Anti-Sense Peptide Recognition of Sense Peptides: Direct Quantitative Characterization with the Ribonuclease S-Peptide System Using Analytical High-Performance Affinity Chromatography†

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ABSTRACT: The ability of peptides coded by the anti-sense strand of DNA to interact specifically with peptides coded by the sense strand has been evaluated. The sense peptide examined, ribonuclease S-peptide, was immobilized on a coated silica affinity chromatographic matrix. Anti-sense peptides were synthesized on the basis of the anti-sense DNA sequence for the S-peptide region in native pancreatic ribonuclease A. The interaction of synthetic anti-sense peptides with sense peptide was quantitated from the degree of retardation during chromatographic elution on the sense peptide affinity matrix in buffers with and without soluble competing sense peptide. Sense/anti-sense peptide interactions were found to occur with significant affinities with each of two anti-sense 20-residue peptides of opposite amino-to-carboxyl orientations and to weaken progressively with decreasing length of anti-sense peptide. The substantial chromatographic retardation of anti-sense peptides was specific, since it decreased as expected with increasing concentration of the soluble competing S-peptide, could not be mimicked by the elution of several control peptides (including S-peptide itself) on the S-peptide matrix, and did not occur with a blank chromatographic matrix (no S-peptide attached). The stoichiometry of anti-sense peptide binding to immobilized sense peptide was found to be far greater than 1:1, and at least 4–5:1, for the two 20-mer anti-sense peptides. In sum, the analytical affinity chromatographic experiments have established quantitatively that anti-sense peptide binding to sense peptides occurs in the ribonuclease S-peptide case and have identified some structural elements that govern these interactions. Nonetheless, while the implications are strong for the potential usefulness of anti-sense peptides to learn about principles of native peptide and protein recognition and as tools in biotechnology, the generality and structural mechanisms of anti-sense peptide binding remain to be more fully understood.

The sequence and consequent higher order properties of native polypeptides and proteins are determined by the sense strand of DNA, through transcription (from 3′ to 5′) to mRNA (5′ to 3′, respectively) and translation of the latter to protein (amino to carboxyl terminal, respectively). Yet, recently, peptides corresponding to the usually ignored anti-sense DNA code have been reported to have properties of potential biological and biotechnological importance. Anti-sense peptides to ACTH,[1] denoted "HTCA" and containing amino acid sequences defined by the base sequence code of the anti-sense strand of cloned DNA (read either 3′ to 5′ or 5′ to 3′ for peptide amino to carboxyl), can bind to ACTH as judged by assays in which HTCA was attached to microtiter plate wells, and ACTH bound noncovalently to the attached HTCA was measured either by enzyme immunoassay (Bost et al., 1985a) or by the amount of radiolabel of ^{125}I-ACTH attached (Blalock & Bost, 1986). The microtiter plate assay method also was used to detect binding of δ-endorphin to its anti-sense peptides (Bost et al., 1985a). Furthermore, on the basis of the possibility that anti-sense and sense peptides might have complementary molecular structures, antibodies to HTCA (read 5′ to 3′) were elicited; these were found to mimic ACTH in stimulating steroidogenesis in mouse adrenal cells (Bost et al., 1985a) and subsequently have been immobilized and used in immunoaffinity chromatography to isolate ACTH receptors (Bost et al., 1985a; Bost & Blalock, 1986). Anti-sense peptide binding to sense peptides has been correlated with the pattern of hydropathy of amino acid residues (Kyte & Doolittle, 1982) coded by sense and anti-sense DNA (Blalock & Smith, 1984; Bost et al., 1985b). Such data certainly raise provocative questions about the nature of peptide and protein recognition generally and suggest potential biotechnological uses of synthetic anti-sense peptides as selective macromolecular interactors. Nonetheless, at present, little understanding has been obtained experimentally of the structural nature of sense/anti-sense peptide recognition.

To obtain the direct understanding of anti-sense peptide interactions needed to evaluate the underlying bases for interaction and to develop such peptides as designed recognition molecules, we have used the experimental approach of analytical high-performance affinity chromatography. Previous work (Swaisgood & Chaiken, 1985, 1986; Abercrombie & Chaiken, 1985; Winzor, 1985; Chaiken, 1986; Fassina et al., 1986) has established that analytical affinity chromatography, including analytical HPAC, can be used to measure interactions quantitatively between immobilized and mobile molecules, in a direct manner and without the need for the interaction to produce a second-order effect (spectroscopic, enzymatic, or otherwise). Thus, the extent of retardation of a mobile molecule in isocratic elutions under binding conditions

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[1] Abbreviations: ACTH, adrenocorticotrophic hormone; HPAC, high-performance affinity chromatography; PAM, phenylacetamidomethyl; Boc, *tert*-butyloxycarbonyl; HPLC, high-performance liquid chromatography; RNase, ribonuclease; TFA, trifluoroacetic acid.

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and in elutions in the presence of soluble molecules that compete with immobilized molecules can be used to calculate equilibrium binding constants for mobile molecule–immobilized molecule and mobile molecule–soluble competitor complexes. With this methodological background, the analytical HPAC approach was used to determine equilibrium binding characteristics of anti-sense to sense peptides, with the bovine pancreatic RNase S-peptide case as a test system. Native S-peptide was immobilized on a silica affinity matrix, and the retardation of S-peptide-related anti-sense peptides of varying sequence was measured. The chromatographic elution characteristics allow a quantitative evaluation of affinity and specificity of anti-sense peptide interaction with sense peptide in the RNase S-peptide case and establish a basic experimental paradigm of general use for the further characterization and design of anti-sense peptides.

