

Peptide Purification

Chromatographic procedures included reverse phase on a C_{18} resin with a gradient of 20–100% acetonitrile in 0.1% trifluoroacetic acid; ion exchange on SP-C25 resin with a gradient of 0–1 M NaCl in 10 mM HCl or acetate buffer (pH 4.5 or pH 6.1); and gel filtration on Sephadex G-25 in 10 mM HCl or 10 mM acetate, pH 4.5.

The amino acid content and purity of the peptides was confirmed by HPLC, NMR and amino-acid analysis. Table I gives an example. Deamidination was not a problem in the glutamine-containing peptides. A small amount of oxidized methionine was detected by NMR in peptide I and was reduced, as previously described,[19] with N-methylmercaptoacetamide[20] kindly provided by C.H. Li.

Peptide Design

Shoemaker et al.[10] have synthesized a series of C-peptide analogues with a wide range of helix contents, dependent on the nature of the N-terminal residue, but these were not designed to bind to S-protein. Conversely, the tight-binding S-peptide (1–20) is a poor helix former and an unsatisfactory starting point for generating a series of peptides that would show a large, reliably measurable, range of helix contents. The basic consideration here was to make all possible substitutions that stabilize the isolated peptide helix but do not interfere with strong binding to S-protein.

We have made use of the extensive literature (e.g., by Berger, Chaiken, Hofmann, Scoffone, Richards, and colleagues) on the effects of substitutions and chemical modifications in S-peptide on binding to S-protein and on enzymatic activity of reconstituted RNaseS (see reviews by Richards and Wyckoff[21] and by Blackburn and Moore[22]).

The sequences of S-peptide, the C-peptide analogues of Shoemaker et al.,[10] and the peptides synthesized here are shown in Table II. The last five residues of S-peptide (1–20) are not needed for binding or for enzymatic activity.[13,14,23–25] Therefore, 15-mers were made. Position 1 was chosen as the residue to be varied as in Shoemaker et al.[10] It was assumed (see beginning of this paper) that changes at position 1 would not directly affect binding. Deletion of Lys1 decreases binding only four- to fivefold.[24] The Glu9 → Leu change decreases binding threefold[26] but increases helix content in C-peptide analogues.[19] The substitution Gln11 → Glu is a conservative change for binding[27] and has been shown to be helix stabilizing in C-peptide analogues (Osterhout and Carey,

TABLE I. Amino-Acid Analysis of Peptide VI* (sucAla1, Gln11)†

Amino-acid	Found	Expected*
Asp	0.96	1
Glx‡	2.03	2
Ser	1.02	1
His	0.99	1
Arg	1.20	1
Thr	1.04	1
Ala	3.70	4
Met	0.90	1
Leu	0.98	1
Phe	1.11	1
Lys	1.12	1

*See Table II for sequence.
†The composition and purity of all synthetic peptides was checked by NMR and HPLC.
‡Amino acid hydrolysis cannot distinguish between Glu and Gln. The presence of Gln in the Gln11 peptides V and VI was confirmed by the absence of any material comigrating on HPLC with the corresponding Glu11 peptide (I and IV, respectively).

TABLE II. Sequences of Peptides Referred to in This Study

Peptide	Sequence
S-peptide (1–19)*	KETAAAKFERQHMDSSTSA†
S-peptide (1–15)	KETAAAKFERQHMDS
I	KETAAAKFLREHMDS
II	AETAAAKFLREHMDS
III	Acetyl-AETAAAKFLREHMDS
IV	Succinyl-AETAAAKFLREHMDS
V	KETAAAKFLRQHMDS
VI	Succinyl-AETAAAKFLRQHMDS
Peptides of Shoemaker et al.[10]	
RN16	KETAAAKFLRAHA
RN22	AETAAAKFLRAHA
RN21	Acetyl-AETAAAKFLRAHA
RN24	Succinyl-AETAAAKFLRAHA

*It has been observed previously [14,39] that the material purified from Sigma S-peptide is the 19-residue fragment from the N-terminus of RNaseA, rather than the 20-residue fragment. The purified peptide used here was assumed to be S-peptide (1–19).
†Both natural S-peptides have a free carboxy-terminus whereas all synthetic peptides have a carboxy-terminal amide group.

unpublished data). Both Met13 and Asp14 are needed for binding. Asp14 has to be retained for binding despite its probable destabilizing interaction with the helix dipole. The free α-carboxyl group, however, was blocked by amidination since it is strongly helix-destabilizing in C-peptide[7]: all previous binding studies have been performed with peptides having a free α-carboxyl group.

The synthetic peptides contain all of the residues that are invariant in this region[22] (Glu2, Ala5, Phe8, Arg10, Gln11, His12, Asp14) except for Gln11, which is needed for good catalytic activity but not for binding. To regain catalytic activity and to investigate the role of Glu11 in stabilizing the helix, two peptides (V and VI) were synthesized with Gln in position 11.

Binding Assay

The association of peptides to S-protein was measured by the method of Filippi et al.[11] This assay exploits the fact that, while the peptides are only partially helical in isolation, they adopt their full helical conformation when bound to S-protein. The increase in ellipticity caused by peptide helix formation upon binding to S-protein is measured at a wavelength at which there is no signal from conformational changes in the S-protein.[28] The ellipticity of a fixed concentration of S-protein and varying concentrations of peptide in a tandem cell was measured with the solutions first separated, then mixed. Assays were performed at pH 5.3.

