Antisense Peptide Recognition of Sense Peptides: Sequence Simplification and Evaluation of Forces Underlying the Interaction

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ABSTRACT: Structural principles were studied which underlie the recognition of sense peptides (sense DNA encoded) by synthetic peptides encoded in the corresponding antisense strand of DNA. The direct-readout antisense peptides corresponding to ribonuclease S-peptide bind to an affinity matrix containing immobilized S-peptide with significant selectivity and with dissociation constants in the range of 10^{-6} M as judged by analytical affinity chromatography. Synthetic, sequence-modified forms of antisense peptides also exhibit substantial binding affinity, including a "scrambled" peptide in which the order of residue positions is changed while the overall residue composition is retained. The antisense mutants, as the original antisense peptides, bind at saturation with greater than 1:1 stoichiometry to immobilized S-peptide. The data suggest significant sequence degeneracy in the interaction of antisense with sense peptide. In contrast, selectivity was confirmed by the inability of several control peptides to bind to immobilized S-peptide. The idea was tested that the hydropathic pattern of the amino acid sequence serves to induce antisense peptide recognition. A hydropathically sequence-simplified mutant of antisense peptide was made in which all strongly hydrophilic (charged) residues were replaced by Lys, all strongly hydrophobic residues by Leu, and all weakly hydrophilic and hydrophobic residues by Ala, except Gly which was unchanged. This "KLAG" mutant also binds to immobilized S-peptide, with an affinity only an order of magnitude less than that with the original antisense peptide and with multiple stoichiometry. Mutants of the KLAG model, in which the hydropathic pattern was changed substantially, exhibited a lower binding affinity for S-peptide. The conclusion from these data, that hydropathic patern recognition is a major factor inducing the sense/antisense peptide interaction, helps explain why scrambled antisense peptide binds, since the latter when simplified to contain only K, L, A, and G has substantial homology to the KLAG model of original antisense peptide. The data fit with a model for sense/antisense peptide interaction in which the peptides are elongated and perhaps flexible chains and bind by multiple contacts induced by a form of generic hydropathic pattern recognition between the chains.

Native peptides and proteins encoded in the sense strand of DNA normally interact through well-defined and complementary recognition surfaces formed by folding into compact conformations. This view of recognition does not readily fit the observations recently made of antisense peptide binding. The antisense strand of DNA normally is not expressed as functional protein in most cells, although in-frame transcription of antisense DNA has been reported (Adelman et al., 1987) and in-frame translation of antisense DNA has been observed in vitro (Julkunen et al., 1988). Yet, when made by chemical synthesis, peptides encoded in the antisense DNA strand frequently have been found to possess interaction properties related to sense peptides. Such antisense peptides bind directly to corresponding sense peptides, with appreciable affinity and specificity (Bost et al., 1985a; Blalock & Bost, 1986; Shai et al., 1987; Knutson, 1988; Brentani et al., 1988; Chaiken, 1988). In addition, for several cases, including ACTH[1] (Bost et al., 1985a; Bost & Blalock, 1986) γ-endorphin (Carr et al., 1986), LHRH (Mulchahey et al., 1986), fibronectin (Brentani et al., 1988), and angiotensin II (Elton et al., 1988) antibodies raised against antisense peptides were found to recognize the receptors of the corresponding sense peptides and proteins. Other observations of antisense peptide interactions also have been made (Knigge et al., 1988; Kang et al., 1988).

While these recurrent observations of selective binding properties of antisense peptides have rather profound implications for antisense peptides as recognition molecules in research and technology, the structural features underlying their interactions have been difficult to understand. Upon inspection of amino acid sequences of sense and antisense peptides, a general hydropathic pattern has been observed (Blalock & Bost, 1986; Blalock & Smith, 1986; Bost et al., 1985b). When a hydrophilic residue is present in a sense strand, the antisense DNA sequence for that residue most frequently encodes a hydrophobic residue. And the reverse is also true—for hydrophobic residues in a sense strand and hydrophilic residues in an antisense strand. Yet, it is difficult to envision how such a pattern of side chains would give rise to the type of complementary recognition surfaces, involving compactly folded molecules, expected for native peptide and protein interactions. Furthermore, one would not expect direct interactions of side

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[1] Abbreviations: ACTH, adrenocorticotrophic hormone; LHRH, luteinizing hormone releasing hormone; S-peptide, residues 1–20 of bovine pancreatic ribonuclease; S-protein, residues 21–124 of ribonuclease; RNase, ribonuclease; HPLAC, high-performance liquid affinity chromatography; HPLC, high-performance liquid chromatography; PAM, (phenylacetamido)methyl; Boc, *tert*-butyloxycarbonyl; TFA, trifluoroacetic acid; HOBT, hydroxybenzotriazole; DCC, dicyclohexylcarbodiimide.

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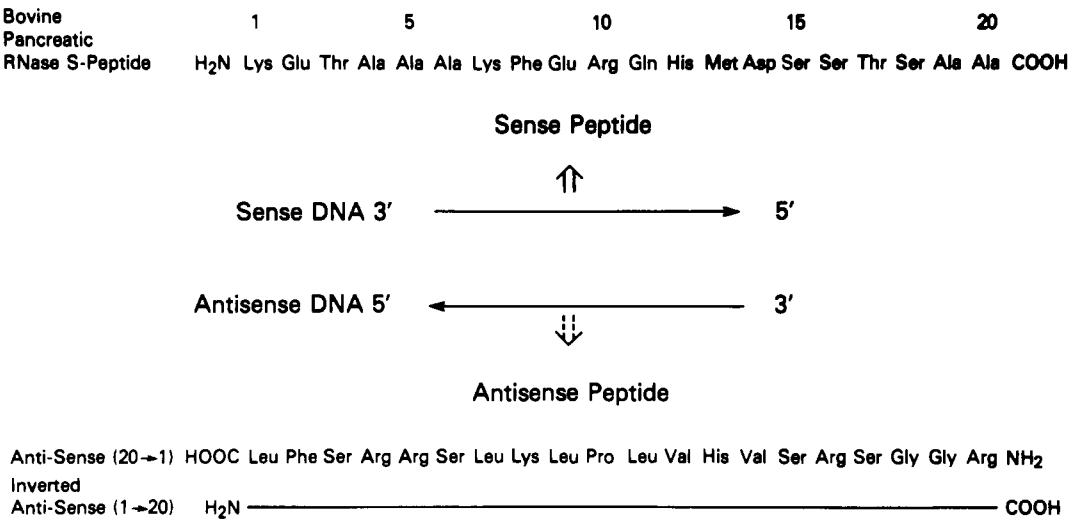

FIGURE 1: Relationship between sequences of AS and IAS peptides and the orientation of RNA encoding them.

chains of hydropathically opposite residues in the antisense versus sense peptides, hydrophilic interacting with hydrophobic.