Materials and Methods

General Materials and Instrumentation. The subtilisin fragments of bovine pancreatic RNase A, S-peptide (containing residues 1–20), and S-protein (containing residues 21–124) were purchased from Sigma Chemical Co. (St. Louis, MO). Activated affinity chromatographic supports, based on a silica backbone and containing a 6-carbon spacer with a terminal *N*-hydroxysuccinimide ester for immobilizing molecules through their amino groups, were kindly granted to us by Waters Chromatography Division, under the trade name Accell 78 (Milford, MA). Glass columns for packing affinity matrices were purchased from Rainin Instrument Co. (Woburn, MA). PAM resins for solid-phase peptide synthesis were purchased from Applied Biosystems (Foster City, CA), and Boc amino acids from Peninsula Laboratory (Belmont, CA).

Chemical Characterization of Peptides and Proteins. Amino acid analyses were performed at the National Institutes of Health, by Angela Corigliano Murphy, with a Beckman Model 6300 amino acid analyzer with ninhydrin detection. Sequencing of synthesized peptides was performed at Triton Biosciences (Alameda, CA) with an ABI gas-phase sequencer, Model 470A. Reverse-phase HPLC analyses and separations were performed on a Gilson HPLC system, composed of a Model 811 dynamic mixer, a Model 802B manometrics module, two Model 303 pumps, and a Model 116 variable-wavelength absorbance detector, and an NEC Model PC-8023A-C recorder, all programmed by an Apple IIc computer. Analytical HPAC was carried out with an LKB HPLC system, composed of a Model 2150 ceramic head pump, a Model 2152 HPLC controller, and a Model 2151 variable-wavelength absorbance monitor, coupled to a Kratos Spectroflow Model 757 recorder.

Peptide Synthesis and Purification. Anti-sense peptides were synthesized by the solid-phase approach on PAM resins (Mitchell et al., 1978). After cleavage from resins, deprotection, and preliminary extraction, all synthesized peptides were purified to a high degree of chromatographic homogeneity, 95 to >99%, by reverse-phase HPLC on a semipreparative μBondapak C_{18} column (Waters Millipore Corp.) as follows: solvent A, 0.1% TFA in water; solvent B, 0.1% TFA in acetonitrile; elution was with a linear gradient from 10% to 40% solvent B in 35 min and from 40% to 80% solvent B in the next 5 min; the flow rate was 3 mL/min and detection was by absorbance at 226 nm. Both anti-sense 20-mer peptides (see Figure 1) were purified further by affinity chromatography on a high-capacity immobilized S-peptide matrix (see below).

High-Performance Affinity Chromatography. Immobilization of S-peptide on Accell 78 was carried out by shaking 2 g of matrix in a 10-mL solution containing 1 mg (except where noted) of S-peptide in 0.5 M NaCl–0.1 M $NaHCO_3$, pH 8.0, for 1.5 h. The reacted matrix was washed with 2 volumes of the applied buffer and then end-capped by mixing with 10 mL of 0.3 M aminoethanol in the same buffer for 1 h. The matrix then was washed with 5 volumes of the reaction buffer and finally with 5 volumes of HPLC-grade water. Amino acid analysis of the matrix product showed a loading of 0.35 mg of S-peptide/g of matrix (70% overall yield); this converts to an overall concentration of immobilized S-peptide of 9.1×10^{-5} M. A 1.5-g portion of the product was packed in a glass column (6.6 × 100 mm), with a final bed volume of 2.30 mL. A second portion of 0.2 g was packed into another glass column (3 × 50 mm), with a final bed volume of 0.32 mL.

A preparative, high-capacity affinity matrix also was prepared by reacting 3.7 mg of S-peptide with 2 g of Accell 78. For this and the more dilute S-peptide affinity matrices, the number of attachment sites of S-peptide to Accell 78 was not determined explicitly. However, Gawronski and Wold (1972a) have made such an estimate for S-peptide immobilized at pH 7.5 through its amino groups to cyanogen bromide activated agarose and found the predominant linkage to be through Lys-1 rather than Lys-7 by a ratio of about 3:1. Whatever the attachment mode, all of the S-peptide matrices used were found to bind ribonuclease S-peptide with a binding affinity similar to that for the interaction in solution (see below).

A blank matrix was prepared by reacting 2 g of Accell 78 with 10 mL of 0.3 M aminoethanol in 0.5 M NaCl–0.1 M $NaHCO_3$, pH 8.0, for 1 h, followed by washing and packing as before.

Analytical HPAC. Dissociation constants for interaction of synthetic anti-sense peptides with S-peptide were obtained by two methods: zonal elution (Swaisgood & Chaiken, 1985, 1986; Abercrombie & Chaiken, 1985; Chaiken, 1986) and continuous (broad zone) elution (Swaisgood & Chaiken, 1985; Winzor, 1985). In the zonal elution method, the column of immobilized S-peptide was preequilibrated with an elution buffer of 0.2 M NH_4OAc, pH 5.7, at a flow rate of 1 mL/min monitored by UV absorbance at 226 nm, until a constant base line was observed. Then a 10-μL zone containing 5–300 μg of the appropriate anti-sense peptide in 0.2 M NH_4OAc, pH 5.7, was injected, and the elution profile was recorded. The variation of the elution volume V with the concentration of mobile peptide [P] was evaluated by the equation

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

where V = experimental elution volume of mobile interactor (here, anti-sense peptide), V_0 = unretarded elution volume (determined with bovine neurophysin, oxytocin, and S-peptide elutions), $[M]_T$ = total concentration of immobilized (matrix-bound) S-peptide, V_m = mobile-phase volume (determined with dextran blue elution), [P] = concentration of mobile peptide in the chromatographed zone, and $K_{M/P}$ = dissociation constant of the complex of matrix-bound sense peptide (M) and the mobile anti-sense peptide (P). During zonal elution, [P] changes continuously and is not determined. However, when V was measured at various initial (injected) zonal concentrations of P, namely $[P]_0$, and extrapolated to $[P]_0 = 0$, $K_{M/P}$ was determined from the y-intercept value of $1/(V - V_0)$ by using eq 1 and [P] = 0. It should be noted that, since no assumptions were made about the mechanism of anti-sense peptide binding and therefore about the stoichiometry of the complexes formed, the $K_{M/P}$ values obtained are taken as