Unless otherwise stated, the buffer system used throughout was 1 mM borate and 1 mM citrate with either (as stated) 1 mM phosphate or 100 mM NaCl or 100 mM NaH_2PO_4.

Helical content and stability were measured by C.D. (see reference 19) in a Jasco J-500A spectropolarimeter in the laboratory of J.T. Yang (University of California Medical School, San Francisco).

Thermal denaturation studies were performed in a Cary 118 spectrophotometer; a microprobe in the sample gave continuous monitoring of temperature.

RESULTS

Helix Formation in the Isolated Peptides

All of the peptides synthesized were good helix formers (Fig. 1). Despite the required differences between these peptides and those of Shoemaker et al.,[10] the Glu11 peptides (I–IV) had at least as high a helix content as the C-peptide analogues studied[10] and considerably higher than either S-peptide (1–20) (Fig. 2a) or S-peptide (1–15) (Fig. 2b). The helix content of each peptide at 3°C (Fig. 1) correlates with its thermal stability (Fig. 2), as is the case for the C-peptide analogues.[10] No concentration dependence of helix formation was observed for any of these peptides in the 7–70-μM range, and an apparent decrease at lower concentrations in some of the peptides is thought to be caused by surface adsorption.

All the peptides (Fig. 1) showed the same type of pH-dependent helix formation, with an optimum

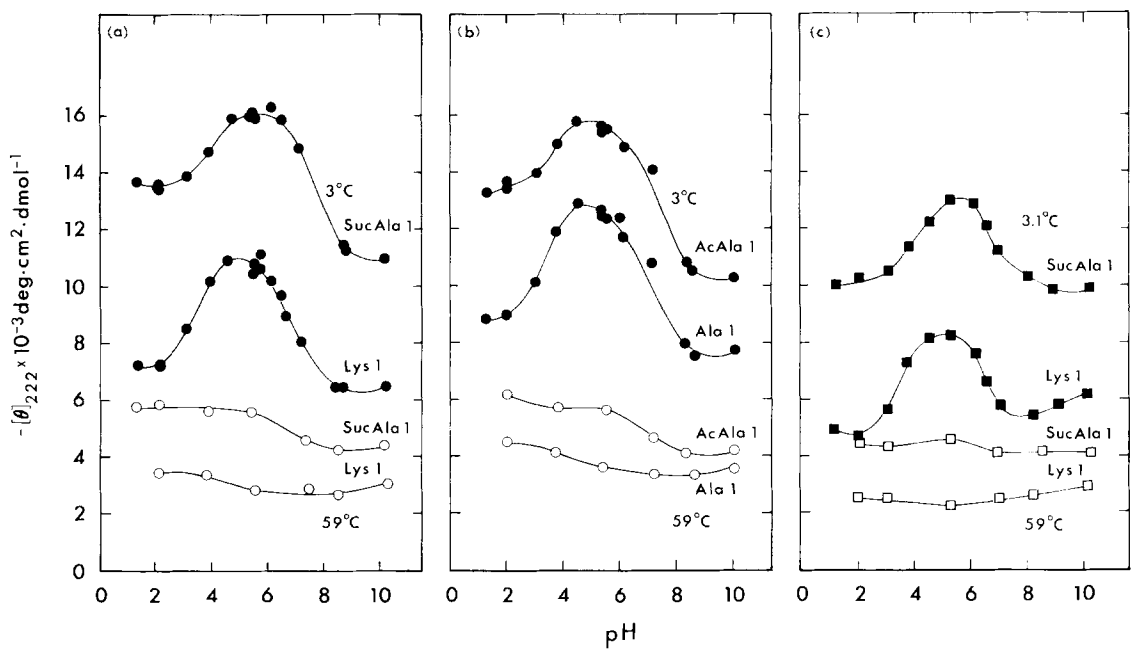
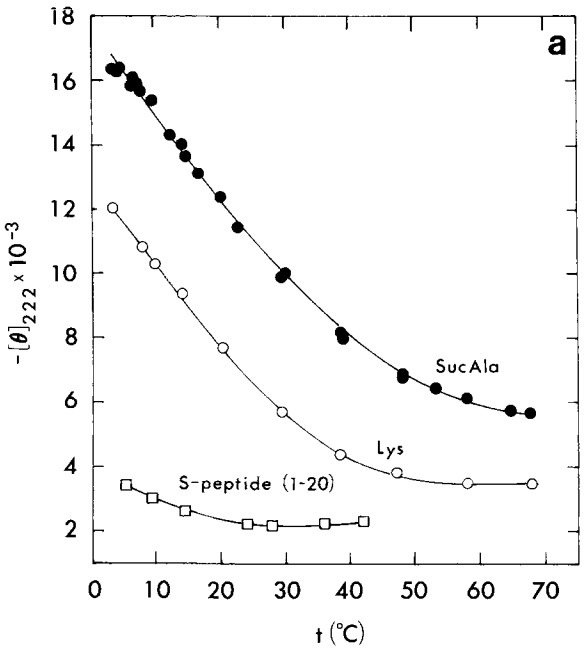


Fig. 1. Peptide helix formation as a function of pH at 3°C and at 59°C, 0.1 M NaCl, 1 mM phosphate buffer, 12–20 μM peptide concentration. [θ] is the mean residue ellipticity at 222 nm. (a),(b) Peptides with Glu11. (c) Peptides with Gln11.