Recently (Shai et al., 1987), we reported that antisense peptides encoded in the antisense strand of DNA for bovine pancreatic ribonuclease S-peptide have significant affinity for the 20-residue sense S-peptide. Synthetic antisense 20-mer, AS[SPep(20→1)], was found to bind both immobilized and soluble S-peptide as judged by analytical affinity chromatography. Zonal elution experiments showed that both antisense 20-mer [read 3′ to 5′ in antisense DNA for amino to carboxyl in peptide (Figure 1)] and inverted antisense 20-mer (the same amino acid order, but with amino to carboxyl orientation reversed) bind to native S-peptide in a specific peptide-directed manner. Several unrelated control peptides did not bind, including those with similar size and net charge. Shortened antisense and inverted antisense peptides, representing different segments along the sequence, also bind sense S-peptide. The binding pattern of shortened peptides, along with the close to equal affinities of antisense and inverted antisense peptides, suggested that the conformational specificity was somewhat degenerate. A multisite model of binding was proposed, with peptide partners interacting as rather elongated molecules at many residues along the sequences. This mechanistic hypothesis was consistent with the greater than 1:1 stoichiometry of binding measured chromatographically by broad-zone elutions and frontal analysis. Somewhat unexpectedly, we found a stoichiometry of at least four to six molecules of AS[SPep(20→1)] or IAS[SPep(1→20)] bound per molecule of immobilized native S-peptide. Overall, in the first antisense peptide case we tested, results were obtained which argue for selectivity in antisense peptide interaction but also for complexity in this interaction.

On the basis of these results, we embarked on an effort to learn more about the driving forces responsible for antisense peptide recognition in the S-peptide case. We have varied the sequence of antisense peptides and examined what aspects of sequence appear most important. Several specific questions were posed. (1) How do affinity and stoichiometry vary with sequence modification along the antisense 20-mer chain? (2) Does the stoichiometry vary with chain shortening? (3) On the basis of effects of sequence variation, can a simplified antisense S-peptide be designed which retains structural features predicted to be important for binding?

Materials and Methods

Routine materials and affinity matrices were prepared as described before (Fassina et al., 1986; Shai et al., 1987). Amino acid compositions were obtained by Angela Corigliano Murphy at NIH with a Beckman Model 6300 amino acid analyzer. Sequencing of selected synthesized peptides was carried out by Dr. Richard Harkins at Triton Biosciences (Alameda, CA) with an ABI gas-phase sequencer, Model 470A.

Peptide Synthesis and Purification. Peptides were synthesized by the solid-phase approach on PAM resins (Mitchell et al., 1978) as before (Shai et al., 1987) but with one important exception. Here, instead of symmetrical anhydrides being used in double or triple coupling steps at room temperature (Mitchell et al., 1978; Heath & Merrifield, 1986), coupling was carried out with freshly prepared hydroxybenzotriazole (HOBT) active esters of the Boc amino acids. On the basis of previous studies on HOBT active ester stability (Shai et al., 1985), these esters were prepared 1 h prior to each coupling step by mixing together Boc amino acid, DCC, and HOBT in a molar ratio of 1:1:1 at 0 °C and adding 2–3 equiv of HOBT active esters/equiv of peptide elongation sites on the PAM resin. After cleavage from resin and deprotection with liquid HF, the peptides were extracted with TFA and then precipitated by addition of ether. The synthetic peptides were purified to a chromatographic homogeneity of 95 to >99% by reverse-phase HPLC on a semipreparative μBondapak C_{18} column (Waters-Millipore Corp., Bedford, MA) using a linear gradient from 10% acetonitrile in 0.1% TFA at zero time to 80% acetonitrile in 0.1% TFA at 50 min.

Analytical High-Performance Liquid Affinity Chromatography (HPLAC). Dissociation constants for the interactions of synthetic antisense peptides and mutants with sense S-peptide were obtained by affinity chromatographic analysis as previously described for antisense peptides (Shai et al., 1987), with both zonal elution (Swaisgood & Chaiken, 1986, 1987, Abercrombie & Chaiken, 1985; Chaiken, 1986) and continuous (broad-zone) elution (Winzor, 1985; Swaisgood & Chaiken, 1987). These analyses utilized immobilized S-peptide matrix (9.1×10^{-5} M total peptide) in columns of either 0.32- or 2.3-mL bed volume. Variation of elution volume V with concentration of mobile peptide $[P]_0$ was evaluated from zonal elutions on the basis of

$$\frac{1}{V - V_0} = \frac{K_{M/P}}{[M]_T(V_0 - V_m)} + \frac{[P]_0}{[M]_T(V_0 - V_m)} \quad (1)$$

In eq 1, V is the experimental elution volume of mobile interactor (here, antisense peptide); V_0 is the unretarded elution volume (determined by elution of bovine neurophysin, oxytocin,

