

- R. B. (1978) *J. Org. Chem.* 43, 2845-2852.
 Mulchahey, J. J., Neill, J. D., Dion, L. D., Bost, K. L., & Blalock, J. E. (1986) *Proc. Natl. Acad. Sci. U.S.A.* 83, 9714-9718.
 Ohlson, S., Lunblad, A., & Zopf, D. (1988) *Anal. Biochem.* 169, 204-208.
 Shai, Y., Jacobson, K., & Patchornik, A. (1985) *J. Am. Chem. Soc.* 107, 4249-4252.
 Shai, Y., Flashner, M., & Chaiken, I. M. (1987) *Biochemistry* 26, 669-675.
 Swaisgood, H. E., & Chaiken, I. M. (1986) *Biochemistry* 25, 4148-4155.
 Swaisgood, H. E., & Chaiken, I. M. (1987) in *Analytical Affinity Chromatography* (Chaiken, I. M., Ed.) pp 105-115, CRC Press, Boca Raton, FL.
 Taylor, J. W., & Kaiser, E. T. (1986) *Pharmacol. Rev.* 38, 291-319.
 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.

Recognition Properties of Antisense Peptides to Arg⁸-vasopressin/Bovine Neurophysin II Biosynthetic Precursor Sequences

Giorgio Fassina,^{†§} Moreno Zamai,^{‡||} Michael Brigham-Burke,[⊥] and Irwin M. Chaiken^{*,†,⊥}

National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892, and Department of Macromolecular Sciences, Smith Kline & French, Research and Development, King of Prussia, Pennsylvania 19406

Received February 24, 1989; Revised Manuscript Received June 22, 1989

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 Arg⁸-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

* Address correspondence to this author at the Department of Macromolecular Sciences, Smith Kline & French, Research and Development, 709 Swedeland Road, P.O. Box 1539, King of Prussia, PA 19304-0939.

† National Institutes of Health.

‡ Present address: Tecnogen SpA, via Ampere 56, 20131 Milano, Italy.

|| Present address: Laboratory of Genetic Engineering, Eniricerche, via S. Salvo 1, 20097 San Donato, Milanese (MI), Italy.

⊥ Smith Kline & French, Research and Development.

of this phenomenon has remained unclear. To provide further insight into this problem, we undertook an examination of a second system, the amino terminal region of the Arg⁸-vasopressin/bovine neurophysin II biosynthetic precursor (proAVP/BNPII).¹ This polypeptide case has the advantage of providing the possibility to study cross-specificity in a meaningful way. The proAVP/BNPII precursor contains, from the amino terminus, the nine-residue sequence of the neurohypophysial hormone AVP, the tripeptide spacer Gly-LysArg, the 93-residue sequence for the hormone binding neurohypophysial protein BNPII, an Arg spacer, and a C-terminal glycoprotein. Several antisense peptides directed toward the N-terminal 20-residue region of proAVP/BNPII were synthesized and their interactions studied by analysis of retardation properties on affinity columns containing immobilized BNPII and AVP. Moreover, the antisense peptides could be immobilized on a solid support and binding properties of the corresponding sense peptides investigated, again by analytical high-performance liquid affinity chromatography (HPLAC). The degree of cross-recognition among AVP- and BNPII-related peptides was evaluated. In addition, forces underlying the interaction were evaluated, by studying solvent effects and effects of sense peptide sequence variation on recognition of AS peptides. The results again reflect the type of sequence degeneracy in sense/antisense peptide recognition previously observed with S-peptide-related antisense peptide sequence simplification (Shai et al., 1987b). But, the data also emphasize the consistent observation that antisense peptides can bind to sense peptides with significant affinity and selectivity.

EXPERIMENTAL PROCEDURES

Materials. Authentic AVP, LVP, OT, and AVT were obtained from Sigma Chemical Co. (St. Louis, MO). In addition, a sample containing an approximately equimolar mixture of native AVP and OT was obtained as the "hormone fraction" by acid extraction of bovine posterior pituitaries and fractionation on Sephadex G-75 in 1 N formic acid (Breslow et al., 1971). BNPI and BNPII were obtained from bovine posterior pituitaries as before (Chaiken et al., 1984). Sequential-grade trifluoroacetic acid, dimethylformamide, and triethylamine were obtained from Pierce Chemical Co. (Rockford, IL). HPLC-grade water, acetonitrile, and ethyl acetate were from Fisher Scientific (Lexington, MA). Activated affinity supports, based on a silica backbone and containing a six-carbon spacer with a terminal *N*-hydroxy-succinimide ester for immobilizing molecules through their

amino groups, were kindly provided to us by Waters Chromatography Division, under the trade name ACCELL 78 (Milford, MA). Ethyl acetimidate and MetTyrPheNH₂ were from Sigma Chemical Co. Ethyl [1-¹⁴C]acetimidate hydrochloride (50 mCi/mmol) was obtained from Amersham (Arlington Heights, IL). Glass (Omni) columns for packing affinity matrices were purchased from Rainin Instruments Co. (Woburn, MA). Scintillation counting was performed with a SEARLE MARK III 6880 liquid scintillation system.

Solid-Phase Peptide Synthesis. Synthetic vasopressinyl peptides and antisense proAVP/BNPII peptides were produced, except as noted, by manual solid-phase peptide synthesis (Mitchell et al., 1978) on Boc-Arg(Tos)-, Boc-Ala-, and Boc-Gly-oxy-methyl (phenylacetamido)methyl (PAM) resins (0.5 mmol/g) from Applied Biosystems (Foster City, CA) as described before (Fassina & Chaiken, 1988). In the case of AS[proAVP/BNPII(20-1)] and AS[proAVP/BNPII(20-13)], the DNP group on His at position 14 was removed prior to hydrogen fluoride cleavage of peptidyl resin by treating 1 g of resin suspended in 5 mL of DMF with 0.1 mL of thiophenol for 1 h at room temperature with agitation; the resin was washed thoroughly with DMF, water, and ethanol-dichloromethane and then vacuum dried. Synthetic peptides were fractionated by gel filtration on Sephadex G-25 and then purified by reverse-phase HPLC on an Axxiochrom C-18 semipreparative column (25 × 1 cm i.d.) with a linear gradient from 0.1% TFA in 10% acetonitrile at zero time to 0.1% TFA in 35% acetonitrile at 35 min and then to 0.1% TFA in 80% acetonitrile at 50 min, under a flow rate of 3.0 mL/min. In the cases of AS[proAVP/BNPII(20-1)] and AS[proAVP/BNPII(12-1)] peptides containing (CHO)Trp in position 7, the formyl group was removed prior to HPLC purification, by raising the pH to 11 with 2 N NaOH for a few seconds and then rapidly returning it to neutrality with HCl. Removal of the formyl group was monitored as the loss of absorbance at 300 nm. Identity of synthetic peptides was confirmed by amino acid analysis after hydrolysis with constant-boiling HCl, at 110 °C for 24 h in vacuo.