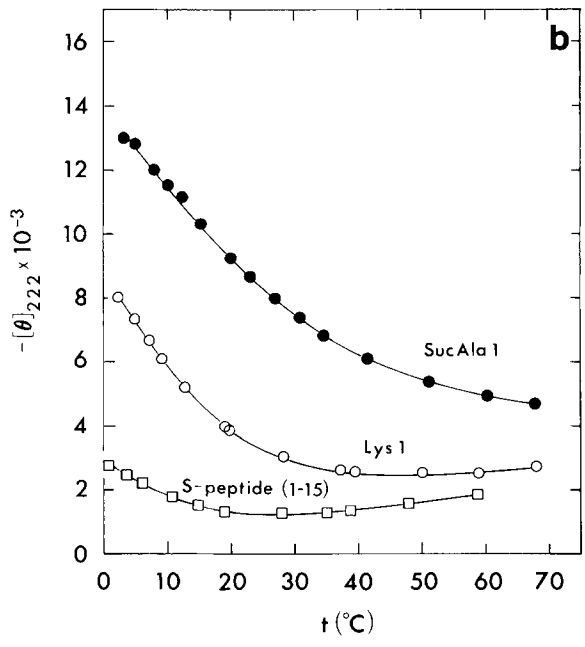


Fig. 2. Peptide helix formation as a function of temperature at pH 5.3, 0.1 M NaCl, 1 mM phosphate buffer, 12–20 μM peptide. $[\theta]$ is the mean residue ellipticity at 222 nM; units are deg cm^2 dmol^{-1}. Values for S-peptide (1–20) are taken from reference 7, but with $[\theta]$ multiplied by (20/15) for comparison with the 15-residue peptides studied here; residues 16–20 do not participate in helix formation.[6,35] In a, the curves for peptide II and III (not shown) are intermediate between those of I and IV. (a) Peptides (1–15) with Glu11. Residue 1 is denoted in the figure. (b) Peptides (1–15) with Gln11.

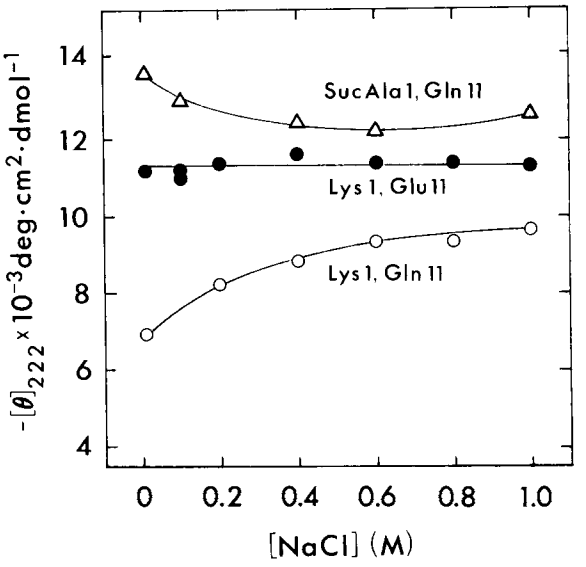


Fig. 3. NaCl dependence of peptide helix formation at 3°C, pH 5.4, 1 mM phosphate buffer, 18–33 μM peptide. Values for the other peptides at an intermediate and at a high NaCl concentration are as follows; $-[\theta]_{222}$ is given in deg cm^{2}dmol^{-1}.

Peptide	0.1 M NaCl	1.0 M NaCl
II (Ala 1, Glu11)	12,800	11,500
III (acAla 1, Glu11)	15,800	13,400
IV (suc Ala 1, Glu11)	16,200	12,500

around pH 5.3, ascribed earlier to ionization of Glu2 and His12.[19] The helix content is also dependent on the charged nature of the N-terminal amino acid, as observed in the C-peptide analogues.[10] The range of helix content between the best (suc-Ala1) and the worst (Lys1) helix former is much less here, however, than in the C-peptide analogues, independent of whether residue 11 is Glu or Gln. The difference must somehow be caused by the two additional C-terminal residues.

The effect on helix content of the charge on residue 1 is salt-dependent in the C-peptide analogues studied by Shoemaker et al.; the salt dependence agrees with the predictions of the helix dipole model.[10] The Glu11-containing peptides I–IV show similar salt dependences except for I (Lys1, Glu11). At pH 5.3 the helix content of this peptide is independent of salt concentration (Fig. 3), in striking contrast to the behavior of the C-peptide analogue (RN16) with Lys1 and Ala11. Peptide V, with Lys1 and Gln11, does show a salt dependence (Fig. 3) like that of the C-peptide analogue RN16.