Table I: Sequences of IAS[SPep(1→20)] and Several Synthetic Peptide Mutants[a]

designation	sequence (one-letter code)
AS(1→20)	LFSRRSLKLPLVHVSRSGGR
AS(3→20)	SRRSLKLPLVHVSRSGGR
[K¹E²]IAS(1→20)	KESRRSLKLPLVHVSRSGGR
[E¹K²]IAS(1→20)	EKSRRSLKLPLVHVSRSGGR
[E¹E²]IAS(1→20)	EESRRSLKLPLVHVSRSGGR
[P³,⁶,¹³,¹⁵]IAS(1→20)	LFPRRPLKLPLVPVPRSGGR

[a] Residue replacements in mutants are underlined.

and S-peptide, none of which interact with immobilized S-peptide or blank matrix); $[M]_T$ is the total concentration of immobilized (matrix-bound) S-peptide; V_m is the volume outside the affinity beads (determined with dextran blue elution); $[P]_0$ is concentration of mobile peptide in the initial chromatographed zone; and $K_{M/P}$ is the dissociation constant of the complex of matrix-bound sense peptide (M) and mobile antisense peptide (P). For competitive zonal elutions, data were evaluated by

$$\frac{1}{V - V_0} = \frac{K_{M/P}}{[M]_T(V_0 - V_m)} + \frac{K_{M/P}[L]_T}{K_{L/P}[M]_T(V_0 - V_m)} \quad (2)$$

In eq 2, $[L]_T$ is the total concentration of competing ligand, here sense peptide (S-peptide); $K_{L/P}$ is the dissociation constant for solution interaction between mobile antisense peptide and mobile competing sense peptide; and the rest of the parameters are as before. From the variation of V at different $[L]_T$, $K_{M/P}$ and $K_{L/P}$ can be determined with the same experimental data. On the basis of the previously observed multiple stoichiometries and antisense peptide binding to sense peptide (Shai et al., 1987), chromatographically measured $K_{M/P}$ and $K_{L/P}$ values for antisense peptides are taken as apparent (functional) rather than microscopic dissociation constants.

For continuous-elution analytical affinity chromatography, solutions containing various concentrations of the synthesized antisense peptide were eluted through the affinity column continuously, and elution volumes were determined by frontal analysis as $\bar{V}$, the volume at half-maximal absorbance in the elution front. The variation of $\bar{V}$ was evaluated with

$$\frac{1}{\bar{V} - V_0} = \frac{K_{M/P}}{M_T} + \frac{[P]_0}{M_T} \quad (3)$$

In eq 3, M_T $[=[M]_T(V_0 - V_m)]$ is total amount of mobile peptide bound to immobilized S-peptide at saturation and $[P]_0$ is initial concentration of mobile antisense peptide (the initial and plateau values of $[P]_0$ are equivalent in broad-zone elution). By use of plots of $1/(\bar{V} - V_0)$ vs $[P]_0$, values of $1/M_T$ were calculated from the slope and of $K_{M/P}$ from the intercept. Stoichiometry, the moles of mobile peptide bound per mole of immobilized peptide at saturation, was determined at the ratio of M_T to the matrix S-peptide content determined by amino acid analysis.

Results

Site-Directed Analogues of IAS[SPep(1→20)]. Several families of sequence variants of antisense peptides were made in the present study. Table I shows the set of amino-terminal mutants which were synthesized, along with previously reported IAS[SPep(1→20)] (Shai et al., 1987). Mutants were made to test the contribution of charged versus hydrophobic residues as well as backbone conformation in antisense peptide recognition. Peptide 2, IAS[SPep(3→20)], retains the same net charge as IAS[SPep(1→20)], while removing the first two highly hydrophobic residues, Leu and Phe. In peptides 3 and 4, the two N-terminal hydrophobic residues were replaced by two hydrophilic residues, Lys and Glu, thus increasing hydrophilicity with no change in the net charge of these mutants. In peptide 5, the Leu-Phe sequence was replaced by Glu-Glu, reducing the net charge of the molecule to +4 (at the elution conditions used for chromatographic binding analysis). In peptide 6, three Ser residues and His were replaced by four prolyl residues, the latter of which were expected to restrict backbone conformational potential substantially.

Quantitative Interaction Properties of Antisense Peptides and Site-Directed Mutants. The interactions of antisense peptides and mutants with immobilized native S-peptide were evaluated by zonal elution of varying amounts of mobile peptides. As shown by the examples in Figure 2, linear variations of $1/(V - V_0)$ vs P were observed for all interacting peptides, a behavior consistent with eq 1 and allowing $K_{M/P}$ values to be calculated from $1/(V - V_0)$ values at y intercepts ($P = 0$). The $K_{M/P}$ values obtained are summarized in Table II. Peptides found to have low affinities were evaluated on the large bed volume (2.3 mL) column, as opposed to the 0.32 mL bed volume column used for those with relatively high affinity. Table II also lists, for comparison, the value of $K_{M/P}$ obtained for the interaction of mobile native S-protein with immobilized S-peptide as well as the results with several

Table II: Experimental Dissociation Constants ($K_{M/P}$) for Complexes of Immobilized S-peptide with AS, IAS, and IAS Mutant Peptides As Determined by Zonal-Elution Analytical HPLAC[a]

peptide designation	no. of residues	retention time on RP-HPLC (min)	net positive charge	bed volume of column used (mL)	extrapolated $V - V_0$ (mL)[b]	$K_{M/P}$	$pK_{M/P}$
IAS(1→20)	20	23.6	6	0.32	12.98	8.0×10^{-7}	6.1
IAS(3→20)	18	23.6	6	0.32	9.26	1.0×10^{-6}	6.0
[K¹E²]IAS(1→20)	20	23.2	6	0.32	6.25	1.5×10^{-6}	5.8
[E¹K²]IAS(1→20)	20	23.2	6	0.32	6.33	1.5×10^{-6}	5.8
[P³,⁶,¹³,¹⁵]IAS(1→20)	20	29.6	5	0.32	7.87	1.2×10^{-6}	5.9
S-protein	104	31.3	7	0.32	7.51	1.2×10^{-6}	5.9
AS(20→1)	20	30.1	6	0.32	20.57	4.0×10^{-7}	6.4
AS(14→1)	14	21.8	4	2.3	5.32	1.2×10^{-5}	4.9
AS(20→8)	13	22.1	4	2.3	2.86	2.2×10^{-5}	4.7
AS(10→1)	10	28.4	3	2.3	2.33	2.7×10^{-5}	4.6
IAS(5→20)	16	24.2	5	2.3	7.35	8.4×10^{-6}	5.1
[E¹E²]IAS(1→20)	20	23.2	4	2.3	1.68	3.7×10^{-5}	4.4