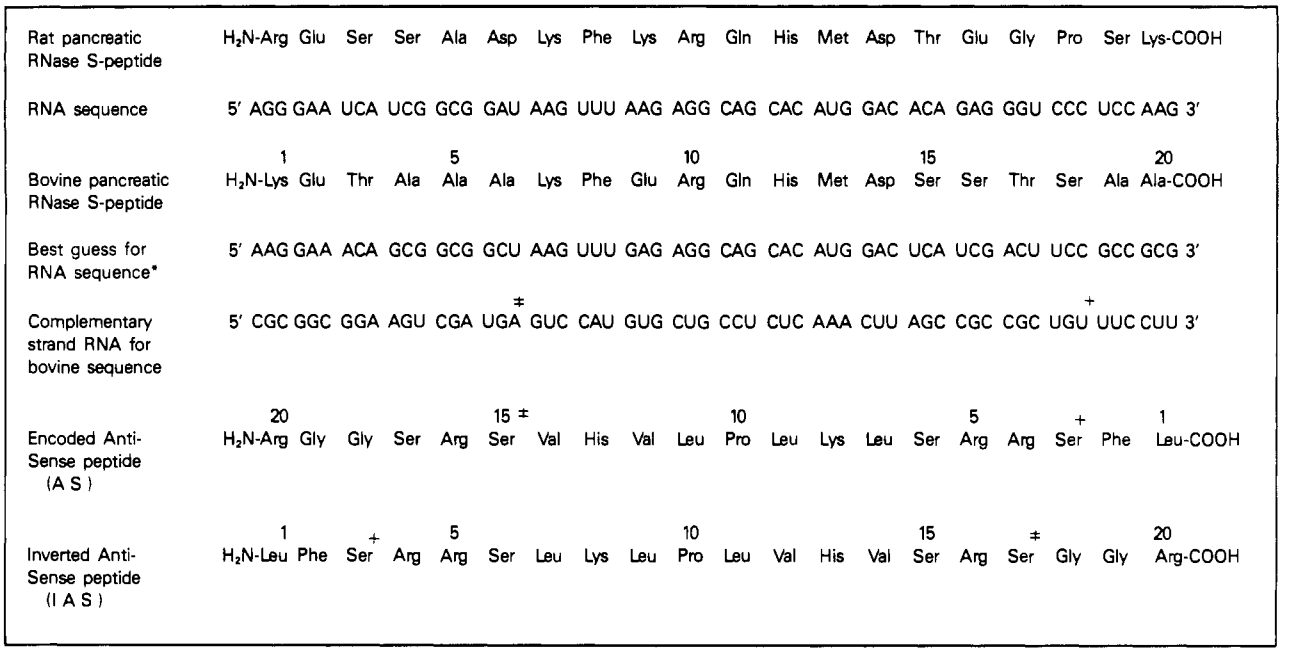

FIGURE 1: Amino acid sequence of anti-sense and inverted anti-sense peptides as deduced from the corresponding complementary RNA of bovine pancreatic RNase S-peptide. The diagram denotes the relationship of anti-sense peptides (AS, defined by anti-sense RNA, read 5′ to 3′) and inverted anti-sense peptides (IAS, the same sequence as anti-sense peptides but with amino-to-carboxyl orientation inverted) to the sense peptide sequence. Thus, amino-terminal Leu of IAS and carboxyl-terminal Leu of AS both correspond to the residue encoded by the anti-sense anticodon CUU (read 5′ to 3′) that matches up to the sense anticodon AAG for residue 1 (Lys) of native S-peptide. (*) For residues 1, 3, 4, 6, 9, 15, 18, and 19, the anticodon is different from rat anticodon by one base; for residues 17 and 20, the anticodon is different from rat anticodon by two bases. (+) UGU is the code for Cys; Ser is placed in position 3 to avoid complications of the free SH group in synthetic anti-sense peptides. (±) UGA is the anticodon for chain termination; Ser is placed in position 15 of synthetic anti-sense peptides. Further details of the notation are given in the text.

apparent (functional) rather than microscopic dissociation constants.

In cases where competitive elutions were carried out, zones of anti-sense peptides were injected with injection and elution buffer solutions containing different S-peptide concentrations, $[L]_T$, ranging from 7.9×10^{-6} to 3.9×10^{-5} M. The variation of V with $[L]_T$ was evaluated by the equation

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

where $[L]_T$ = total concentration of competing ligand, here sense peptide (S-peptide), $K_{L/P}$ = dissociation constant between mobile anti-sense peptide and mobile competing sense peptide, and the rest of the parameters are as before.

Thus, when V was measured at different $[L]_T$, $K_{M/P}$ and $K_{L/P}$ were determined simultaneously. Again, as noted above, no assumptions are made about stoichiometry and mechanism of anti-sense peptide interactions, and $K_{M/P}$ and $K_{L/P}$ thus are interpreted as apparent (functional) and not microscopic dissociation constants. Small and nonsystematic variations in peak tailing in zonal elution profiles did not affect calculated V values as judged by the reproducibility of the latter in repeat experiments.

In the continuous elution analytical affinity chromatography method, using frontal analysis to evaluate V, the column was prewashed with 0.1 M AcOH after each run, prior to equilibration with 0.2 M NH_4OAc, pH 5.7. After equilibration, solutions containing different concentrations of the synthesized anti-sense peptide were passed through the column continuously and monitored by UV absorbance (λ = 226 nm) until a plateau of maximum absorbance was observed. V values were determined as $\bar{V}$, the volume at half-maximal absorbance in the elution front, which was corrected for the small shoulder of UV-absorbing material observed at V_0. The latter V_0 shoulder apparently derived from contaminant material from the column and eluted at the peptide–buffer front after column flow was stopped to change the eluting buffer alone to buffer with peptide.