The basic conclusion from these studies of helix stability is that we have been able to synthesize a series of S-peptide (1–15) analogues that have signifi-

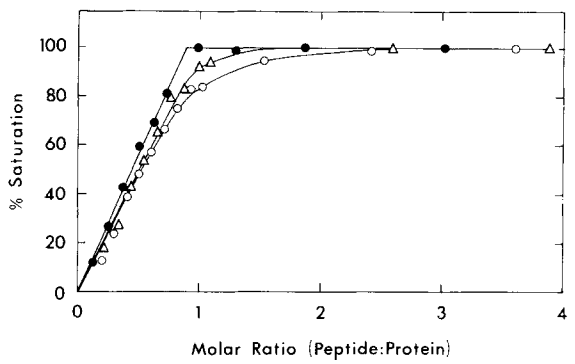


Fig. 4. Titration curves for the binding of peptide IV (sucAla1, Glu11) to S-protein (see text), at different temperatures, pH 5.3, in 0.1 M NaCl, 1 mM phosphate buffer, 3.6–4.5 μM S-protein. 20°C (●), 30°C (Δ), 35°C (○). The increment of ellipticity is expressed as a percentage of the maximum value determined at saturation.

cant and systematically variable helix stability in aqueous solution.

Binding of S-Peptide Analogues to S-Protein

The affinity of the peptides for S-protein was assayed[11] at pH 5.3, the optimal pH for helix formation in the isolated peptides. A strong temperature dependence for binding, previously reported for S-peptide,[29] was also observed here (Fig. 4, Table III). Binding was routinely assayed at 35°C, the minimum temperature which allowed accurate determi nation of the association constant by this method. Data were analyzed by a double reciprocal plot (Fig. 5), as in Filippi et al.[11] The plot is based on the work of Levit and Berger.[24] The slope of the plot gives the S-protein concentration and the intercept on the y-axis gives the association constant. Exact determination of the low concentrations of S-peptide and peptides used in this method is difficult because of surface adsorption, and possibly also for other reasons. This explains why in Figure 4 the stoichiometry of binding at 20°C is not quite 1:1, and in Figure 5 the straight lines "tail off" at the end. The K_a values calculated by the procedure used here are insensitive to these effects. The tabulated values of K_a were calculated from several individual points lying between 10 and 90% saturation on the double reciprocal plots of the binding curves.

Table III shows that all of the synthetic peptides bind to S-protein and that they do so with greater affinity than either S-peptide (1–19) or S-peptide (1–15). In addition, the affinity increases with the helix stability of the isolated peptide: the best helix former, peptide IV, binds ten times more strongly than S-peptide (1–19) at 35°C, 0.1 M NaCl, pH 5.3.

The affinity of S-peptide for S-protein increases approximately 70-fold in the presence of the substrate 2′, 3′ cCmp[24] and, in the RNaseS crystal structure, Gln11 interacts with the phosphate moiety of a dinucleotide substrate. Table III shows that phosphate alone increases K_a both for S-peptides (1–19) and (1–15) and for the synthetic peptides. Phosphate is known to stabilize S-protein alone[30] and it could act as a partial substrate analogue.

S-peptide (1–19) was found to bind slightly better than S-peptide (1–15) in either 0.1 M NaCl or 0.1 M phosphate, pH 5.3 (Table III).

Ellipticity of Bound Peptides

The binding assay is based on observing the increase in ellipticity when the partially helical pep-

TABLE III. Association Constants for Peptide Binding to S-Protein at pH 5.3*

	0.1 M NaCl buffer †		0.1 M phosphate buffer‡	
Peptide	Temp. (±0.2°C)	$K_a(10^6\ M^{-1})$	Temp. (±0.2°C)	$K_a(10^6\ M^{-1})$
I (Lys1, Glu11)	35.0	4.99 ± 1.30	35.0	⩾20
		5.20 ± 1.07		
II (Ala1, Glu11)	35.0	6.32 ± 1.09		
III (acAla1, Glu11)	35.0	6.45 ± 2.29		
IV (sucAla1, Glu11)	35.0	10.00 ± 2.13		
	30.0	24.6 ± 12.2		
	39.0	3.98 ± 0.81		
V (Lys1, Gln11)	35.0	2.19 ± 0.47	39.0	⩾10
VI (sucAla1, Gln11)	35.0	4.03 ± 1.05		
S-peptide (1–19)	35.0	1.05 ± 0.12	35.0	13.9 ± 7.5
	39.0	0.202 ± 0.011	39.0	4.72 ± 1.12
S-peptide (1–15)	35.0	0.865 ± 0.030	20.0	⩾10
			30.0	16.2 ± 5.9
			39.0	2.64 ± 0.46

*S-protein concentration 4–5 μM. K_a values were calculated from several individual points on the double reciprocal plots of the binding curves (Figs. 4, 5) and the S.D. are shown. See text.
†Buffer composition: 0.1 M NaCl, 1 mM phosphate, 1 mM borate, 1 mM citrate, ph 5.3.
‡Buffer composition: 0.1 M NaH_2PO_4, 1 mM borate, 1 mM citrate, pH 5.3.