For the AS[proAVP/BNPII(12-1)] material used to make ACCELL-immobilized 12-mer for separating AVP and OT (see below), the peptide synthesis was carried out on a Bioscience 9600 peptide synthesizer using a protocol similar to that used above for manual synthesis.

Preparation of High-Performance Affinity Columns. Immobilization of sense and antisense peptides to ACCELL 78 was carried out by general procedures described before (Fassina et al., 1986). Dry resins were packed in 50 × 3.0 mm i.d. or 100 × 6.6 mm i.d. Omni HPLC glass columns.

Oriented Immobilization of AVPGlyLysArg. To preserve binding properties toward BNPII, AVPGlyLysArg was immobilized in the form of its complex with BNPII. This favors attachment through the ϵ -amino group of Lys 11 in AVPGlyLysArg, since the α -amino group is involved in strong ionic interaction with neurophysin (Breslow et al., 1973) and is protected from reacting with the matrix. The BNPII used in this procedure was first reacted with ethyl acetimidate to protect free amino groups and prevent attachment of the protein to the matrix during peptide immobilization. Briefly, BNPII (3 mg) was dissolved in 300 μ L of 0.5 M NaHCO₃, pH 10.3, with stirring. Ethyl acetimidate (50 mg in 10 μ L of 2 N NaOH) was added, and the mixture was incubated at room temperature. After 1.5 h, 300 μ L of glacial acetic acid was added, and BNPII was purified from residual reagents. A small amount of BNPII was inactivated by this procedure and was removed by affinity chromatography on a Met-

¹ Abbreviations: AS, antisense; AUFS, absorbance unit full scale; AVP, Arg⁸-vasopressin; AVT, Arg⁸-vasotocin; BNPI, bovine neurophysin I; BNPII, bovine neurophysin II; Boc, *tert*-butoxycarbonyl; Boc₂O, *tert*-butoxycarbonyl anhydride; Bzl, benzyl; CHO, formyl; Cl-Z, chlorobenzyl; Cl₂-Bzl, dichlorobenzyl; DCC, dicyclohexylcarbodiimide; DNP, dinitrophenyl; EDTA, ethylenediaminetetraacetic acid; HOBT, hydroxybenzotriazole; HPLAC, high-performance liquid affinity chromatography; HPLC, high-performance liquid chromatography; *K*_{M/P}, chromatographically derived equilibrium dissociation constant of complex of mobile interactant (P) with immobilized (M) interactant; *V*, experimental elution volume of mobile interactant; *V*₀, unretarded elution volume; [*M*]_T, total concentration of immobilized peptide or protein interactant; [*P*]₀ (eq 2), initial concentration of mobile peptide; *V*_m, volume outside affinity beads; *M*_T (eq 2), total amount of mobile peptide bound to immobilized matrix at saturation; LVP, Lys⁸-vasopressin; MYFamide, MetTyrPheamide; OT, oxytocin; PAM, (phenylacetamido)methyl; pCH₃-Bzl, *p*-methylbenzyl; proAVP/BNPII, biosynthetic precursor of AVP and BNPII; pro⁸AVP/BNPII, semisynthetic precursor of AVP and BNPII, containing the sequence, from the amino terminus, AVP-GlyLysArg-NPII (Fassina & Chaiken, 1988b); RNase S, ribonuclease S; TFA, trifluoroacetic acid; Tos, tosyl or toluenesulfonyl.

	20	13
AS[ProAVP/NP(20→13)]	NH ₂ - Ser-Gln-Leu-Gln-Val-Gly-His-Gly - COOH	
		12
AS[ProAVP/NP(12→1)]		NH ₂ -Pro-Leu-Ala-Ala-Pro-Trp-Ala-Val-Leu-Glu-Val-Ala - COOH
	20	1
AS[ProAVP/NP(20→1)]	NH ₂ - Ser-Gln-Leu-Gln-Val-Gly-His-Gly-Pro-Leu-Ala-Ala-Pro-Trp-Ala-Val-Leu-Glu-Val-Ala - COOH	
COMPLEMENTARY STRAND RNA	5' UCU-CAG-CUC-CAG-GUC-GGA-CAU-GGC-CCU-CUU-GCC-GCC-CCU-UGG-GCA-GUU-CUG-GAA-GUA-GCA 3'	
RNA FOR BOVINE SEQUENCE	5' UGC-UAC-UUC-CAG-AAC-UGC-CCA-AGG-GGC-GGC-AAG-AGG-GCC-AUG-UCC-GAC-CUG-GAG-CUG-AGA 3'	
	1	20
BOVINE ProAVP/NP(1→20)	NH ₂ - Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-Gly-Lys-Arg-Ala-Met-Ser-Asp-Leu-Glu-Leu-Arg - COOH	
	1	12
AVP	NH ₂ - Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-Gly-Lys-Arg - COOH	
	1	12
A1([Ala 4,5,7,8,9]AVP-GKR)	NH ₂ - Cys-Tyr-Phe-Ala-Ala-Cys-Ala-Ala-Ala-Gly-Lys-Arg - COOH	
	1	12
A2([Ala 3,4,5,7,8,9]AVP-GKR)	NH ₂ - Cys-Tyr-Ala-Ala-Ala-Cys-Ala-Ala-Ala-Gly-Lys-Arg - COOH	
	1	16
A3([Ala 4,5,7,8,9]AVP-GKRAMGA)	NH ₂ - Cys-Tyr-Phe-Ala-Ala-Cys-Ala-Ala-Ala-Gly Lys-Arg-Ala-Met-Gly-Ala - COOH	
	1	16
A4([Ala 3,4,5,7,8,9]AVP-GKRAMGA)	NH ₂ - Cys-Tyr-Ala-Ala-Ala-Cys-Ala-Ala-Ala-Gly-Lys-Arg-Ala-Met-Gly-Ala - COOH	
	1	16
A5(AVP-GKRAMGA)	NH ₂ - Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-Gly-Lys-Arg-Ala-Met-Gly-Ala - COOH	
	1	3
MYFamide	NH ₂ - Met-Tyr-Phe-NH ₂	

FIGURE 1: Amino acid sequence of antisense peptides as deduced from the corresponding complementary RNA of BNPII precursor. The diagram denotes the relationship of AS peptides to the sense peptide sequence. Sequences also are shown of several AVP sequence variants used in this study.

TyrPhe-Affi-Gel 102 column, equilibrated with 0.4 M NH₄Ac, pH 5.7. Fractions of 30 drops were collected, and triacim BNPII was eluted with 0.2 M HAc. Fractions containing the protected protein were pooled, frozen, and lyophilized. For the preparation of [AVPGlyLysArg]ACCELL, an equimolar solution prepared by dissolving 2.5 mg of AVPGlyLysArg and 24.5 mg of triacim BNPII in 20 mL of 0.1 M NaHCO₃-0.5 M NaCl, pH 7.0, was incubated with 2 g of resin and subsequently washed as described above. Dried resin was packed in a 100 × 6.6 mm i.d. Omni column. Prior to use, the column was washed with 200 mL of 0.2 M HAc, to disrupt the complex between immobilized AVPGlyLysArg and triacim BNPII.