[a] Elution was on an analytical (0.45 × 100 cm) C18 column with a linear gradient from 10% acetonitrile in 0.1% TFA at zero time to 80% acetonitrite in 0.1% TFA at 50 min. [b] No significant retardation was observed on immobilized S-peptide with S-peptide itself; the somewhat hydrophobic oxytocin (CYIQNCPLG-amide); the small acidic protein bovine neurophysin II; three peptides corresponding to neurophysin II fragments except for Ser replacement of $^1/_2$-Cys where underlined (LGSFVGTA, residues 32–39; RQSLPSGPGGKGR, residues 8–20; NYLPSPSQSGQK, residues 48–59); the highly charged peptide KEKEKLEFIL; and the basic peptide GRGLSLSR.

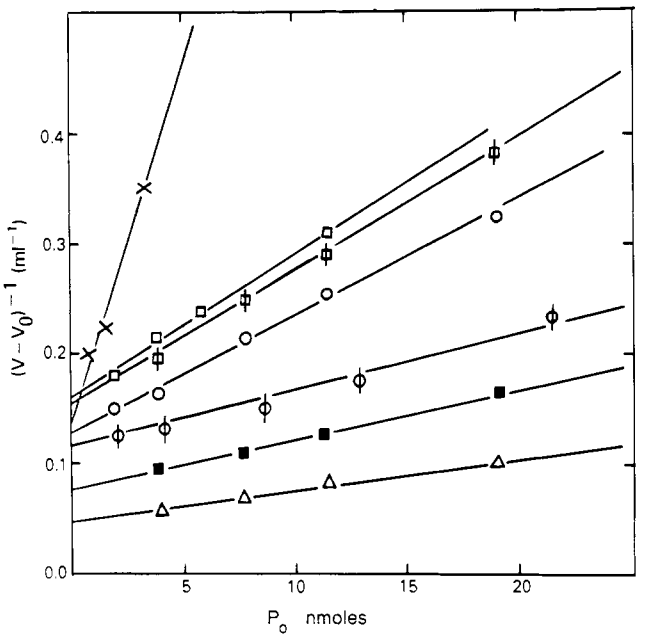

FIGURE 2: Zonal-elution analytical HPLAC determination of RNase S-peptide binding for a family of high-affinity synthetic antisense and inverted antisense 20-mers, shortened fragments, and site-directed mutants using a low-volume immobilized S-peptide column. (△) AS(20→1); (■) IAS(1→20); (ϕ) IAS(3→20); (○) [P^{3,6,13,15}]IAS-(1→20); (ϕ) [E^{1}K^2]IAS(1→20); (□) [K^{1}E^2]IAS(1→20); (×) S-protein. In all cases zones of antisense peptides containing the denoted nanomoles of peptides were eluted on the 0.32 mL bed volume column of immobilized S-peptide in 0.2 M NH_4OAc, pH 5.7. Elution volumes are plotted as $1/(V - V_0)$; extrapolated values at nanomoles = 0 are used to calculate the $K_{M/P}$ values, from eq 1, that are reported in Table I.

peptides which did not bind to the affinity matrix. The elution behavior of S-protein, a known interactor to immobilized S-peptide, was measured with the same matrix and elution conditions as for antisense peptides. The agreement of the $K_{M/P}$ value of S-protein with values expected from previous solution and chromatographic experiments argues that the immobilized S-peptide used here can attain a close-to-native state and that no significant nonspecific interactions with affinity matrix occur under the elution conditions used.

Variation of charge/hydropathic character at the amino terminus of IAS[SPep(1→20)] has some effects on binding. IAS[SPep(3→20)], which lacks two N-terminal hydrophobic residues, has a slightly lower calculated affinity toward immobilized S-peptide than does full-sequence IAS[SPep(1→20)], consistent with our previous observation (Shai et al., 1987) that chain shortening causes reduction in affinity. Upon replacement of the two N-terminal hydrophobic residues by two charged residues, affinities are reduced versus those of the original IAS[SPep(1→20)] and IAS[SPep(3→20)]. For no change in net charge, in the cases of [K^{1}E^2] and [E^{1}K^2]-IAS[SPep(1→20)], the affinity decrease is rather modest, roughly 2-fold compared to that of IAS[SPep(1→20)]. There is no significant difference in the $K_{M/P}$ values between these two mutant peptides.

A more dramatic increase in $K_{M/P}$, close to 100-fold versus that of IAS[SPep(1→20)], was found with [E^{1}E^2]IAS-[SPep(1→20)]. The apparent affinity decrease here can be correlated with the decrease in net positive charge (from +6 to +4). Indeed, among all the peptides tested, there is a general tendency toward a decrease in binding affinity upon decreasing net positive charge (Figure 3). However, it is likely that charge is not the only governing factor in affinity decrease, since comparison of the affinities of several different peptides

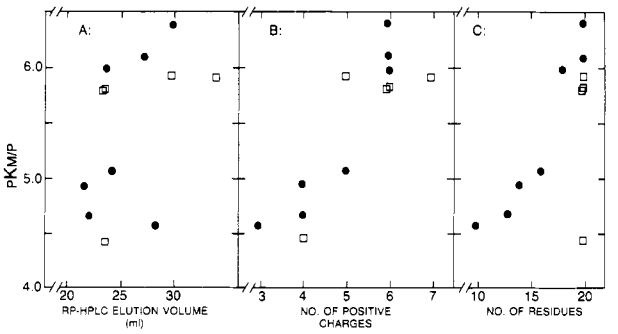

FIGURE 3: Relationship between p$K_{M/P}$ values (of the interaction of AS and IAS peptides (●) and mutants (□) with immobilized S-peptide) and antisense peptide characteristics. (A) RP HPLC elution volumes (see Table II). (B) Number of positive charges (at the elution conditions). (C) Number of residues.