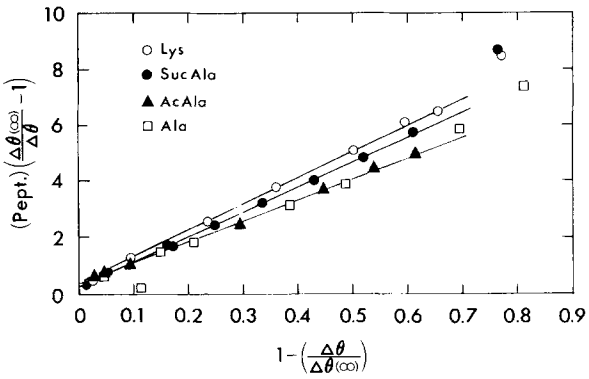


Fig. 5. Binding of Glu11 peptides to S-protein, plotted by the equation of Levit and Berger[24] (see also reference 11). Conditions are 35°C, pH 5.3, 0.1 M NaCl, 1 mM phosphate buffer, 3.6–4.5 μM S-protein. Compare the data for IV in Figure 4. Residue 1 is denoted in the figure.

tides become fully helical on being bound to S-protein. By working at saturating concentrations of the peptide we can calculate the increase in molar ellipticity of the peptide when it binds to S-protein (Table IV). The ellipticity increment increases with temperature as the helix formed by the isolated peptide melts out and then, at temperatures (35°C, 39°C) where the resultant RNaseS begins to unfold (see below), the ellipticity increment decreases. This effect, which is shown in Table IV for peptide IV, was seen for all the Glu11-containing peptides. As expected, the lower the ellipticity of the isolated peptide, the larger the increase on binding to S-protein.

By measuring the ellipticity of the peptide in isolation and the increase in ellipticity on binding, we obtain by simple addition the molar ellipticity of the peptide in its fully helical conformation in the ribonuclease. The results (Table IV) show close agreement between the values obtained for the different peptides at temperatures where the reconstituted RNaseS is stable (see below). This agreement indicates that all the synthetic peptides and also S-peptide (1–15) adopt similar helical conformations when bound to S-protein. S-peptide (1–19) in RNaseS also gives a similar molar ellipticity[31] when corrected for the difference in peptide length.

Using the temperature-dependent baseline value (0% helix formation) for $[\theta]_{222}$ obtained by K.R. Shoemaker from studies of P-peptide (1–8) (unpublished results), we can calculate values for the change in the mean residue ellipticity ($\delta[\theta]_{222}$; as calculated for 15 residues) for these peptides when residues 3–13 undergo a zero to 100% helix transition. The $\delta[\theta]_{222}$ value calculated for peptides I–IV is $-29{,}200 \pm 500$ deg cm^2dmol^{-1} peptide residue and for S-peptide (1–15) it equals $-29{,}200 \pm 600$ deg cm^2dmol^{-1} peptide residue. This value does not take into account any temperature dependence of the ellipticity of the fully formed peptide helix in native RNaseS.[28,32] By using this value for the change in $[\theta]$ corresponding to 100% helix formation, the helix content of the synthetic Glu11-containing peptides (3°C, 0.1 M NaCl, pH 5.3) is calculated as 45% for (I) and 63% for (IV), as compared to 15% for S-peptide (1–19).

The Glu11-containing peptides (I–IV) give reduced enzymatic activity compared to natural RNaseS, as expected from previous work[33]: < 4% activity with 2′, 3′ cCMP as a substrate and approximately 15% activity with RNA as a substrate. RNaseS reconstituted from the Gln11-containing peptides (V and VI) gave slightly more than 100% activity when compared to S-peptide (1–15) or (1–20), showing that the synthetic peptides bind to S-protein in such a way as to yield catalytically active RNaseS.

Themostability of Reconstituted Ribonucleases

The thermal unfolding of RNaseS can be followed by the decrease in absorbance on exposure of buried Tyr residues. Unfolding of the reconstituted RNaseS was carried out at pH 5.3, the optimum pH for helix formation by the isolated peptide. A high RNaseS concentration was used (>100 μM, with 30% mole excess peptide) to be in the range[34] where the T_m is independent of concentration; data not shown. In all cases, fully reversible and approximately parallel transitions were observed (Fig. 6). All the semisynthetic ribonucleases unfold at higher temperatures than native RNaseS. For parallel transitions such as these, the T_m of the transition is a measure of the relative stability of the semisynthetic RNaseS. In the best case, we have produced a semisynthetic protein which has a T_m 6°C above that of the natural RNaseS.

The fact that the stability of the isolated helix contributes to determining the stability of the reconstituted RNaseS is also shown by the salt dependence of thermal stability (Table VI). For the peptides with Gln11, helix stability is strongly salt-dependent (Fig. 3), as expected if the N-terminal charge affects stability by interacting with the helix dipole.[10] Peptide V (Lys1, Gln11) shows an increase in helix stability with increasing M NaCl, whereas peptide VI (suc-Ala1, Gln11) shows a decrease in helix stability with increasing M NaCl. This behavior is parallel to the salt dependence of the difference in thermal stability in the reconstituted RNaseS formed by these two peptides: δT_m decreases from 1.8° at 0.01 M NaCl to 1.3° at 0.1 M NaCl, and vanishes at 0.6 M NaCl.

An unexpected and as yet unexplained observation is that RNaseS containing S-peptide (1–15) is more stable than RNaseS with S-peptide (1–19) (Table V). Possibly the free α-COO^- group of S-peptide (1–15) makes a stabilizing interaction with positive charge(s) in S-protein. It is known that in the isolated peptide a free α-COO^- in C-peptide is strongly helix-destabilizing,[7] probably because of its interaction with the