S-Methylation of AVPGlyLysArg. Conversion of half-cystine residues in AVPGlyLysArg to their S-methyl derivatives was achieved by reduction with β-mercaptoethanol and methylation with methyl *p*-nitrobenzenesulfonate (Heinrikson, 1971). S-Methylated peptide was purified by reverse-phase HPLC as described above for synthetic peptides.

¹⁴C Labeling of AS[proAVP/BNPII(20→1)]. Peptide (0.5 mg) was dissolved in 200 μL of sodium bicarbonate, pH 10.3, and ethyl [1-¹⁴C]acetimidate (0.1 mg in 10 μL of H₂O) was added. The solution was stirred for 1 h at 0 °C. Unlabeled ethyl acetimidate (1.5 mg in 50 μL of H₂O) was added to assure complete protection of amino groups. After 1-h further reaction, the mixture was acidified with a few drops of glacial acetic acid. The [¹⁴C]AS[proAVP/BNPII(20→1)] was purified by semipreparative reverse-phase HPLC as described above for synthetic peptides. The specific activity of final product was 1200 cpm/μg.

Protection of α- and ε-Amino Groups with Boc₂O. Peptides were dissolved at 5 mg/mL in 1:1 (v/v) water-dioxane with stirring. For each 1 mL of solution, one drop of *N*-ethylmorpholine was added and then 200 μL of Boc₂O. The extent of reaction was followed by reverse-phase HPLC, with the same elution conditions as described above for purification of synthetic peptides. Protected peptides were purified by semipreparative reverse-phase HPLC, as described above.

Analytical HPLC. Dissociation constants for the interactions of immobilized with mobile peptides and proteins were obtained by isocratic zonal (Swaisgood & Chaiken, 1986;

Fassina & Chaiken, 1987) and frontal elutions (Winzor, 1985; Ohyama et al., 1985) as described in the previous paper (Shai et al., 1989). The equations used are for zonal elution

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

and for frontal elution

$$\frac{1}{(\bar{V} - V_0)} = \frac{K_{M/P}}{[M_T]} + \frac{[P]_0}{[M_T]} \quad (2)$$

The extent of the retardation, \bar{V} , is the volume at the midpoint of the elution front, calculated from the volume at half-maximal absorbance in the front. In both zonal and frontal elution analyses, determined elution volumes are estimated to be reliable to ±10%, corresponding to a ±10% limit of reliability of $K_{M/P}$ values.

RESULTS

Design of Antisense and Sense Peptides. Three peptides encoded in the complementary mRNA corresponding to bovine AVP/BNPII precursor sequences 1–20, 1–12, and 13–20 were made by solid-phase peptide synthesis and purified to chromatographic homogeneity by reverse-phase HPLC. Identity of purified peptides with intended sequences was confirmed by amino acid analysis and spectroscopic determination of tryptophan content. Antisense peptides were denoted as follows (Figure 1): AS[proAVP/BNPII(20→1)], the AS peptide corresponding to residues 1–20 of the precursor (thus corresponding to AVP, the tripeptide spacer GlyLysArg, and the first eight amino-terminal residues of BNPII); AS[proAVP/BNPII(12→1)], corresponding to precursor residues 1–12, namely, AVPGlyLysArg; and AS[proAVP/BNPII(20→13)], corresponding to precursor residues 13–20, the first eight residues of BNPII.

A set of native (sense) peptides also were synthesized, corresponding to residues 1–12 and 1–16 of the N-terminal portion of the precursor, together with sequence-simplified and modified forms of these. They were purified and their abilities

Table I: Dissociation Constants of Soluble (Mobile) Antisense Peptides with Immobilized AVPGlyLysArg As Determined by Zonal Analytical Affinity Chromatography^a

mobile interactant	[NH ₄ Ac] (mM)	[AVPGKR] (mM)	$K_{M/P}$ (mM)
AS[proAVP/BNP(20-1)]	50		3.5
[¹⁴ C]AS[proAVP/BNP(20-1)]	50		6.9
[¹⁴ C]AS[proAVP/BNP(20-1)]	50	0.1	>30
AS[proAVP/BNP(12-1)]	50		7.3
AS[proAVP/BNP(20-13)]	50		>30 ^b
BNP(12-1)	400		0.016
S-peptide	50		>30 ^b
RNase S	50		>30 ^b
AVPGlyLysArg	50		>30 ^b

^a $K_{M/P}$ calculated by extrapolation of $1/(V - V_0)$ at $[P]_0 = 0$ by zonal elution chromatography at pH 5.7 and by use of $M_T = 10.5 \times 10^{-8}$ mol obtained from frontal elution of BNP(12-1) on the [AVPGlyLysArg]ACCELL column. ^b Value of $K_{M/P}$ corresponding to $(V - V_0) \leq 0.1$ mL, or 1/10 of V_0 . The latter is considered the limit of accuracy of the experimental measurement of $1/(V - V_0)$ with [AVPGlyLysArg]-ACCELL matrix.

to bind BNP(12-1) verified by analytical HPLAC (Fassina & Chaiken, 1988).

Selectivity and Quantitation of Binding of Antisense to Immobilized Sense Peptides. To evaluate antisense peptide binding to sense peptide in the AVP/BNP case, both zonal and frontal elutions were carried out with immobilized AVPGlyLysArg and immobilized BNP(12-1). The AVPGlyLysArg and BNP(12-1) affinity matrices used here both were found to be functionally active, that is, able to bind to complementary sense component—BNP(12-1) and hormone, respectively. In the case of support-bound AVPGlyLysArg, column capacity determined from frontal analysis with BNP(12-1) was $M_T = 1.05 \times 10^{-7}$ mol for a column bed of 80×6.6 mm i.d. The $K_{M/P}$ values for the interaction between immobilized AVPGlyLysArg and mobile BNP(12-1) were calculated to be $16 \mu\text{M}$ by zonal and $15.5 \mu\text{M}$ by frontal elutions. These agree within a factor of 2 with $K_{M/P} = 8 \mu\text{M}$ calculated for the interaction between immobilized BNP(12-1) and mobile AVPGlyLysArg by zonal elution.

When both AS[proAVP/BNP(20-1)] and its [¹⁴C]acim derivative were eluted zonally on the [AVPGlyLysArg]ACCELL column (80×6.6 mm i.d.), binding was observed as chromatographic retardation. The $K_{M/P}$ values calculated for the AS 20-mer peptides and a set of control peptides were determined from the corrected elution volumes, $V - V_0$, with eq 1 and are given in Table I. The affinity obtained here for the AS 20-mer is quite low. However, it is measurable and significantly greater than that for several control peptides eluted under the same chromatographic conditions.