(Table II) shows that binding affinity does not respond in a 1:1 fashion with net charge. As an example, IAS[SPep(5→20)] and [P^{3,6,13,15}]IAS[SPep(1→20)] have the same net charge, yet the latter has about a 10-fold higher affinity than the former. The reduction of stoichiometry with decreased antisense peptide size also argues against simple ion exchange as the mechanism governing antisense/sense peptide binding (see below).

The proline mutant, [P^{3,6,13,15}]IAS[SPep(1→20)], was designed to test the degree of conformational degeneracy in antisense peptide binding. In spite of the conformational restrictions imposed by four prolyl residues, this analogue retains relatively strong affinity, with a $K_{M/P}$ value only 1.5 times higher than that of IAS[SPep(1→20)]. This supports the conclusion of conformational degeneracy (Shai et al., 1987) based on comparing AS[SPep(20→1)] with IAS[SPep(1→20)].

Effect of Sequence Scrambling. The results obtained from site-directed mutants suggest a significant degree of both sequence and conformational degeneracy in the structural code which drives antisense peptide binding to sense peptide. In order to test this possibility further, we synthesized a scrambled antisense peptide, keeping the residues of antisense 20-mer but changing their order. The scrambled sequence was GSVRKRLSLRVSFSPGLRLH. This peptide also was found to bind to immobilized S-peptide. Zonal competitive elution analysis yielded a $K_{M/P}$ value of 1.1×10^{-6} M, 3 times greater than (but still surprisingly close to) that for AS[SPep(20→1)]. Moreover, the scrambled peptide also binds to soluble S-peptide as judged by the extent of competition by the latter; $K_{L/P} = 2.3 \times 10^{-6}$ M. As found previously for AS[SPep-(20→1)] (Shai et al., 1987), the apparent dissociation constant of scrambled antisense peptide is larger with soluble than with immobilized S-peptide.

Response of Stoichiometry to Sequence Variation and Truncation. The stoichiometry of binding of antisense peptide analogues to immobilized S-peptide was measured by evaluating the functional capacity of the S-peptide matrix with continuous (broad zone) elution analytical affinity chromatography. Solutions of various concentrations $[P]_0$ of mobile antisense peptides and mutants were eluted on either the 0.32 mL or the 2.3 mL bed volume column as noted in Table III. Elutions were continued with peptide solutions until saturation was achieved and the eluate had the same concentration of mobile peptide as the initial applied solution as judged by UV absorbance. As described before (Shai et al., 1987), the variation of $1/(V - V_0)$ vs $[P]_0$ gave a straight line with a slope equal to $1/M_T$ and thus was used to calculate M_T, the functional capacity of the matrix for mobile, antisense peptide.

Table III: Evaluation of Stoichiometry and $K_{M/P}$ Values of AS and IAS Peptides and Mutants Using the Frontal Analysis Method (Data Taken from Figure 4)

peptide designation	slope ($mmol^{-1}$)	M_T (nmol) [column volume (mL)]	$K_{M/P}$	ratio of frontal $K_{M/P}$ to zonal $K_{M/P}$	stoichiometry ratio
AS(20→1)[a,b]	5.2×10^3	190 [0.32]	1.4×10^{-5}		6.6
IAS(1→20)[a,b]	7.75×10^3	129 [0.32]	2.0×10^{-5}		4.4
[P[3,6,13,15]]IAS(1→20)[b]	1.06×10^4	94 [0.32]	1.5×10^{-5}	12.4	3.2
AS(14→1)[c]	3.08×10^3	324 [2.3]	6.0×10^{-5}	5.2	1.6
AS(20→8)[c]	2.17×10^3	459 [2.3]	1.8×10^{-4}	8.1	2.2
AS(10→1)[c]	2.98×10^3	334 [2.3]	1.5×10^{-4}	5.5	1.6
IAS(5→20)[c]	1.3×10^3	881 [2.3]	1.3×10^{-4}	15.3	4.2
[E[1]E[2]]IAS(1→20)[c]	3.62×10^3	276 [2.3]	1.7×10^{-4}	4.6	1.3

[a] Data were taken from previous work. [b] Stoichiometry was calculated with a 0.32 mL bed volume column. [c] Stoichiometry was calculated with a 2.3 mL bed volume column.

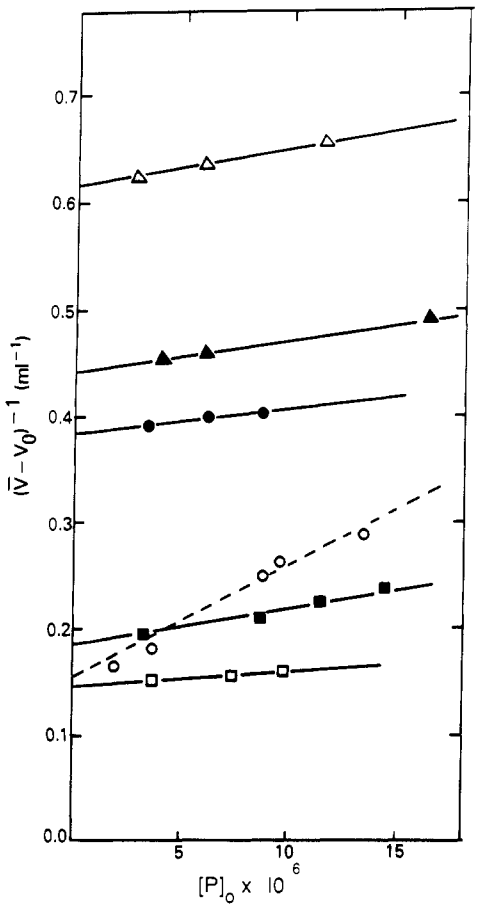

FIGURE 4: Frontal elution analysis of shortened and site-directed mutants of AS and IAS peptides. Solutions of peptides were eluted, at varying concentrations ($[P]_0$), on the 2.3 mL bed volume column (except for [P[3,6,3,15]]IAS[SPep(1→20)] on the 0.32 mL bed volume column) of S-peptide–Accell. (□) IAS(5→20); (○) [P[3,6,13,15]]IAS(1→20); (■) AS(14→1); (●) AS(20→8); (▲) AS(10→1); (△) [E[1]E[2]]IAS(1→20). Elution volumes are plotted as $1/(\bar{V}-V_0)$ vs $[P]_0$. The values of $K_{M/P}$ and M_T shown in Table III were calculated with eq 3.