The variation of elution volume $\bar{V}$ was plotted according to the equation

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

where $\bar{V}$ = volume at which the affinity matrix is half-saturated, V_0 = void volume, $K_{M/P}$ = dissociation constant of complex of the immobilized S-peptide and anti-sense peptide, M_T = total amount of immobilized S-peptide $[=[M]_T(V_0 - V_m)]$, and $[P]_0$ = initial concentration of mobile anti-sense peptide (the initial and plateau values of [P] are equivalent in broad zone elution). Thus, from a plot of $1/(\bar{V} - V_0)$ vs. $[P]_0$, $1/M_T$ can be determined from the slope and $K_{M/P}$ from the intercept.

RESULTS

Design of Anti-Sense Peptides. Peptides corresponding to the complementary mRNA of bovine pancreatic RNase S-peptide [obtained from comparison to rat pancreatic RNase (MacDonald et al., 1982) with minimal base change], read in the direction 5′ → 3′, were synthesized (Figure 1). These peptides were purified by reverse-phase HPLC as described under Materials and Methods; the final products were generally greater than 99% homogeneous as judged by observation of a single major reverse-phase chromatographic peak. The structures of all peptides were verified by amino acid analysis after reverse-phase HPLC. In addition, for the representative case of inverted anti-sense 20-mer, the structure was confirmed by automated amino acid sequencing of material obtained from combined reverse-phase HPLC and preparative affinity chromatography.

Two families of peptides were synthesized (see Figure 1). The first set of peptides, designated anti-sense (AS), corresponds to the normal reading of the anti-sense mRNA from 5′ to 3′, with the amino terminus at the 5′ end. The full-length anti-sense peptide sequence is denoted AS (20 → 1), signifying that the N-terminal residue (to the left of the arrow) of the anti-sense peptide corresponds to the anti-sense code of residue 20 (the carboxyl-terminal Ala) of native S-peptide, while the carboxyl-terminal residue (to the right of the arrow) corresponds to the anti-sense code of residue 1 (the amino-terminal Lys) of native S-peptide. The second set of peptides synthesized is designated inverted anti-sense (IAS). IAS (1 → 20) is the peptide with the same sequence as AS (20 → 1) but with the amino-to-carboxyl orientation inverted, so that the amino-terminal residue (to the left of the arrow) corresponds to Lys-1 of native S-peptide and the carboxyl-terminal residue corresponds to Ala-20. The shorter peptides contain fragments of AS (20 → 1) or IAS (1 → 20) with the residue numbers defined in parentheses referring to those shown in Figure 1. In all cases, the numbers in the peptide designations correspond to the residue position in native S-peptide.

Quantitative Interaction Properties of 20-mer Anti-Sense Peptides. The interactions of both AS (20 → 1) and IAS (1 → 20) with soluble and immobilized S-peptide were evaluated by the zonal competitive elution affinity chromatography approach. Similar amounts of 20-mer AS or IAS peptides were eluted on a column of 0.32-mL total bed volume in 0.2 M NH_4OAc, pH 5.7, containing varying concentrations of the soluble S-peptide $[L]_T$. In these zonal elutions, >99% of each anti-sense 20-mer applied was recovered in a specifically retarded chromatographic peak, as judged by reverse-phase HPLC analysis of applied and affinity column eluted material. The elution profiles obtained, shown in Figure 2, confirm that both 20-mers bind to the affinity matrix as judged by their retardation and that V decreases as $[L]_T$ increases. Elution volumes determined at each value of $[L]_T$ were plotted as $1/(V - V_0)$ vs. $[L]_T$, according to eq 2. As shown in the Figure 2 inset, the variation of $1/(V - V_0)$ with $[L]_T$ is linear. The data demonstrate that there is a defined affinity between immobilized S-peptide and AS or IAS peptides. Values of apparent $K_{M/P}$ values were calculated graphically from the intercepts of the $1/(V - V_0)$ vs. $[L]_T$ plots. In both cases a relatively high affinity was determined (see Figure 2). By comparison, with the same S-peptide affinity matrix, the value of $K_{M/P}$ determined for the S-peptide–S-protein interaction (Fassina et al., 1986) by elution of S-protein on the S-peptide column was 7.0×10^{-7} M; the $K_{L/P}$ value for the S-protein–S-peptide interaction in solution, determined by competitive elution with soluble S-peptide, was 1.9×10^{-7} M.

The dissociation constants ($K_{L/P}$) for the interaction of AS or IAS 20-mer peptides with mobile S-peptide were calculated from the slope of the $1/(V - V_0)$ vs. $[L]_T$ plots of Figure 2, again according to eq 2. These values were 3.6×10^{-5} M for AS (20 → 1) and 4.4×10^{-5} M for IAS (1 → 20). The $K_{L/P}$ values are larger than those obtained for $K_{M/P}$. This difference may be due largely to the stoichiometric features of the anti-sense/sense peptide interaction (see below), although the possibility cannot be ruled out that mechanistic differences exist between the solution interaction and that on the matrix. More generally, both K_d values for AS (20 → 1) are smaller than the corresponding values for the 20-mer IAS, suggesting a somewhat higher affinity for AS toward S-peptide. Nonetheless, the affinities are very close for the AS and IAS peptides.