Similar behavior (Table II) was seen for antisense 20-mer elution on the [BNP(12-1)]ACCELL column. This latter matrix had a functional content of BNP(12-1), as judged by elution with oxytocin, of $M_T = 1.44 \mu\text{mol}$ on a column bed of 95×10 mm. Both here and for immobilized AVPGlyLysArg, modification of antisense 20-mer by introduction of the radiolabeled acetimidyl group apparently does not significantly alter the binding affinity to AVPGlyLysArg and BNP(12-1).

When the antisense subfragments AS[proAVP/BNP(20-13)] and AS[proAVP/BNP(12-1)] were eluted on AVPGlyLysArg and BNP(12-1) affinity matrices, experimentally significant retardation differences were observed versus the full-sequence peptide (Tables I and II). AS[proAVP/BNP(20-13)], the antisense peptide directed against the first eight residues of the BNP(12-1) sequence domain of precursor, was retarded significantly on BNP(12-1) but not so on immobilized AVPGlyLysArg. The reverse was true for AS[proAVP/

Table II: Dissociation Constants of Soluble (Mobile) Antisense Peptides with Immobilized BNP(12-1) Determined by Zonal Analytical Affinity Chromatography^a

mobile interactant	[NH ₄ Ac] (mM)	pH	$K_{M/P}$ (mM)
AS[proAVP/BNP(20-1)]	50	5.7	2.9
[¹⁴ C]AS[proAVP/BNP(20-1)]	50	5.7	2.2
AS[proAVP/BNP(20-13)]	50	5.7	1.0
AS[proAVP/BNP(20-13)]	10	5.7	0.5
AS[proAVP/BNP(12-1)]	50	5.7	>30 ^b
BNP(12-1)	400	5.7	0.108
AVPGlyLysArg	400	5.7	0.008
S-peptide	50	5.7	>30 ^b
RNase S	50	5.7	>30 ^b

^a $K_{M/P}$ calculated by extrapolation of $1/(V - V_0)$ at $[P]_0 = 0$ by zonal elution chromatography at pH 5.7 and by use of $M_T = 1.44 \mu\text{mol}$ obtained from frontal elution of AVPGlyLysArg on the [BNP(12-1)]ACCELL column. ^b Limit of experimentally measurable $K_{M/P}$ with the [BNP(12-1)]ACCELL column.

BNP(12-1)], corresponding to the AVPGlyLysArg sequence, which had significant affinity for immobilized AVPGlyLysArg, very close to that found for full-sequence AS[proAVP/BNP(20-1)], but interacted only weakly if at all with immobilized BNP(12-1). Thus, the antisense peptide interactions with sense peptide and protein matrices exhibit a selectivity consistent with the hypothesis that antisense peptides have a directed affinity for their corresponding sense peptide sequences. To evaluate further the specificity of antisense 20-mer interaction, [¹⁴C]-radiolabeled AS[proAVP/BNP(20-1)] was subjected to competitive elution on the [AVPGlyLysArg]ACCELL column equilibrated with buffer containing 0.1 mM AVPGlyLysArg. Disruption of binding was observed (Table I).

Recognition Properties of Immobilized AS[proAVP/BNP(20-1)]. Given the ability of antisense 20-mer to bind both AVPGlyLysArg and BNP(12-1) affinity matrices, we examined whether the 20-mer could be immobilized and itself used as an affinity chromatographic matrix to bind and fractionate sense peptides and proteins to which the antisense sequences were "targeted". AVPGlyLysArg was injected on a column prepared with AS[proAVP/BNP(20-1)], and its observed interaction was evaluated by zonal elution at a series of peptide concentrations and yielded the calculated equilibrium dissociation constant of $5.9 \mu\text{M}$. Interaction data were obtained for a series of sequence-modified forms of AVPGlyLysArg and several controls, with the results given in Figure 2 and Table III. Generally, the measured affinities of sense peptides on immobilized antisense 20-mer are greater than those of antisense peptide on sense peptide columns. The reason for this is not known at present but may reflect variation in the types of complexes formed depending on which of the interacting peptide partners (antisense vs sense) is immobilized. Nevertheless, observed binding of sense peptide to immobilized antisense peptide confirms the observation of sense/antisense interactions evaluated above with immobilized sense peptide/protein affinity systems. Chromatographic dissociation constants reported in Table III were calculated from values of column capacity determined by frontal elutions of AVPGlyLysArg on the AS[proAVP/BNP(20-1)]ACCELL column (50×3.0 mm i.d.). Variation of the extent of retardation of the front on peptide concentration (eq 2) gave $K_{M/P}(\text{frontal}) = 7.4 \mu\text{M}$ and $M_T = 2.0 \times 10^{-8}$ mol. Determination of column capacity also was determined with soluble BNP(12-1), yielding $M_T = 0.39 \times 10^{-8}$ mol. The differential M_T data reflect a greater accessibility of the dodecapeptide AVPGlyLysArg than the 10-kDa protein BNP(12-1) for immobilized AS[proAVP/BNP(20-1)].

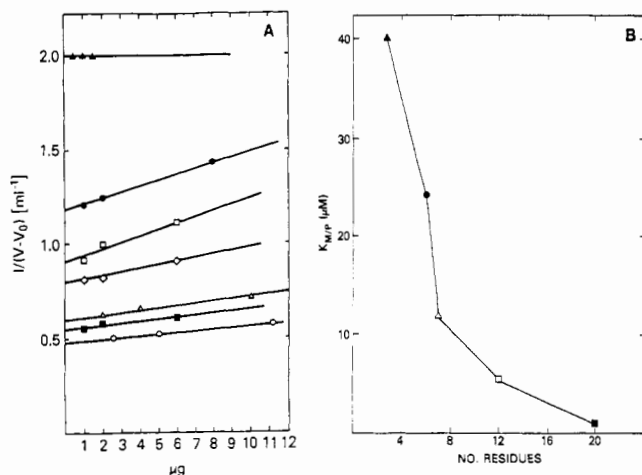


FIGURE 2: Effect of sequence simplification, truncation, and charge depletion of AVPGlyLysArg on the recognition with immobilized AS[proAVP/BNP(20-1)]. (A) The AS column (50×3.0 mm i.d.) was equilibrated at a flow rate of 1.0 mL/min with 50 mM NH_4Ac , pH 5.7. Zones ($20 \mu\text{L}$) containing different amounts of mutant peptides were injected, and the extent of retardation was plotted against the amount applied according to eq 1. (O) A5; (■) A3; (▲) A1; (◇) A4; (□) diBocAVPGlyLysArg; (●) A2; (▲) MYFamide. Extrapolated values at $[P]_0 = 0$ were used to calculate equilibrium binding constants. (B) Dependence of $K_{M/P}$ values on extent of truncation and simplification of sense peptide. Data in the plot correspond to (■) semisynthetic proAVP/BNP(20-1) (given as "20 residues" for residues expected to be involved in interaction; whole semisynthetic precursor is 105 residues), (□) AVPGlyLysArg, (▲) A1, (●) A2, and (▲) MYFamide (see also Table III).