Figure 4 contains the data obtained for several peptides. The linearity of Figure 4 plots argues that saturation occurs in the frontal elutions. If increasing concentrations led to progressively increasing values of M_T (peptide bound), the plots of Figure 4 would be expected to be curvilinear. The calculated values of M_T are given in Table III along with stoichiometries determined as ratios of M_T to the total amount of immobilized S-peptide determined by amino acid analysis of the matrix. In general, the data reveal that, while stoichiometries vary among the different peptides analyzed, they are all greater than 1. The stoichiometry generally decreases with decreasing peptide size for the truncated peptides, although the trend is not rigorous. The shortest peptide tested, AS[SPep(10→1)], has a value of 1.6. As found before, the 20-mers have stoichiometries of 4–6. Two 20-mer mutants tested, [E[1]E[2]]-IAS[SPep(1→20)] and [P[3,6,13,15]]IAS[SPep(1→20)], both exhibit stoichiometries of greater than 1 but less than that of 20-mer, especially the former case.

Broad-zone elution data also allowed determination of $K_{M/P}$ values from y intercepts of the plots $1/(\bar{V}-V_0)$ vs $[P]_0$. These values also are given in Table III. The $K_{M/P}$ values determined by continuous elution are generally greater by 5–15-fold than the values obtained by zonal elutions. In general, the larger the capacity (M_T) of the antisense peptide for immobilized S-peptide, the greater the disparity in $K_{M/P}$'s from continuous versus zonal elutions. As surmised before (Shai et al., 1987), the lower values for $K_{M/P}$ found with the zonal approach appear due to the fact that this approach treats the multiple interactions of mobile antisense peptide with immobilized S-peptide as an average interaction and therefore can overestimate the affinity of a single sense/antisense peptide/peptide binding event.

Correlation of $pK_{M/P}$ Values with Antisense Peptide Characteristics. To gain further insight into possible patterns of affinity variation for the site-directed mutants, $pK_{M/P}$ values were correlated with several peptide properties, including those related to hydrophobicity, charge, and size (sequence length). As shown in Figure 3A, there is no direct relationship between $pK_{M/P}$ and retention on reverse-phase (C18) matrix. Reverse-phase matrix retention has been shown to be directed predominantly by hydrophobic interaction, although the process may well be more complex. Nevertheless, the data in Figure 3A suggest that the interactions of AS peptides and mutants with native S-peptide are not directed in a simple way by peptide hydrophobicity alone. As shown in Figure 3B, the interactions between antisense peptides and immobilized S-peptide also are not simply charge-directed. There is, however, an approximate correlation with positive charge which is more distinct than that with reverse-phase chromatographic retention. However, several peptides with the same net charge have quite different affinities. In addition, the smaller the number of positive charges on the molecules, the smaller the stoichiometry value (the number of molecules of antisense peptide bound per molecule of immobilized S-peptide). This is the opposite of what one might expect if there were a simple set of charge interactions between antisense peptide positive charges and a fixed number of negatively charged binding sites on the affinity matrix. Perhaps the best correlation with $pK_{M/P}$ comes from peptide chain length, Figure 3C. This result is consistent with the argument made previously (Shai et al.,

Table IV: Evaluation of Experimental Dissociation Constants for IAS and AS Sequence-Simplified Peptides and Stoichiometry Ratio for Simplified AS Case

peptide designation	sequence	zonal elution		competitive zonal elution		frontal analysis	
		slope	$K_{M/P}$ (M)	$K_{M/P}$ (M)	$K_{L/P}$ (M)	$K_{M/P}$ (M)	stoichiometry ratio
simplified IAS	LLAKKALKLALLKLAKAGGK	12.7×10^3	2.6×10^{-6}				
simplified AS	KGGAKALKLLALKALKKALL	12.0×10^3	2.2×10^{-6}	3.3×10^{-6}	6.4×10^{-5}	1.7×10^{-5}	3.5

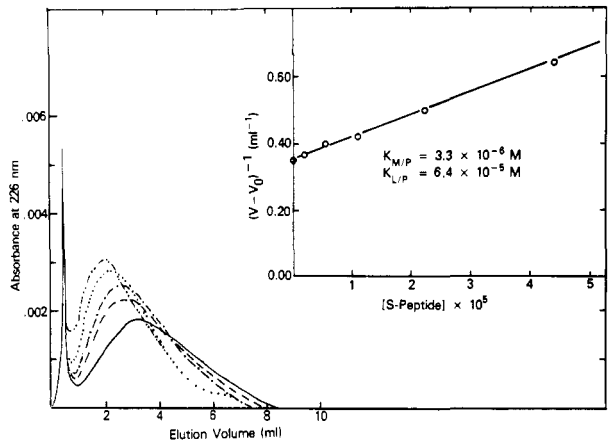

FIGURE 5: Competitive zonal-elution HPLAC analysis of sequence-simplified AS[SPep(20→1)] binding to S-peptide–Accell. Zones: 10 μL of 7.9 nmol of simplified AS peptide, were eluted on the 0.32 mL bed volume column in 0.2 M NH_4OAc, pH 5.7, containing the following molar concentrations of soluble S-peptide: (–··–) 4.38×10^{-5}; (···) 2.19×10^{-5}; (–·–) 1.09×10^{-5}; (––) 0.54×10^{-5}; (—) 0. Elution profiles are shown in the main figure; one profile (for 0.27×10^{-5} nmol) is omitted to avoid clutter. The insets show the variation of V with soluble competitor concentration plotted as $1/(V - V_0)$ vs [S-peptide] according to eq 2. The values of $K_{M/P}$ and $K_{L/P}$ determined from the data with eq 2 are given in the inset.