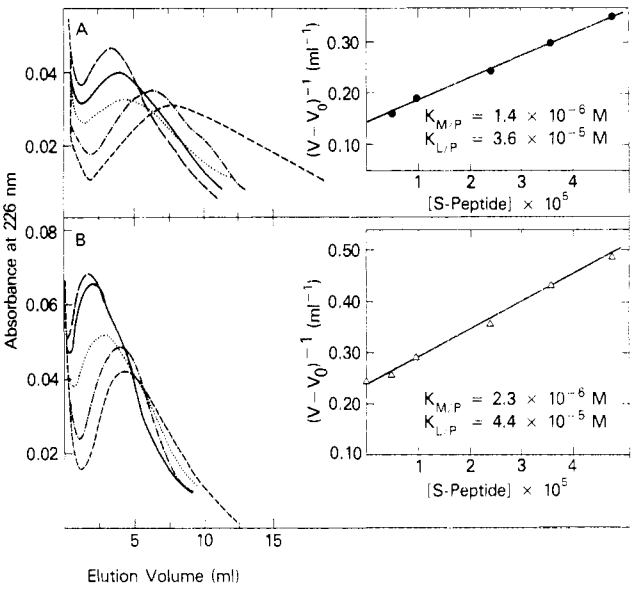

FIGURE 2: Competitive zonal elution HPAC analysis of anti-sense 20-mer peptide binding to RNase S-peptide. Zones of 7.6 nmol of either (A) AS (20 → 1) or (B) IAS (1 → 20) in 10 μL were eluted on the 0.32-mL bed volume column of immobilized S-peptide, $[M]_T = 9.1 \times 10^{-5}$ M, in 0.2 M NH_4OAc, pH 5.7, containing the following molar concentrations of soluble S-peptide: (– –) 0.47×10^{-5}; (—) 0.95×10^{-5}; (···) 2.39×10^{-5}; (–·–) 3.58×10^{-5}; (– · –) 4.78×10^{-5}. Elution profiles are shown in the main figures; the insets show the variation of V with soluble competitor concentration plotted as $1/(V - V_0)$ vs. [S-peptide] according to eq 2. In panel B, the elution profile for [S-peptide] = 0 is omitted to avoid clutter, but the value of $1/(V - V_0)$ is given in the inset along with the other data obtained. The values of $K_{M/P}$ and $K_{L/P}$ determined from the data in eq 2 are given in the insets of panels A and B.

The specificity of binding of AS (20 → 1) and IAS (1 → 20) to immobilized S-peptide was demonstrated by several observations. Under the same conditions at which AS and IAS peptides are retarded, several peptides were not retarded on immobilized S-peptide. These include S-peptide, oxytocin, neurophysin, the Arg-rich synthetic kemptide (Gly-Arg-Gly-Leu-Ser-Leu-Ser-Arg), and a highly charged decapeptide (Lys-Glu-Lys-Glu-Lys-Leu-Glu-Phe-Ile-Leu). Further, neither AS nor IAS peptides were retarded to any large extent on a blank column containing the same aminoethanol end-capped matrix in the same bed volume, but with no immobilized S-peptide. In addition, when AS and IAS peptides were eluted at the same buffer conditions on a column of similar bed volume of high-capacity immobilized S-peptide (see Materials and Methods), the extent of retardation increased to the extent expected from the increased $[M]_T$ according to eq 1. In addition to the above, the specificity of the S-peptide–Accell matrix itself was demonstrated by the elution behavior of S-protein (see above) and the agreement of the $K_{M/P}$ and $K_{L/P}$ values obtained chromatographically with K_d values obtained previously for S-peptide–S-protein interaction in solution and on an S-peptide–agarose affinity matrix (Hearn et al., 1971; Gawronski & Wold, 1972a,b).

Interaction of Shortened Anti-Sense Peptides. The dissociation constants $K_{M/P}$ were determined for a series of shortened anti-sense peptides to immobilized S-peptide, from zonal elutions in the absence of the soluble competitive ligand S-peptide. As with the 20-mers, the overall mass recovery of the shortened anti-sense peptides from affinity columns was >99%. However, given the presence of small amounts of nonbinding peptide that coeluted with some of the shortened peptides during reverse-phase HPLC purification, the mass recovery of applied sample in the retarded affinity chroma-

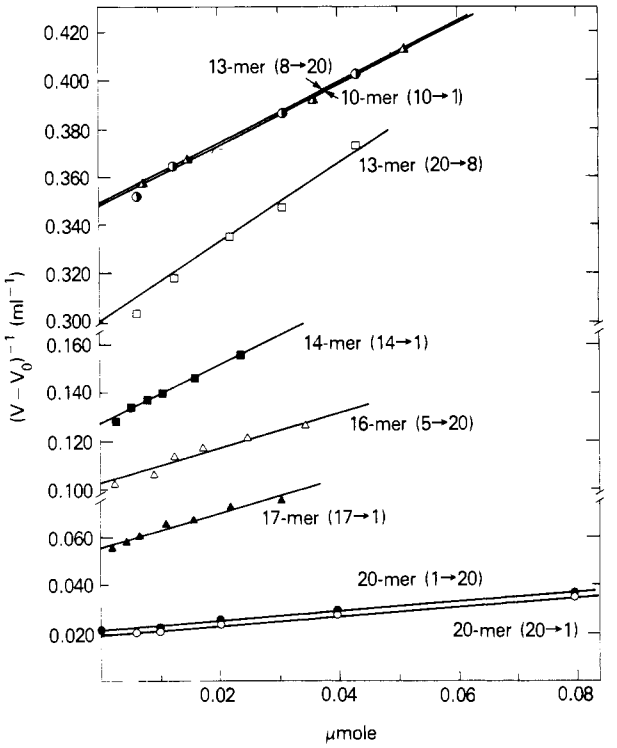

FIGURE 3: Zonal elution analytical HPAC determination of RNase S-peptide binding by synthetic anti-sense and inverted anti-sense 20-mers and shortened fragments. Designation of fragments is according to Figure 1. (○) AS (20 → 1); (●) IAS (1 → 20); (▲) AS (17 → 1); (△) IAS (5 → 20); (□) AS (14 → 1); (□) AS (20 → 8); (▲) AS (10 → 1); (◑) IAS (8 → 20). In all cases, zones of peptides of 10-μL volume containing the denoted micromoles of peptide were eluted on the 2.3-mL bed volume column of immobilized S-peptide in 0.2 M NH_4OAc, pH 5.7. Elution volumes are plotted as $1/(V - V_0)$ vs. micromoles of mobile molecule; the extrapolated $1/(V - V_0)$ values at μmol = 0 are used to calculate $K_{M/P}$ values, from eq 1, that are reported in Table I.