Effect of Mobile-Phase Composition. The sensitivity to solvent characteristics was evaluated for the interaction between immobilized AS[proAVP/BNP(20-1)] and AVPGlyLysArg (Figure 3). Many biologically relevant interac-

Table III: Dissociation Constants of Soluble (Mobile) Sense Peptides with Immobilized AS[proAVP/BNP(20-1)] As Determined by Zonal Analytical High-Performance Affinity Chromatography^a

mobile interactant	net charge ^b	$K_{M/P}$ (μM)
AVPGlyLysArg	+3	5.9
BNP(12-1)	-1 ^c	66
A5	+3	9.6
A3	+2	11
A1	+2	12
A4	+2	18
diBocAVPGlyLysArg	+1	18
A2	+2	24
diBoc-A1	0	>200 ^d
diBoc-A2	0	>200
S-methyl-AVPGKR	+3	4.6
MetTyrPheNH ₂	+1	40
AS[proAVP/BNP(12-1)]	-1	>200
AS[proAVP/BNP(20-13)]	+1	>200
pro ⁵ AVP/BNP(12-1)	0 ^e	1.4
AS[proAVP/BNP(20-1)]	0	>200
S-peptide	+1	>200
RNase S	+8	>200

^a $K_{M/P}$ calculated by extrapolation of $1/(V - V_0)$ at $[P]_0 = 0$ by zonal elution chromatography at pH 5.7 in 50 mM NH_4Ac and by use of $M_T = 2.0 \times 10^{-8}$ mol obtained from frontal elutions of AVPGKR on the AS[proAVP/BNP(20-1)]ACCELL column. ^b Assuming -1 for Asp and Glu and +1 for Lys, Arg, and His. ^c Net charge in 1-8 sequence of BNP(12-1). ^d Limit of experimentally measurable $K_{M/P}$ with the AS[proAVP/BNP(20-1)]ACCELL column. ^e Net charge in 1-20 sequence of proAVP/BNP(12-1).

tions are produced by a combination of hydrophilic, hydrophobic, and ionic effects and thus generally are pH-dependent. Mobile-phase composition thus can affect molecular recognition processes. For example, the presence of organic solvents in the elution buffer considerably reduces hydrophobic interactions (Van Oss et al., 1981; Allenmark & Bomgren, 1982;

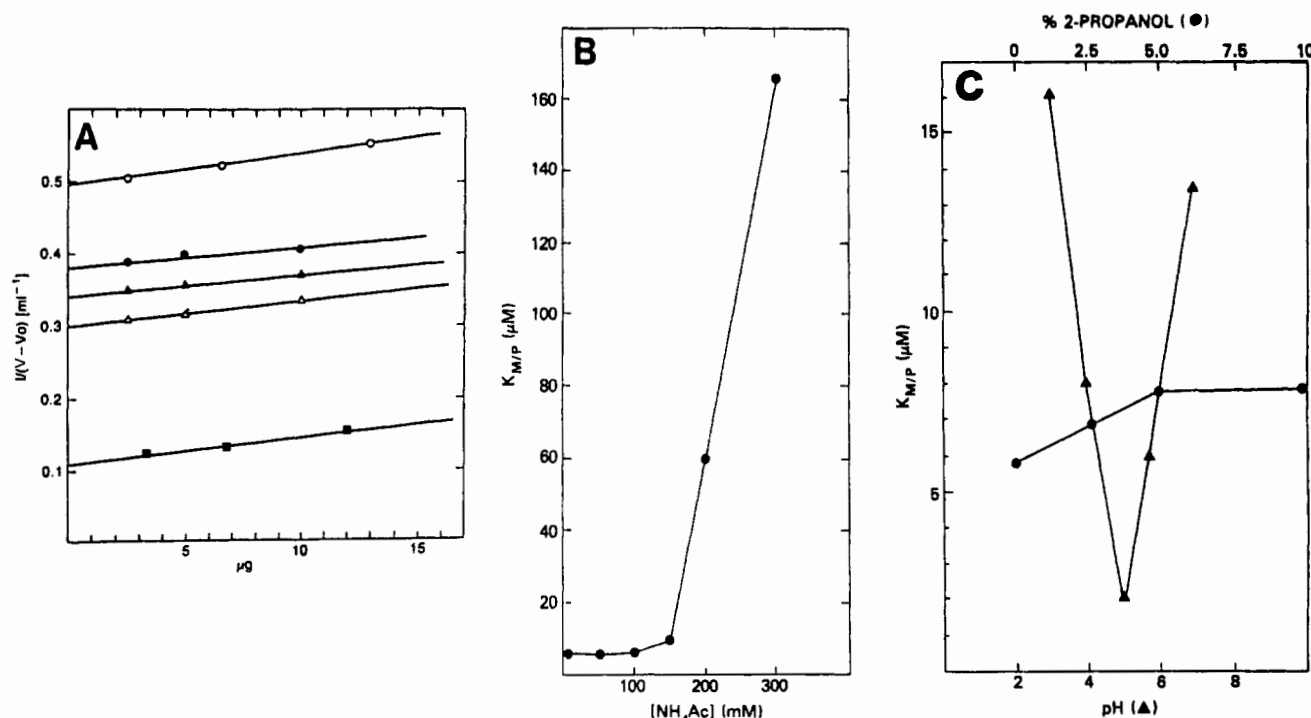


FIGURE 3: (A) Dependence of chromatographically calculated equilibrium dissociation constants on mobile-phase composition for the interaction of immobilized AS[proAVP/BNP(20-1)] with AVPGlyLysArg. Elution data at buffer compositions as follows: (■) 50 mM NH_4Ac , pH 5.0; (▲) 50 mM NH_4Ac , pH 5.7; (●) 100 mM NH_4Ac , pH 5.7; (○) 50 mM NH_4Ac , pH 5.7, 2.5% 2-propanol; (◇) 150 mM NH_4Ac , pH 5.7. Data are plotted according to eq 1 and extrapolated to values at $[P]_0 = 0$ to determine equilibrium dissociation constants. (B) Dependence of chromatographically calculated equilibrium dissociation constant ($K_{M/P}$) for the AVPGlyLysArg interaction with AS[proAVP/BNP(20-1)] on the NH_4Ac concentration at pH 5.7 (see also Table III). (C) Dependence of chromatographically calculated equilibrium dissociation constant for the interaction of AVPGlyLysArg with immobilized AS[proAVP/BNP(20-1)] on pH (▲) and on the presence of 2-propanol in the eluting buffer (○) (see also Table III).

Fassina et al., 1987), while increasing salt concentrations promote hydrophobic and diminish ionic effects.