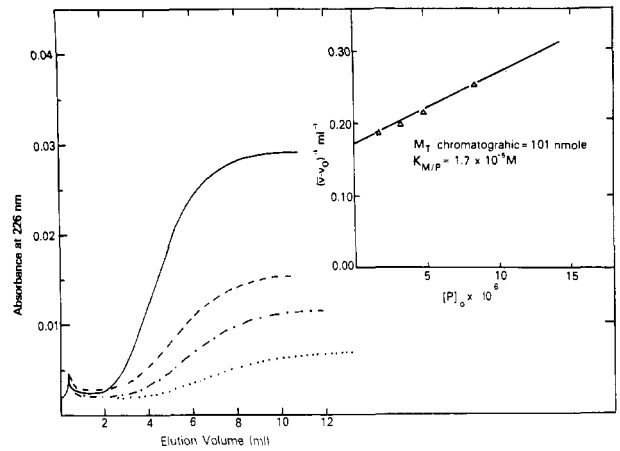

FIGURE 6: Evaluation of stoichiometry of simplified AS[SPep(20→1)] binding to immobilized S-peptide by frontal analysis. Solutions of simplified AS(20→1) were eluted at varying concentrations ($[P]_0$), on the 0.32 mL bed volume column. The elution profiles obtained are shown into the plateau regions. $[P]_0$ values: (—) 0.83×10^{-5} M; (––) 0.48×10^{-5} M; (–·–) 0.31×10^{-5} M; (···) 0.16×10^{-5} M. Inset: Plot of $1/(V - V_0)$ vs $[P]_0$. The values of M_T and $K_{M/P}$ shown in the inset were calculated with eq 3.

1987) that many residues along the chain contribute to affinity.

Sequence-Simplified IAS[SPep(1→20)] and AS[SPep(20→1)]. To improve the possibility to ultimately define mechanism, we attempted to design sequence-simplified models of AS and IAS 20-mers. Sequence simplification of native peptides has proven useful to identify the way amino acid residues contribute to binding affinity and conformation (Komoriya & Chaiken, 1982; Fassina & Chaiken, 1988). For the antisense S-peptide cases, simplification was accomplished by use of four amino acid residues to conserve the hydropathic distribution along the chain. Leu replaced all the highly hydrophobic residues, Lys replaced positively charged residues, Ala replaced weakly hydrophilic and weakly hydrophobic residues, and Gly was retained unaltered. Sequences of the simplified peptides are given in Table IV. Peptide binding characteristics of simplified antisense peptides were evaluated on the 0.32 mL bed volume column. From both zonal and frontal analyses (Figures 5 and 6; Table IV), the simplfied peptides exhibited binding affinities and, as determined for the AS case, multiple stoichiometry quite similar to those found for the parent AS and IAS peptides.

Discussion

Affinity chromatographic analysis of antisense peptide recognition of sense peptides has made it possible to define several quantitative properties of these interactions. That the interaction process is of relatively high affinity and of at least modest specificity, defined here as selectivity, has been established in the S-peptide case. The interaction has been observed both on the solid phase and in solution. Importantly, on the solid phase, the interaction is saturable [as seen from linear $1/(\bar{V} - V_0)$ vs $[P]_0$ plots], and the stoichiometry is generally greater than 1:1. How this interaction occurs, whether it can be modulated by synthetic sequence changes, and what relevance, if any, it has to intra- and intermolecular interactions of natural peptides and proteins remain intriguing questions.

Data obtained here for site-directed sequence mutants of S-peptide-related antisense peptides strongly suggest that a primary feature which promotes binding to sense peptides is the hydropathic pattern of amino acid sequence. Changes in sequence which decrease either net charge or hydrophobic residue content weaken interaction, as judged from variations in dissociation constants, and lead to changes in stoichiometry of antisense/sense complexation, at least on the solid phase. However, considerable site-directed sequence changes are tolerated without full loss of binding even with mutants in which significant charged–hydrophobic residue switches are made. The data argue that the sense/antisense interaction is sequence-degenerate. This conclusion was reached tentatively from the observation that both AS and IAS peptides bind nearly equally to S-peptide (Sahi et al., 1987). It is supported further by the observation in the current work that sequence-scrambled antisense peptide exhibits substantial binding affinity. The observation that the multi-proline antisense peptide mutant also binds substantially strongly argues from conformational degeneracy as well.

What other features besides hydropathic pattern help control sense/antisense peptide recognition? When variation of binding affinity is correlated with several peptide properties, as shown in Figure 6, perhaps the best correlation occurs with size (peptide length). Taken together with previous data (Shai et al., 1987), this result suggests a sequence-matchup model in which peptides in elongated configurations interact at multiple points along their sequences. The peptides would not acquire compact configurations for binding. In such a model, hydropathic pattern could place multiple combinations of in-

teracting residues close to one another. This model allows for considerable sequence degeneracy for recognition, including incorporation of prolyl residues, because elongated peptide configurations could be flexible, and not necessarily fixed, and because contacts could be provided by any residues which would supply adequate stabilizing free energy. This model also allows for multiple stoichiometry. Concerning the latter, the trend to lower stoichiometry with reduced antisense peptide length still is difficult to explain unequivocally. Two alternative models may be considered, one involving interaction of antisense peptide aggregates with sense peptide and a second involving interaction of multiple antisense peptides with different short segments along the sense peptide chain. Considering the latter possibility, antisense peptides as short as six residues have been found to have significant affinities and selectivities, as in the case of insulin (Knutson, 1988). In either case, the phenomenon of decreasing stoichiometry with decreasing size argues against ion exchange as a trivial explanation for the chromatographic retardation of antisense peptides on immobilized sense peptides. If simple ionic interaction were the mechanism, then reducing size might be expected to allow more, not less, mobile peptide molecules to bind to matrix sites, and the stoichiometry would increase with decreasing antisense peptide size.