TABLE IV. Change in Peptide Ellipticity* on Binding to S-Protein

Peptide	Temp. (0.2°)	Peptide ellipticity (deg cm^2dmol^{-1}) $-[\theta]_{222}$	Ellipticity increase on binding (deg cm^2dmol^{-1}) Measured† $-[\theta]_\lambda$	Ellipticity increase on binding (deg cm^2dmol^{-1}) Calculated‡ $-[\theta]_{222}$	Ellipticity change for bound peptide (deg cm^2dmol^{-1})*** $-[\theta]_{222}$
(A) In 0.1 M NaCl buffer, pH 5.3					
I (Lys1, Glu11)	10.0	10,200	14,800	17,400	29,400
II (Ala1, Glu11)	10.0	11,100	14,200	16,800	29,700
III (acAla1, Glu11)	10.0	14,700	11,300	13,300	29,800
IV (sucAla1, Glu11)	10.0	16,000	9,100	10,700	28,500
	20.0	12,400	12,900	15,200	28,900
	30.0	10,000	15,500 ± 500 (2)	18,300 ± 600	29,100 ± 600
	35.0	9,200	14,300 ± 400 (3)	16,800 ± 400	26,600 ± 400
	39.0	8,300	13,500	15,900	24,600
(B) In 0.1 M phosphate buffer, pH 5.3					
S-peptide (1–15)	20.0	1,100	23,600**	26,200	28,800
	30.0	1,300	27,000	27,600	29,800
	39.0	1,400	25,800	27,300	29,200

*Concentration 4–5 μM S-protein, 17–25 μM peptide. Where multiple determinations were performed, the SD is given and the number of determinations is given in parentheses. The mean residue molar ellipticity is calculated on the basis of 15 residues/peptide.

†Measured in presence of saturating amounts of peptide, at the observed isodichroic point for thermal unfolding of S-protein, i.e., 226 nm in 0.1 M NaCl, 224 nm in 0.1 M phosphate buffer.

‡Calculated using observed ratios for synthetic peptides in strongly helical conditions: 3°C, 0.1 M NaCl, pH 5.3; $[\theta]_{226} = 0.850 \times [\theta]_{222}$, $[\theta]_{224} = 0.946 \times [\theta]_{222}$, $[\theta]_{225} = 0.903 \times [\theta]_{222}$.

**Measured at 225 nM.

***Obtained by adding columns 1 and 3 and then correcting for a temperature-dependent baseline for 0% helix formation, based on studies of P-peptide (1–8) (Shoemaker, unpublished results).

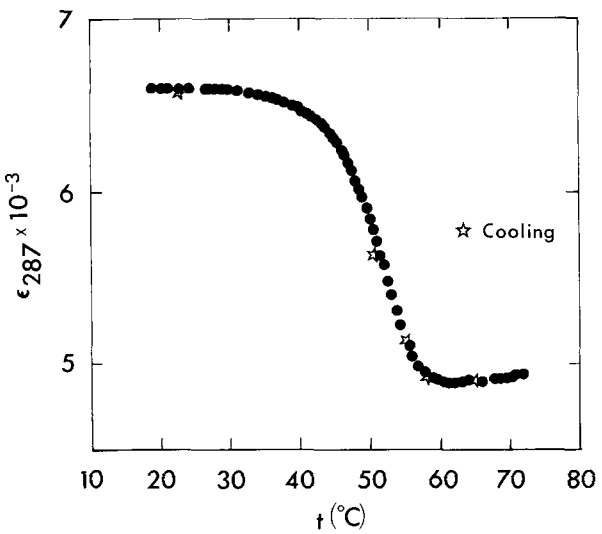


Fig. 6. Reversible thermal unfolding of semisynthetic RNaseS containing peptide III (acAla1,Glu11). Conditions: 127 μM S-protein and 170 μM peptide III, pH 5.3, 0.1 M NaCl, 1 mM phosphate buffer. The values measured on cooling start from 73°C. E_{287} is the extinction coefficient at 287nm ($M^{-1}cm^{-1}$).

negative pole of the helix dipole.[19] S-peptide (1–19), therefore, is thought to be a better reference peptide for the synthetic peptides, which have a blocked C-terminus, than S-peptide (1–15) because the free α-COO^- group of (1–19) is farther removed from the peptide helix. The use of S-peptide (1–19) also avoids any complicating effects of an α-COO^- group in or near the S-peptide:S-protein binding site.

Inorganic phosphate is known to stabilize both RNaseS and S-protein;[30] 0.1 M phosphate has a large stabilizing effect on the reconstituted proteins studied here, whether they were formed by using S-peptide (1–19) or either of the two synthetic peptides I (Lys1, Glu11) or V (Lys1, Gln11) (Table VI). Despite the possible contribution of Gln11 to phosphate binding (see above), these results suggest that phosphate stabilization does not depend on the presence of Gln11 and instead reflects stabilization of the S-protein moiety.[30]

DISCUSSION

Rationale for Expecting Peptide Helix Stability to Influence Binding and RNaseS Stability

The binding of S-peptide to S-protein can be represented, in simplified form, by the equations:

$$h + N \rightleftharpoons hN;\ K_1 = (hN)/(h)(N) \quad (1)$$

$$c + N \rightleftharpoons hN;\ K_2 = (hN)/(c)(N) \quad (2)$$

$$c \rightleftharpoons h;\ K_3 = (h)/(c) = K_2/K_1 \quad (3)$$

where h and c represent the helical and random-coil forms of the peptide and N and hN denote folded S-protein and RNaseS, respectively. The helix-coil equilibrium in a peptide and the binding to S-protein are more complex than indicated above and K_1, K_2 and K_3 are apparent equilibrium constants. Since $K_3 = K_2/K_1$ there should be a direct relation between the helix content of S-peptide and its affinity for S-protein, with the following provisos. First, the stability-enhancing changes made in the S-peptide should not interfere with binding to S-protein. For this reason we varied systematically only the N-terminal residue of S-peptide (1–15) which is an isolated surface residue in RNaseS. In addition we made use of other helix-enhancing substitutions only if they did not interfere with tight binding, as measured in earlier studies.[11,27] Second, the helix formed by the isolated peptide must be structurally related to the helix in RNaseS and, hence, capable of enhancing binding to S-protein. Earlier studies[6,35] suggest that the helix is localized in the isolated S-peptide (1–20) in much the same way as in RNaseS.