In the AS[proAVP/BNPII(20-1)]/AVPGlyLysArg case, binding was optimal around pH 5.0 (Figure 3C), slightly dependent on the presence of 2-propanol (Figure 3C) and more strongly dependent on buffer ionic strength (Figure 3B). These observations suggest the presence of a mixed-mode type of interaction, with perhaps more dominant ionic/hydrophilic and some hydrophobic contributions. Of note, the lack of a strict correlation of affinity of eluting peptides with their net charges (Table III) argues against a simple ion-exchange process as the cause for peptide interaction with immobilized antisense 20-mer.

Effect of Sequence Simplification. In the ribonuclease S-peptide case (Shai et al., 1987a), significant sequence variation can be tolerated in the antisense peptide with only partial loss of affinity for sense S-peptide. Similarly in the present study, sequence simplification by substitution of several residues in the sense peptide AVPGlyLysArg with Ala was tolerated. Significant binding was still observed in the highly simplified peptide A2 (Figure 2A and Table III), in which only six of the original amino acids were preserved in the sequence (Figure 1). Elongation of the sequence in peptides A3, A4, and A5 did not affect binding affinity noticeably, nor did removal of the disulfide bridge between residues 1 and 6. In contrast, deletion of two charges in AVPGlyLysArg by Boc protection of amino groups decreased the affinity by 3-fold, while similar charge reduction in mutants A1 and A2 disrupted the recognition almost completely. Such sequence degeneracy is not inconsistent with interaction specificity. Substantial sequence simplification can be tolerated in several naturally occurring peptide recognition systems, including S-peptide/S-protein (Komoriya & Chaiken, 1982) and AVP/BNPII (Fassina & Chaiken, 1988). For the latter case, the entire set of AVPGlyLysArg sequence-simplified peptides used in the current study was previously found to interact with BNPII with a hierarchy of affinity (Fassina & Chaiken, 1988, 1989; Fassina et al., 1988) curiously similar to that for interaction with AS[proAVP/BNPII(20-1)].

Effect of Sense Peptide Sequence Truncation. A multisite contact hypothesis of sense/antisense peptide interaction was suggested by, among other data, the observed decrease in binding affinity of shortened antisense peptide (Shai et al., 1987b). In the current study, interaction affinity of immobilized AS[proAVP/BNPII(20-1)] was found to be dependent on the length of the sense peptide (Figure 2B). Related to this, the extent of retardation of semisynthetic proAVP/BNPII (Fassina & Chaiken, 1989) was higher than that for either AVPGlyLysArg or BNPII alone, with a calculated $K_{M/P} = 1.4 \mu\text{M}$ (Table III). This result argues that AS[proAVP/BNPII(20-1)] can bind simultaneously to BNPII and AVPGlyLysArg sequence domains. Sequence truncation has a profound effect on the net charge of the resulting peptide, but, as above, a better correlation exists between affinity and peptide length (Figure 2B) than between affinity and net charge (Table III).

Use of Immobilized Antisense Peptides as Selective Recognition Agents. The differential affinities of AVPGlyLysArg and BNPII on AS[proAVP/BNPII(20-1)]ACCELL (Table III) suggested that the immobilized antisense peptide could be used as an affinity agent to fractionate sense peptides from biological sources. Crude acid extract of bovine posterior pituitaries, containing AVP, OT, BNPI, BNPII, and presumably many other acid-soluble molecules, was eluted on the AS[proAVP/BNPII(20-1)] column (80 × 6.6 mm i.d.) pre-

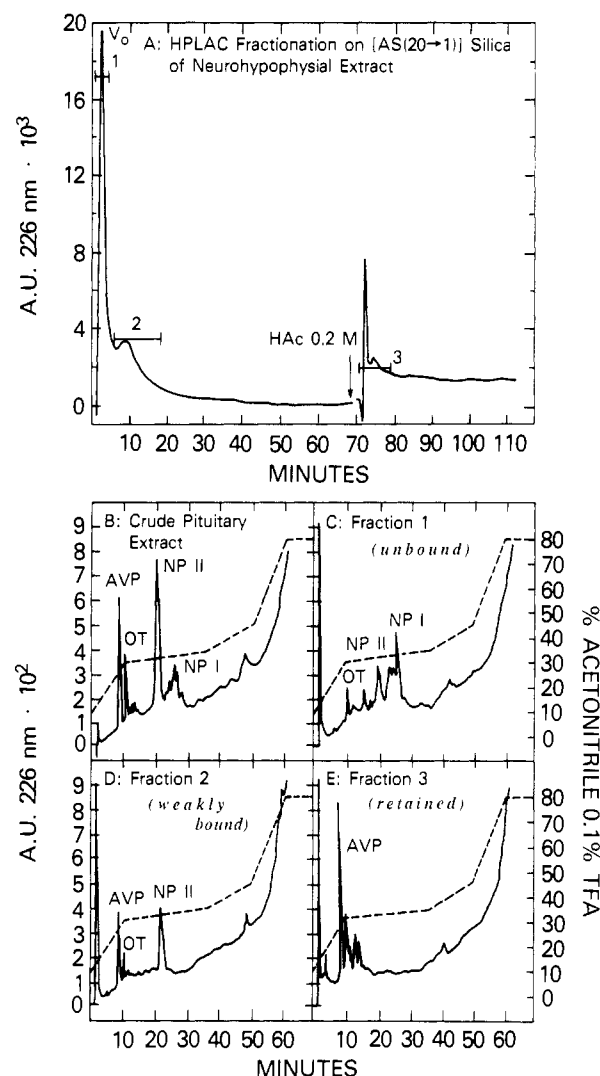


FIGURE 4: (A) Elution profile of crude pituitary extract applied to AS[proAVP/BNPII(20-1)]ACCELL. The column (80 × 6.6 mm i.d.) was equilibrated at a flow rate of 1.0 mL/min with 50 mM NH_4Ac , pH 5.7. Crude pituitary extract (50 μg) was injected in a 20- μL zone, and the effluent was monitored at 280 nm, 0.1 AUFS. After 70 min, the eluent was changed to 0.2 M HAc. Fractions, collected as indicated, were frozen and lyophilized for subsequent HPLC analysis. (B) Reverse-phase HPLC analysis of crude pituitary extract. A 50- μg amount of crude pituitary extract was injected onto the Axxiochrom RP C18 column (100 × 4.6 mm i.d.) equilibrated with 15% CH_3CN , 85% H_2O , and 0.1% TFA at a flow rate of 0.9 mL/min. Gradient as indicated in the figure. Effluent was monitored by UV absorbance at 226 nm, 0.1 AUFS. (C) RP-HPLC analysis of fraction 1 of (A). (D) RP-HPLC analysis of fraction 2 of (A). (E) RP-HPLC analysis of fraction 3 of (A).