In an effort to improve chances to "decode" the forces which drive the sense/antisense peptide interaction amid data suggesting substantial sequence degeneracy, synthetic simplification was carried out. The simplified antisense peptides made were on the basis of the multipoint–hydropathic pattern model discussed above. The twenty-residue model peptides consisting of KLAG (Lys, Leu, Ala, Gly) bind to immobilized S-peptide with affinity and multiple stoichiometry substantially similar to those of parent AS and IAS 20-mers. The interaction of simplified antisense peptides with sense peptide apparently also occurs in solution as judged by the competitive elution data. The success of sequence simplification has several ramifications for understanding the sense/antisense peptide interaction process. First, the result itself is at least consistent with the multisite–hydropathic pattern model of peptide recognition. Second, the peptides can serve as starting points for mutation studies which, based on simplified sequences, should allow relatively straightforward questions to be asked about the importance of particular structural elements for interaction.

The simplification results may help explain the interaction found for scrambled AS 20-mer. If the latter is converted into a four-residue sequence on the basis of the KLAG simplification scheme, significant homology is noted with the simplified AS and IAS 20-mers. More identity is seen with the IAS sequence, including five of eight residues in the region of residues 4 through 11:

```
                    ↓ ↓ ↓     ↓   ↓
AS parent:      KGGAKALKLLALKLAKKALL
Scrambled:      GALKKKLALKLALAAGLKLK
IAS parent:     LLAKKALKLALLKLAKAGGK
                    ↑↑ ↑ ↑ ↑   ↑   ↑
```

These homologies may help explain why the scrambled peptide can mimic parent antisense peptide. Thus, while the scrambled peptide initially was designed to significantly randomize amino acid sequence, the hydropathic pattern was conserved inadvertently to a substantial extent.

Having accumulated considerable confidence in the experimental observation of sense/antisense peptide interaction, one reasonably may ask whether this phenomenon is relevant to the way native peptides and proteins interact with one another, both intra- and intermolecularly. It is recognized that the sequence degeneracy and relatively low affinities often observed for the former can lead to skepticism about just how meaningful these can be. However, it is important to remember that significant peptide sequence simplification can be tolerated in native peptides and protein fragments with retention of binding and function (Komoriya & Chaiken, 1982; Taylor & Kaiser, 1986; Fassina & Chaiken, 1988). Furthermore, biologically relevant protein interactions can be of low affinity, as for lectins (Anderson & Walters, 1986) and monoclonal antibodies (Ohlson et al., 1988). These considerations lead us to the conclusion that the recurrent observations of antisense peptide interactions should not be dismissed lightly and that their relevance ought to be considered further.

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Recognition Properties of Antisense Peptides to Arg^8-vasopressin/Bovine Neurophysin II Biosynthetic Precursor Sequences

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ABSTRACT: We studied the interaction properties of synthetic antisense (AS) peptides encoded in the antisense strand of DNA corresponding to the N-terminal 20-residue sequence of the biosynthetic precursor of Arg^8-vasopressin (AVP) and its binding protein bovine neurophysin II (BNPII). Binding affinities of sense polypeptides AVP and BNPII with AS peptides were measured by analytical affinity chromatography, in each case by the extent of chromatographic retardation of a soluble polypeptide interactor on an affinity matrix containing the other interactor as the immobilized species. Chromatographically calculated dissociation constants ranged from 10^{-3} to 10^{-6} M. Experiments were carried out to define the selectivity and underlying forces involved in the AS peptide interactions. For AS peptide elutions on sense peptide affinity supports, reduced binding affinity with increasing 1-propanol concentration and ionic strength suggested the presence of both ionic and hydrophobic contributions to AS peptide/immobilized sense peptide recognition. This same conclusion was reached with the antisense peptides as the immobilized species and measurement of elution of sequence-simplified, truncated, and charge-depleted forms of sense peptides. Immobilized AS 20-mer affinity matrix differentially retarded AVP versus oxytocin (OT) and BNPII versus BNPI (the neurophysin related biosynthetically to OT) and was used to separate these polypeptides from acid extracts of bovine posterior pituitaries. In addition, immobilized AS 12-mer corresponding to AVP-Gly-Lys-Arg could be used to separate AVP from OT. The results confirm that antisense peptides recognize sense peptides with significant selectivity in the AVP/BNPII precursor case. The data also confirm the contribution of both hydrophilic and hydrophobic elements to the interaction and raise the intriguing potential to use antisense peptides in affinity technology, by a form of general ligand affinity separation which we would define as "pattern recognition affinity chromatography".

The observation of interactions between peptides encoded by the antisense strand of DNA and those coded by the corresponding sense strand (Bost et al., 1985b; Blalock & Bost, 1986; Shai et al., 1987a; Brentani et al., 1988; Knutson, 1988) has raised questions about what forces are responsible for sense/antisense peptide recognition and whether antisense peptides could be used as guides to willfully design synthetic molecules which recognize natural peptides and proteins. Direct measurements have been made for the interaction of RNase S-peptide with antisense peptide (Shai et al., 1987a,b, 1989). On the basis of the results, it was proposed that interacting peptides may be elongated rather than compactly folded and that affinity may well depend on a matchup of hydropathic patterns formed along the chains of both antisense and sense partners. A general pattern of opposing hydrophilic and hydrophobic residues in sense versus antisense sequences has been observed by inspection of the genetic code (Blalock et al., 1984; Bost et al., 1985a,b). Although this pattern in itself does not suggest a direct matchup of complementary interacting residues, a more elaborate hydropathic pattern recognition could be occurring (Chaiken, 1989).

While the S-peptide study [see Shai et al. (1989)] has provided quantitative support for the hypothesis that antisense peptide can interact with sense peptide, the degree of generality

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