tographic peaks for some anti-sense peptides was as low as 85%, with the remaining peptide eluting close to the void volume. The presence of nonbinding contaminants has no effect on the determination of $K_{M/P}$ values from extrapolation to $P_0 = 0$. Varying amounts of the peptides were injected in zones of constant volume. As shown in Figure 3, a linear variation of $1/(V - V_0)$ vs. P_0 was found for all peptides, a behavior consistent with eq 1 and allowing $K_{M/P}$ values to be calculated from $1/(V - V_0)$ at the y intercepts. These values, corrected for the small degrees of retardation observed on a blank column ($M_T = 0$), are given in Table I. For AS (20 → 1) and IAS (1 → 20), elutions using the 2.3-mL bed volume column gave $K_{M/P}$ values of 1.2×10^{-6} M for AS (20 → 1) and 1.3×10^{-6} M for IAS (1 → 20), which are close to those obtained by competitive elution on the 0.32-mL bed volume column. By comparison, as shown in Figure 3 and Table I, affinity generally decreases ($K_{M/P}$ increases) with peptide shortening, although most of the shorter peptides still retain substantial affinity. In addition, in cases for which peptides contain similar sequences but in inverted order [e.g., AS (20 → 1) vs. IAS (1 → 20) and AS (20 → 8) vs. IAS (8 → 20)], the anti-sense form has a higher calculated affinity than the inverted anti-sense peptide, but the difference is rather small and its significance cannot be generalized at the present time.

Stoichiometry. While the zonal affinity chromatographic elution method has provided a measure of the affinity for anti-sense peptide binding to immobilized S-peptide, determining the stoichiometry for this interaction required measuring the functional capacity of the S-peptide matrix by the

Table I: Dissociation Constants of S-Peptide Interactions with Anti-Sense Peptides As Determined by Analytical Affinity Chromatography

peptide designation[a]	extrapolated elution vol[b] (mL)	$K_{M/P}$[c] (M)	corrected $K_{M/P}$[d] (M)
AS (20 → 1)	51.5 (0.75)	1.2×10^{-6}	1.2×10^{-6}
IAS (1 → 20)	47.6 (0.75)	1.3×10^{-6}	1.3×10^{-6}
AS (17 → 1)	18.0 (0.60)	3.4×10^{-6}	3.5×10^{-6}
IAS (5 → 20)	9.7 (0.40)	6.3×10^{-6}	6.5×10^{-6}
AS (14 → 1)	7.8 (0.55)	7.7×10^{-6}	8.3×10^{-6}
AS (20 → 8)	3.3 (0.10)	1.8×10^{-5}	1.8×10^{-5}
IAS (8 → 20)	2.8 (0.30)	2.1×10^{-5}	2.3×10^{-5}
AS (10 → 1)	2.9 (0.30)	2.1×10^{-5}	2.3×10^{-5}
IAS (11 → 20)	<1.0	$>5.9 \times 10^{-5}$	
AS (16 → 8)	<1.0	$>5.9 \times 10^{-5}$	

[a] Designation relates to Figure 1. [b] Determined from extrapolation of zonal elution data in Figure 3 to μmol = 0 (see eq 1) and expressed as $V - V_0$; elution volumes on blank column, expressed as $V - V_0$, are given in parentheses. [c] Determined from extrapolated values of $V - V_0$ by use of eq 1. [d] Calculated from extrapolated $V - V_0$ values corrected for the small degrees of retardation observed for peptides on the blank column.

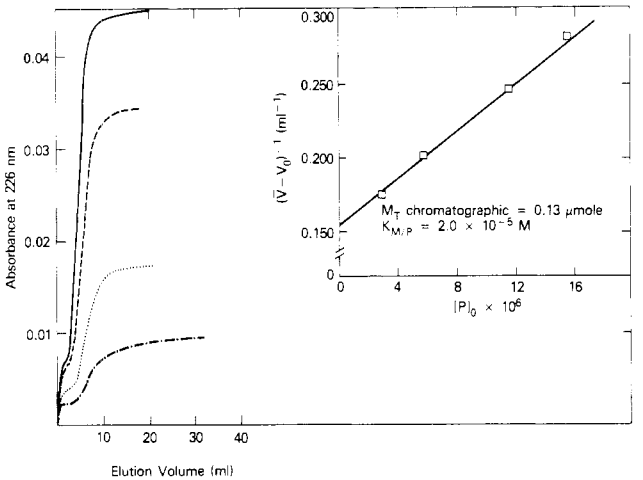

FIGURE 4: Evaluation of stoichiometry of inverted anti-sense (1 → 20) binding to immobilized S-peptide by frontal analysis. Solutions of IAS (1 → 20) were eluted, at varying concentrations ($[P]_0$), on the 0.32-mL bed volume column of S-peptide–Accell, and the elution profiles obtained are shown to the plateau regions. $[P]_0$ values: (—) 1.53×10^{-5} M; (- - -) 1.15×10^{-5} M; (···) 0.57×10^{-5} M; (–·–) 0.28×10^{-5} M. Inset: Plot of $1/(\bar{V} - V_0)$ vs. $[P]_0$. The values of M_T and $K_{M/P}$ shown in the inset were calculated by using eq 3.

continuous (broad zone) elution method. Solutions of different concentrations, $[P]_0$, of mobile AS (20 → 1) and IAS (1 → 20) were passed through the affinity column, in each case until saturation was achieved and the eluate had the same concentration of mobile peptide as the initial applied solution. From eq 3, it is expected that the plot of $1/(\bar{V} - V_0)$ (where $\bar{V}$ is the elution volume for half-saturation) vs. $[P]_0$ will be a straight line with a slope equal to $1/M_T$. V_0 is an experimentally determined constant, so M_T (functional capacity of the matrix) can be calculated. Figure 4 contains the elution profiles for the IAS (1 → 20) case and the corresponding straight line plot of $1/(\bar{V} - V_0)$ vs. $[P]_0$. The results with AS (20 → 1) are not shown but are rather similar. The values of M_T determined by this procedure were 190 nmol for AS (20 → 1) and 129 nmol for IAS (1 → 20). The total amount of S peptide immobilized on the matrix was determined by amino acid analysis to be 29 nmol. These data therefore indicate that at least 6.5 and 4.4 molecules of AS (20 → 1) and IAS (1 → 20), respectively, can bind per molecule of immobilized S-peptide. It is quite possible that the actual

stoichiometries of 20-mer AS (20 → 1) and IAS (1 → 20) binding at saturation are in fact larger than those determined chromatographically, since some of the immobilized S-peptide could be inaccessible or inactive for binding. The observation of greater than 1:1 stoichiometry is consistent with an observation, made by zonal elution, that saturation of the S-peptide matrix could not be accomplished with up to a 4-fold excess of anti-sense peptide.