pared with the same types of matrix used for the analytical evaluation of binding constants. The elution profile obtained with 50 μg of crude extract is shown in Figure 4A. Three fractions were collected: (1) an unretarded fraction eluting at the void volume, (2) retarded material, and (3) a retained fraction eluted with 0.2 M HAc. Reverse-phase HPLC elution profiles of these fractions (Figure 4C-E) were compared to the elution profile of original crude extract applied to the affinity column (Figure 4B). While the void volume peak contains the family of BNPI isoforms, oxytocin, and some material eluting close to BNPII, the retarded fraction is enriched with BNPII and AVP. The fraction eluted with 0.2 M HAc contains mainly AVP and a background of other UV-absorbing material. The antisense peptide column thus can discriminate between molecules of close structure and sequence, such as between the pairs AVP versus OT and

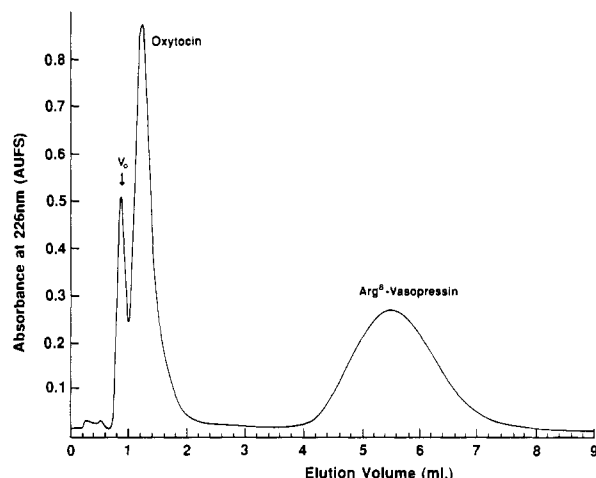


FIGURE 5: Separation of AVP and OT on AS[proAVP/BNPII(12-1)]ACCELL. A sample of bovine pituitary acid extract fraction containing 100 μ g of a mixture of AVP and OT and dissolved in 0.1 M ammonium acetate, pH 5.5, was injected onto an immobilized 12-mer column of 3 mm \times 15 cm ($M_T = 0.223 \mu\text{mol}$), and elution (400 $\mu\text{L}/\text{min}$) was monitored at 226 nm, 1.0 AUFS. Peak identities were verified by comparison with authentic peptide elution positions and by amino acid analysis. The affinity chromatographic peptide separations on immobilized 12-mer were carried out on a Beckman System Gold liquid chromatograph.

BNPII versus BNPI. In contrast, separations cannot be achieved with conventional affinity supports, with OT and AVP eluting with very similar affinity on immobilized BNPII and BNPI and BNPII with similar affinity on immobilized AVP.

Differential retardation also was found for AVP and OT on immobilized AS[proAVP/BNPII(12-1)]ACCELL, corresponding to the AVPGlyLysArg sequence (Figure 1). Elution on immobilized 12-mer column (matrix bed 150 \times 3 mm, $M_T = 0.223 \mu\text{mol}$, in 0.1 M ammonium acetate, pH 5.5) showed selective affinities for AVP ($K_{M/P} = 5.3 \times 10^{-5} \text{ M}$) $>$ AVT ($K_{M/P} = 8.6 \times 10^{-5} \text{ M}$) $>$ LVP ($K_{M/P} = 1.1 \times 10^{-4} \text{ M}$) $>$ OT ($K_{M/P} = 5.3 \times 10^{-4} \text{ M}$). The differential affinity of AVP vs OT could be used to obtain base-line separation of these two neuropeptides from tissue extracts (Figure 5). Again, this type of selectivity is essentially unattainable on conventional sense peptide affinity supports.

DISCUSSION

At present there is no complete understanding of mechanisms underlying antisense peptide recognition of sense peptides. A major goal of the current study was to evaluate whether the antisense/sense peptide binding phenomenon can be considered to occur generally and to improve our understanding of just how selective this recognition process is. All of the antisense peptides made here, namely, the full-sequence AS[proAVP/BNPII(20-1)] and the two fragments 12-1 and 20-13, do have measurable affinity for the sense polypeptides AVP and BNPII. These molecular interactions could be observed with both immobilized sense and antisense peptide matrices. Interaction with the expected partners occurred at conditions in which several control peptides did not interact. When taken with the success of our first attempt with S-peptide (Shai et al., 1987a, 1989) at quantitatively verifying the sense/antisense peptide interaction phenomenon, the data argue strongly that these interactions are likely to occur for a rather extended range of polypeptide and protein cases and are not anomalies for one or a few particular cases only.

The data obtained here also reveal a significant degree of selectivity in antisense peptide recognition. While the full

20-mer sequence AS[proAVP/BNPII(20-1)] interacts with both AVP and BNPII affinity matrices, the AS fragments 12-1 and 20-13 bind preferentially to [AVPGlyLysArg]ACCELL and [BNPII]ACCELL, respectively. Further, the selectivity of AS peptides is apparent when these are immobilized and sense polypeptides fractionated. When acid extracts of posterior pituitary were eluted on immobilized AS[proAVP/BNPII(20-1)], the matrix was found to bind AVP and BNPII preferentially versus the respective related molecules OT and BNPI. BNPI and OT were retarded weakly if at all, with the former recovered only in the unretarded fraction and the latter predominantly in the unretarded fraction and to a small extent (and perhaps only as tailing from the unretarded peak) in the retarded fraction. The ability to distinguish BNPII from BNPI and AVP from OT is striking given the sequence similarities within these sets of molecules. Equally striking is the selectivity exhibited by immobilized AS(12-1) between AVP and LVP.

Categorizing antisense peptide recognition of sense peptides as specific is a major challenge of studies of these molecules. Strong and often subjective debate can be made on the meaning of the term "specificity" and the relationship of specificity to magnitude of affinity. It goes beyond the scope of this paper to construct these arguments fully here. However, in the context of antisense peptides, we would propose that the interactions should be viewed as specific if they can be established to be selective in a systematic way—that is, occurring at higher affinity with the intended sense/antisense components than with other combinations of peptides. For the AS[proAVP/BNPII] peptides reported here, selectivity was established by differential chromatographic retardation, which is greater for the 12-1 or AVP-related peptide than for the 20-13 or BNPII-related peptide on the AVP matrix and the reverse on the BNPII matrix (Tables II and III), and by higher affinity binding, on immobilized 20-1, of AVP and BNPII than of OT and BNPI, respectively. There is low-affinity cross-recognition in these cases, but the phenomenon of cross-recognition also is observed with more natural peptide and protein interactions, for example, with antibodies (Inman & Barnett, 1988). Furthermore, while the affinities of sense/antisense peptide interactions can be quite modest, ranging in the current work from millimolar to micromolar values, such weak affinities also are observed for monoclonal antibody/antigen interactions (Ohlson et al., 1988), the latter of which usually are considered "specific". Low affinity and commonly acknowledged "specific" interactions also have been found to occur for carbohydrate with lectins (Anderson & Walters, 1986). Further, for BNPII in its biologically relevant interaction with itself (self-association), the affinity is weak, approximately 10^{-4} M (unliganded), and does not distinguish BNPII from BNPI. And, NP interaction with OT and AVP, while of somewhat higher affinity (10^{-5} M), does not distinguish OT from AVP. Defining these latter interactions as biospecific is not questioned. Thus, specificity or selectivity need not be linked absolutely to the strength of affinity or even the lack of cross-recognition. We therefore would define the low- to modest-affinity interactions of antisense with sense peptides as of great enough selectivity and affinity to consider them specific. Certainly the degeneracy of sequence and conformation tolerated in these interactions calls into question exactly how this specificity is achieved. But, not knowing the mechanism is an insufficient reason to ignore these interactions. Indeed, not knowing the mechanism in the face of repeated observation of selective antisense peptide interactions is a compelling reason to try to understand this phenomenon and

to relate it to the mechanism of interaction of native peptides, proteins, and other biological molecules with one another. Future efforts need to address the detection and characterization of sense/antisense peptide interactions in solution.