TABLE V. Correlation* of Association Constant and Thermostability of Semisynthetic RNaseS With Peptide Helix Content

Peptide	Peptide ellipticity† (3°C) (deg cm^2dmol^{-1})	K_a(35°C)‡ (10^6 M^{-1})	T_m** (°C)
S-peptide (1–15)	2,500	0.865 ± 0.03	48.1 ± 0.9
S-peptide (1–19)	3,500	1.05 ± 0.12	45.9 ± 0.7
V (Lys1, Gln11)	8,200	2.19 ± 0.47	47.5 ± 0.2
I (Lys1, Glu11)	11,100	4.99 ± 1.30	49.3 ± 0.2
		5.20 ± 1.07	
II (Ala1,Glu11)	12,800	6.32 ± 1.09	50.4
VI (sucAla1, Gln11)	13,000	4.03 ± 1.05	48.8 ± 0.1
III (acAla1,Glu11)	15,800	6.45 ± 2.29	51.1
IV (sucAla1, Glu11)	16,200	10.00 ± 2.13	51.9 ± 0.4

*All values refer to 0.1 M NaCl buffer, pH 5.3.

†From Figure 1 $-[\theta]_{222}$. The original S-peptide (1–20) values[7] have been multiplied by (20/15) to take account of the fact that residues 16–20 are outside the 3–13 helix.

‡From Table III.

**T_m measured at a RNaseS concentration between 100 and 200 μM with 30% mole excess peptide. Average values refer to the mean of two or three determinations; +SD.

TABLE VI. Thermostability of Reconstituted Ribonucleases* vs. NaCl Concentration

Peptide	T_m(°C) in pH 5.3 buffer containing: 0.01 M NaCl	0.1 M NaCl†	0.6 M NaCl	0.1 M phosphate
S-peptide (1–19)	48.2 ± 0.1	45.9 ± 0.7 (3)	47.6 ± 0.1	51.6 ± 0.2
I (Lys1, Glu11)		49.3 ± 0.2 (3)		54.8 ± 0.2
V (Lys1, Gln11)	49.3 ± 0.1	47.5 ± 0.2	49.0 ± 0.1	53.1 ± 0.1
VI (sucAla1,Gln11)	51.1 ± 0.1	48.8 ± 0.1	49.0 ± 0.2	

*pH 5.3. Values quoted are the average of two determinations unless otherwise noted in brackets. For details, see Table V.
†From Table V.

Even though the changes in helix stability are probably small (~1–2 kcal/mol), such changes can produce measurable changes in the T_m of RNaseS if the stability of the 3–13 helix contributes directly to that of RNaseS, because the net stability of RNaseS is itself modest (approximately −10 kcal/mol) and an increment of 1–2 kcal represents a significant change.

These are the basic reasons for expecting measurable effects on binding and on RNaseS stability if peptide helix stability contributes to the stability of RNaseS. We expected a direct answer to the question of whether or not peptide helix stability is correlated with RNaseS stability because we can systematically affect the stability of the peptide helix by varying only the N-terminal residue. In this case, helix stabilization or destabilization is believed to occur via a through-space interaction between the charge on the N-terminal residue and the positive pole of the helix dipole, which should be close to residue 3. This interaction can still take place when S-peptide is part of RNaseS. Because the charged groups on Lys1 are solvent-exposed in RNaseS[36] and strong interactions occur only between partly buried charged groups, according to the analysis of Gurd, Matthew, and coworkers,[37] the charges on residue 1 are not expected to interact strongly with other charged sidechains.

Helix-Forming Properties of the Synthetic Peptides

The basic strategy for design of the peptides produced enhanced helix formation as planned. All of the peptides show substantially greater helix content than natural S-peptide (1–15) or (1–19) (Fig. 2), and as a result, we are able to measure changes in helix stability produced by varying the charge on residue 1.

From studies of C-peptide analogues by Shoemaker et al.,[10] we expected that peptide helix stability would increase in the order Lys1 (+2) → Ala1 (+1) → ac-Ala1 (0) → suc-Ala1 (−1). This trend is observed for the synthetic peptides but the effect is smaller than in the C-peptide analogues. If the estimate derived here ($\delta[\theta]_{222} = -29{,}200$ deg cm^{2}dmol^{-1}) for complete helix formation (in residues 3–13) by S-peptide (1–15) is used, the helix content of the Glu11-containing peptides ranges from 45% (Lys1) to 63% (suc-Ala1) helix.