The frontal analysis data (e.g., Figure 4 inset) also allow (eq 3) determination of $K_{M/P}$ values from y intercepts of $1/(\bar{V} - V_0)$ vs. $[P]_0$ and M_T values from slopes of these plots. These calculated values for 20-mer AS (20 → 1) and IAS (1 → 20) were 1.4×10^{-5} M and 2.0×10^{-5} M, respectively. These values are much closer to the values of $K_{L/P}$ determined by zonal competitive elution (Figure 2) and suggest that the lower values of $K_{M/P}$ found with eq 2 (Figure 2) are due to the fact that eq 1 treats the multiple interactions of mobile anti-sense peptide with immobilized S-peptide as one interaction and therefore overestimates the affinity of a single sense/anti-sense peptide–peptide binding event. $[M]_T$ is also underestimated in the calculation of $K_{M/P}$ by eq 1. In this context, the affinity data of Table I for the various-length anti-sense peptides are subject to the same overestimation due to their calculation using the matrix content of S-peptide and not stoichiometric capacity to determine $[M]_T$. Nonetheless, whatever the effect of stoichiometry on the absolute $K_{M/P}$ values in Table I, the data reflect the persistence of binding for most of the shorter peptides.

Discussion

The striking possibility to design and synthesize anti-sense peptides as recognition molecules for detection, separation, and characterization of native peptides and proteins has emphasized the need to establish rigorously the interaction properties of anti-sense peptides, including affinities and structural characteristics. A major goal of the present study was to evaluate the use of analytical affinity chromatography to begin to obtain this understanding for the ribonuclease S-peptide system. An important element in such an analysis was to establish that the interaction of anti-sense peptide with immobilized S-peptide is peptide-directed. This has been demonstrated primarily by zonal elution experiments shown in Figure 2, especially the ability to displace anti-sense peptide with soluble native S-peptide and the linear response of this competition. In addition, control experiments have shown (1) that the extent of retardation and the binding capacity of anti-sense peptides respond linearly with the amount of immobilized S-peptide, (2) that anti-sense peptides do not bind significantly to a blank matrix end-capped with the same moiety (aminoethanol) as the experimental matrix, and (3) that charged control peptides do not bind to the experimental matrix. Thus, both anti-sense 20-mer (20 → 1) and inverted anti-sense 20-mer (1 → 20) recognize native S-peptide and bind to it in a specific manner and with rather significant affinities.

The analytical affinity chromatography experiments also have provided a delineation of some of the structural elements needed for sense/antisense peptide recognition. Since anti-sense and inverted anti-sense 20-mers have different amino-to-carboxyl orientations, they are not likely to have the same conformational tendencies and interactions of both of these with sense peptides are not likely to be dependent on a single compact folded structure, at least of the anti-sense component. Furthermore, while truncation of anti-sense peptides leads to progressive loss of binding affinities, several of the shortened peptides corresponding to different sequences of the sense peptide (S-peptide) still retain affinity. These results suggest that contact of sense with anti-sense peptides may well be multisite, involving several residues along the sequence. One way this could be accomplished is by peptide interaction in elongated rather than compactly folded forms, although a certain amount of ordered local conformation along the peptide certainly could exist in such an elongated peptide. This view may correlate with the hydropathic analyses of Blalock and Smith (1984) and Bost et al. (1985b), which suggest that the complementarity of sense and anti-sense sequences derives from the residue-by-residue pattern of hydrophobic and hydrophilic residues.

While the current data clearly establish that anti-sense peptides interact directly with sense sequences, and with definable and significant affinities, the mechanism of anti-sense peptide interactions remains ill-defined. Observations made in this study suggest that the stoichiometry of sense/anti-sense peptide interaction is greater than 1:1, at least on the affinity matrix of immobilized S-peptide. The continuous (broad zone) analysis (Figure 4) indicates that at least 4 molecules of the anti-sense peptides bind per molecule of immobilized S-peptide. Again, the stoichiometry results fit a model in which anti-sense and sense peptides interact as elongated species.

The present data emphasize the usefulness of analytical HPAC to evaluate anti-sense peptide interactions. Clearly, further mechanistic understanding using anti-sense and sense peptide sequence mutants would be helpful, as would data on the generality of anti-sense/sense peptide interaction for other peptide systems and correlation with interaction properties of anti-sense peptides in solution. It should not be overlooked that the biological significance of anti-sense peptides per se remains open to discussion. Nonetheless, their occurrence as molecular interactors provides a useful tool to evaluate the relationship between amino acid sequence ordered conformation and interaction. Thus, anti-sense peptides may help to learn about the forces that produce peptide and protein recognition in general. Such information also should be a useful basis to design recognition molecules for biotechnological purposes.

References

Abercrombie, D. M., & Chaiken, I. M. (1985) in *Affinity Chromatography—A Practical Approach* (Dean, P. D. G., Johnson, W. S., & Middle, F. A., Eds.) pp 169–189, IRL Press, Oxford.

Blalock, J. E., & Smith, E. M. (1984) *Biochem. Biophys. Res. Commun. 121*, 203–207.