The hierarchies of effective affinity of AS[proAVP/BNP(20-1)]ACCELL for proAVP/BNP(20-1) > AVP > BNP(20-1) > OT \approx BNP(1-20) \approx 0 and of AS[proAVP/BNP(12-1)]ACCELL for AVP > LVP > OT suggest the potential use of antisense peptides in sequence-directed affinity chromatography. Given the significant although not yet certain degree of sequence degeneracy in the antisense peptide interaction with sense peptides (see below), it might be preferable to think of this separation potential as a form of general ligand affinity chromatography. The peptides could serve in the same way as do triazine dyes (Lowe et al., 1986), binding many proteins with overlapping specificity but with sufficiently different affinity to effect separation. Since the mode of antisense/sense peptide binding is viewed as due to hydrophobic pattern recognition, this type of separation could be viewed as pattern recognition affinity chromatography.

After this work was complete, a report was published (Johnson & Torres, 1988) which showed the interaction of a nine-residue antisense peptide with AVP and the positive competition by this peptide of the interferon induction effect of AVP. The antisense peptide in this case had a sequence defined by reading the AVP antisense DNA sequence from 5' to 3' for amino to carboxyl orientation. This is the opposite reading direction to that used to make AS[proAVP/BNP(20-1)] and fragments in the present study. The results of Johnson and Torres (1988) confirm the ability of an AVP-related antisense peptide to interact directly with AVP and also are consistent, through sequence-variation data, with dual contribution of hydrophilic and hydrophobic elements to the antisense/sense peptide interaction.

REFERENCES

- Allenmark, S., & Bomgren, B. (1982) *J. Chromatogr.* 252, 297-300.
- Anderson, D. J., & Walters, R. R. (1986) *J. Chromatogr.* 376, 69-78.
- 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., Smith, E. M., & Blalock, J. E. (1985a) *Biochem. Biophys. Res. Commun.* 128, 1373-1380.
- Bost, K. L., Smith, E. M., & Blalock, J. E. (1985b) *Proc. Natl. Acad. Sci. U.S.A.* 82, 1372-1375.
- Brentani, R. R., Ribeiro, S. F., Potocnjak, P., Pasqualini, R., Lopes, J. D., & Nakaie, C. R. (1988) *Proc. Natl. Acad. Sci. U.S.A.* 85, 364-367.
- Breslow, E., Aanning, H. L., Abrash, L., & Schmir, M. (1971) *J. Biol. Chem.* 246, 5179-5188.
- Breslow, E., Weis, J., & Menendez-Botet, C. J. (1973) *Biochemistry* 12, 4644-4653.
- Chaiken, I. M. (1988) in *Molecular Mimicry in Health and Disease* (Lernmark, A., Dyrberg, T., Terenius, L., & Hokfelt, B., Eds.) pp 351-367, Excerpta Medica, Amsterdam.
- Chaiken, I. M., Abercrombie, D. M., Kanmera, T., & Sequeira, R. P. (1983) *Pept. Protein Rev.* 1, 139-209.
- Chaiken, I. M., Miller, T. H., Sequeira, R. P., & Kanmera, T. (1984) *Anal. Biochem.* 143, 215-225.
- Fassina, G., & Chaiken, I. M. (1987) *Adv. Chromatogr.* 27, 247-297.
- Fassina, G., & Chaiken, I. M. (1988) *J. Biol. Chem.* 263, 13539-13543.
- Fassina, G., & Chaiken, I. M. (1989) *J. Mol. Recognit.* 1, 158-165.
- Fassina, G., Swaisgood, H. E., & Chaiken, I. M. (1986) *J. Chromatogr.* 376, 87-93.
- Fassina, G., Zamai, M., & Fontana, A. (1987) *J. Cell. Biochem.* 35, 9.
- Fassina, G., Lebl, M., & Chaiken, I. M. (1988) *Collect. Czech. Chem. Commun.* 53, 2627-2636.
- Heinrikson, R. L. (1971) *J. Biol. Chem.* 246, 4090-4095.
- Inman, J. K., & Barnett, A. L. (1988) in *Protein Recognition of Immobilized Ligands* (Hutchens, T. W., Ed.) pp 35-44, Alan Liss, New York.
- Johnson, H. M., & Torres, B. A. (1988) *J. Immunol.* 141, 2420-2423.
- Knutson, V. P. (1988) *J. Biol. Chem.* 263, 14146-14151.
- Komoriya, A., & Chaiken, I. M. (1982) *J. Biol. Chem.* 257, 2599-2604.
- Lowe, C. R., Burton, S. J., Pearson, J. C., & Clonis, Y. D. (1986) *J. Chromatogr.* 376, 121-130.
- Mitchell, A. R., Kent, S. B. H., Engelhart, M., & Merrifield, R. B. (1978) *J. Org. Chem.* 43, 2845-2852.
- Ohlson, S., Lundblad, A., & Zopf, D. (1988) *Anal. Biochem.* 169, 204-208.
- Ohyama, Y., Kasai, K.-I., Nomoto, H., & Inoue, Y. (1985) *J. Biol. Chem.* 260, 6882-6887.
- Shai, Y., Flashner, M., & Chaiken, I. M. (1987a) *Biochemistry* 26, 669-675.
- Shai, Y., Flashner, M., & Chaiken, I. M. (1987b) in *Protein Structure and Design* (Oxender, D., Ed.) pp 439-451, Alan Liss, New York.
- Shai, Y., Brunck, T., & Chaiken, I. M. (1989) *Biochemistry* (preceding paper in this issue).
- Swaisgood, H. E., & Chaiken, I. M. (1986) *Biochemistry* 25, 4128-4155.
- Van Oss, C. J., Absolm, D. R., & Neumann, A. W. (1981) in *Affinity Chromatography and Related Techniques* (Gribnau, T., Visser, J., & Nivard, R. J. F., Eds.) pp 29-37, Elsevier, Amsterdam.
- 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.