The difference in helix stability between peptides with Lys1 (+2) and suc-Ala1 (−1) was expected to be a strong function of NaCl concentration, being maximal at low concentration (e.g., 0.01 M) and decreasing strongly near 1 M. This behavior is observed for peptides with Gln11 but is much reduced in peptides with Glu11. The latter effect is probably related to a putative $Lys7^+ \cdots Glu11^-$ salt bridge being studied in this laboratory. As expected, the peptides with Glu11 show higher helix contents than those with Gln11.

The detailed mechanisms of peptide helix stabilization are not yet understood. The successful design of the peptides used here and by Shoemaker et al.[10,19] shows that the interaction between the helix dipole and the charge on N-terminal residue is important, but several effects are not yet understood: for instance, the effect of the Glu9 → Leu substitution. Three changes in the sequence of S-peptide (1–15) produce roughly equal increases in helix content: Lys1 → suc-Ala, Glu9 → Leu, and Gln11 → Glu.

Relation Between Peptide Helix Content and Affinity for S-Protein

As expected (see above), the affinity for S-protein increases as the helix content of the peptide increases. All six analogues of S-peptide (1–15) in Table III show increased affinity for S-protein at 35°C, 0.1 M NaCl, as compared either to S-peptide (1–15) or (1–19). The best helix former (IV, with suc-Ala1, Glu11) shows the highest affinity for S-protein. As the charge on the N-terminus is varied from +2 to −1, the affinity for S-protein increases both in the four peptides with Glu11 and the two peptides with Gln11.

Peptide Helix Stability and T_m of RNaseS

There is a close correlation (Table V) between the helix content of the isolated peptide and the T_m of the semisynthetic RNaseS reconstituted from that peptide. The best helix former, IV (suc-Ala1, Glu11), gives the highest T_m. The increase in T_m for this peptide as compared to S-peptide (1–19) is 6.0°C. The T_m increases as the charge on the N-terminal residue is varied from +2 to −1, both in peptides with Gln11 or with Glu11. The peptides with Glu11 are better helix formers than those with Gln11 and they also produce higher T_ms. An unexpected feature of the T_m results

is that S-peptide (1–15) gives rise to a significantly higher T_m than S-peptide (1–19). This does not fit the correlation between peptide helix content and T_m of RNaseS, since S-peptide (1–19) is a somewhat better helix former than (1–15). Probably the proximity of the free α-COO^- group to the negative pole of the helix dipole makes S-peptide (1–15) a less good helix former than (1–19), but positions the α-COO^- group so as to make a favorable charge interaction with positively charged groups in S-protein. Another unexplained feature of Table V is that peptide VI (sucAla1, Gln11) shows anomalously low values both of K_a and T_m for the high helix content of the isolated peptide.

The T_m values in Table V have been measured in conditions (>100 μM RNaseS, with 30% mol excess peptide) where T_m is independent of concentration. Labhardt[34] noted that the T_m of RNaseS becomes independent of concentration above 70 μM and pointed out that S-peptide must remain associated with S-protein after unfolding in these conditions. This means that the T_m values give additional information about the stability of the semisynthetic RNaseS—information that is not contained in the K_a values.

Additional evidence that T_m is correlated with stability of the peptide helix is given by the [NaCl] dependence of the δT_m values between peptides with Gln11 and either sucAla1 or Lys1. The difference in helix content between these two peptides depends strongly on NaCl (Fig. 3), in the manner expected[10] for an interaction between the charge on residue 1 and the positive pole of the helix dipole. Table VI shows that δT_m for these two peptides behaves in a parallel manner: δT_m is largest at a low NaCl concentration (0.01 M) and minimal at 0.6 M.

Other Properties of the Binding and Thermal Unfolding Reactions

The semisynthetic ribonucleases formed in this work show many of the properties of RNaseS. There is a strong temperature dependence of the S-peptide:S-protein association constant.[29] Inorganic phosphate increases the K_a and T_m.[30] Phosphate is known to stabilize S-protein,[30] as well as RNaseS.

The peptide-specific differences between effects of NaCl on T_m are superimposed on the complex [NaCl] dependence of the T_m of RNaseS (Table VI). The T_m first decreases and then increases again as NaCl is increased above 0.01 M, as observed earlier for RNaseA.[38] This behavior has been ascribed[36] to the opposing effects of (a) screening favorable Coulombic interactions by NaCl and (b) stabilization by Cl^- at the anion-binding site of RNaseA. Because phosphate is known to bind strongly to the anion-binding site (see reference 36 and references therein), the increase in T_m in 0.1 M phosphate over 0.1 M or 0.6 M NaCl (Table VI) shows directly the effect of stabilization by anion binding.

The recovery of full catalytic activity in semisynthetic RNaseS when Gln11 is present strongly suggests that these peptide analogues have a similar conformation to that of S-peptide in RNaseS. Table IV shows that the helical contents of the peptide analogues in reconstituted RNaseS are the same as that of S-peptide, when measured by C.D. It would be very helpful to know the X-ray structures of the reconstituted ribonucleases, to check on these points and on possible other interactions that may contribute to the increase in K_a and T_m.

SUMMARY

Both the affinity of the S-peptide analogues for S-protein and the thermostability of the semisynthetic ribonucleases are greater than in natural RNaseS and increase as the helical stability of the isolated peptide increases. In the best case, the association constant is increased tenfold and the T_m by 6°C over the values found for natural RNaseS.

Therefore, in the case of RNaseS and the S-peptide helix, it possible to stabilize the entire protein by stablizing one piece of its secondary structure.

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