Blalock, J. E., & Bost, K. L. (1986) *Biochem. J. 234*, 679–683.

Bost, K. L., & Blalock, J. E. (1986) *Mol. Cell. Endocrinol. 44*, 1–9.

Bost, K. L., Smith, E. M., & Blalock, J. E. (1985a) *Proc. Natl. Acad. Sci. U.S.A. 82*, 1372–1375.

Bost, K. L., Smith, E. M., & Blalock, J. E. (1985b) *Biochem. Biophys. Res. Commun. 128*, 1373–1380.

Chaiken, I. M. (1986) *J. Chromatogr. 376*, 11–32.

Fassaina, G., Shai, Y., & Chaiken, I. (1986) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 45*, 1944.

Gawronski, T. H., & Wold, F. (1972a) *Biochemistry 11*, 442–448.

Gawronski, T. H., & Wold, F. (1972b) *Biochemistry 11*, 449–455.

Hearn, R. P., Richards, F. M., Sturdevant, J. M., & Watt, G. D. (1971) *Biochemistry 10*, 806–817.

Kyte, J., & Doolittle, R. F. (1982) *J. Mol. Biol. 157*, 105–132.

MacDonald, R. J., Stary, J. S., & Swift, G. H. (1982) *J. Biol. Chem. 257*, 14582–14585.

Mitchell, A. R., Kent, S. B. H., Engelhard, M., & Merrifield,

R. B. (1978) J. Org. Chem. 43, 2845-2852.
Swaisgood, H. E., & Chaiken, I. M. (1985) J. Chromatogr. 327, 193-204.
Swaisgood, H. E., & Chaiken, I. M. (1986) Biochemistry 25, 4148-4155.
Winzor, D. (1985) in Affinity Chromatography—A Practical Approach (Dean, P. D. G., Johnson, W. S., & Middle, F. A., Eds) pp 149-168, IRL Press, Oxford.

Articles

Modification of Synthetic Peptides Related to Lactate Dehydrogenase (231-242) by Protein Carboxyl Methyltransferase and Tyrosine Protein Kinase: Effects of Introducing an Isopeptide Bond between Aspartic Acid-235 and Serine-236†

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ABSTRACT: The possibility that isoaspartyl residues contribute to the substrate specificity of eucaryotic protein carboxyl methyltransferases and/or tyrosine protein kinases has been investigated with two synthetic oligopeptides, Lys-Gln-Val-Val-Asp/isoAsp-Ser-Ala-Tyr-Glu-Val-Ile-Lys, which correspond to amino acids 231-242 of lactate dehydrogenase. One version of the peptide contains the normal amino acid sequence of the chicken muscle M_4 isozyme. The other version contains an isoaspartyl residue in position 235 in place of the normal aspartyl residue; i.e., Asp-235 is linked to Ser-236 via its side-chain β-carboxyl group, rather than via the usual α-carboxyl linkage. The normal peptide corresponds to the sequence around Tyr-238 that is phosphorylated in Rous sarcoma virus infected chick embryo fibroblasts [Cooper, J. A., Esch, F. S., Taylor, S. S., & Hunter, T. (1984) *J. Biol. Chem. 259*, 7835]. Using protein carboxyl methyltransferase purified from bovine brain, we found that the normal peptide did not serve as a methyl-accepting substrate but that the isopeptide served as an excellent substrate, exhibiting a stoichiometry of one methyl group per peptide and a K_m of 0.54 μM. With tyrosine protein kinase partially purified from normal rat spleen, both peptides were found to serve as phosphate acceptors at Tyr-238, exhibiting K_m values of 4.7 and 8.9 mM for the normal and isopeptide versions, respectively. These results support the idea that protein carboxyl methyltransferase selectively methylates the α-carboxyl group of atypical isoaspartyl residues. In contrast, the presence of isoaspartate had a modest negative effect on substrate activity for a tyrosine protein kinase from rat spleen.

The eucaryotic protein carboxyl methyltransferases (PCMTs)[1] and tyrosine protein kinases share the feature that they tend to covalently modify proteins both in vitro and in vivo with a low stoichiometry, usually less than 0.05 mol of methyl or phosphate group per mole of substrate (Kim & Li, 1979; Kloog et al., 1980; Aswad & Deight, 1983; Clarke & O'Connor, 1983; Sefton et al., 1981; Richert et al., 1982; Cooper et al., 1984). Recent experiments with PCMT from bovine brain and human erythrocytes suggest that this enzyme selectively methylates atypical L-isoaspartyl residues and not normal L-aspartyl or L-glutamyl residues (Aswad, 1984; Murray & Clarke, 1984). The possibility that many proteins contain low levels of isoaspartate, resulting from deamidation of asparagine, isomerization of normal aspartyl residues, or errors in protein synthesis, could explain the low stoichiometry of modification exhibited by this enzyme. To date, the only peptide for which stoichiometric methylation has been convincingly demonstrated is an isoaspartate-containing form of ACTH (Aswad, 1984; Murray & Clarke, 1984). It is important to determine if the apparent specificity of PCMT for isoaspartyl residues is a general property of this enzyme and the extent to which the specificity depends on the amino acid sequence surrounding the isoaspartate.

Recent studies on the amino acid sequences surrounding phosphorylation sites recognized by several tyrosine-specific protein kinases indicate that these enzymes may recognize

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[1] Abbreviations: ACTH, adrenocorticotropin; AdoMet, *S*-adenosyl-L-methionine; Boc, *tert*-butyloxycarbonyl; Bpoc, 2-(4-biphenylyl)-2-[(propyloxy)carbonyl]; Bzl, benzyl; cHex, cyclohexyl; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high-performance liquid chromatography; LDH, lactate dehydrogenase; PCMT, protein carboxyl methyltransferase; TFA, trifluoroacetic acid; TLC, thin-layer chromatography; Xan, xanthenyl; Z, benzyloxycarbonyl; all amino acids are the L isomer; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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