SCREENING THE RECOGNITION PROPERTIES OF PEPTIDE HORMONE SEQUENCE MUTANTS BY ANALYTICAL HIGH PERFORMANCE LIQUID AFFINITY CHROMATOGRAPHY ON IMMOBILIZED NEUROPHYSIN

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Dedicated to the memory of Dr Karel Bláha.

Analytical high performance liquid affinity chromatography with immobilized neurophysin was used as a molecular screen to evaluate the effects of peptide hormone structure modification on protein recognition. Immobilization of neurophysin on silica and highly cross-linked agarose occurred with retention of oxytocin and vasopressin binding properties. The effects of one-residue-at-a-time mutation, multi-site sequence simplification, and sequence randomization of critical contact residues were evaluated by extent of binding of the peptides on the affinity matrix. The analytical chromatography method also was used as a stereoselective detector to identify racemic contaminants in peptide hormone preparations.

Evaluation of the effects of structure modification of peptides involved in recognition phenomena can provide improved understanding of the mechanisms responsible for their interactions. While sequence alterations of natural peptides can be accomplished easily by synthetic methods, classical evaluation of peptide–protein interaction by solution methods is often restricted by the size of the interacting components, by the need to observe secondary effects such as spectroscopic or enzyme activity transitions, or by the requirement of expensive or time consuming analytical procedures. Analytical high performance liquid affinity chromatography (HPLAC) has proven to be an effective technique in detecting and measuring molecular interactions[1–3]. In most cases studied, characteristics of interaction processes measured by analytical HPLAC agree well with data from studies carried out fully in solution. Moreover, this technique is extremely rapid, allows characterization of

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interaction processes without major size limitation and over a wide range of affinity, and does not require that the interaction produce spectroscopic or enzymatic transitions.

In the present study, we evaluated the effect of several structure alterations in the hormones oxytocin (OXT) and [Arg[8]]vasopressin (AVP) on the recognition with bovine neurophysin II (BNP II)*. Peptides were produced by synthetic methods and their binding properties evaluated by zonal elutions on high performance affinity columns containing immobilized BNP II.

EXPERIMENTAL

Sequenal grade trifluoroacetic acid, dimethylformamide and triethylamine were obtained from Pierce Chem. Co. (Rockford, IL). HPLC grade water, acetonitrile and ethyl acetate were from Fisher Scientific (Lexington, MA). Thin-layer chromatography was carried out on silica gel plates (Silufol, Kavalier, Czechoslovakia) in the systems: 2-butanol–98% formic acid–water (75:13.5:11.5) (S1), 2-butanol–25% aqueous ammonia–water (85:7.5:7.5) (S2), 1-butanol–acetic acid–water (4:1:1) (S3), and 1-butanol–pyridine–acetic acid–water (15:10:3:6) (S4). Electrophoresis was performed on Whatman 3MM paper in moist chamber (20 V/cm) for 1 h in 1 M acetic acid (pH 2.4) and a pyridine–acetate buffer (pH 5.7). Activated affinity supports, formed on a coated silica backbone and containing a 6-carbon spacer with a terminal N-hydroxysuccinimide ester for immobilizing molecules through their amino groups, were provided to us by Waters Chromatography Division (Milford, MA) under the trade name Accell 78. Glass ("Omni") columns for packing affinity matrices were from Rainin Instrument Co. (Woburn, MA). Reverse phase HPLC separations were performed on a TSK-120T (250 × 4.6 mm) C-18 column (LKB, Bromma) connected to a Gilson HPLC system (Thompson Instruments, Springfield, VA). Analytical HPLAC 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 Model 2210 recorder. [L-Tic[2]]OXT and [D-Tic[2]]OXT were prepared by solid phase peptide synthesis[4].

Solid Phase Peptide Synthesis

Vasopressinyl peptides were synthesized by solid-phase peptide synthesis on Boc-Arg(Tos)-oxymethyl-phenylacetamidomethyl (PAM) resin (1 g, 0.5 mmol/g) from Applied Biosystems (Foster City, CA). Protected amino acids were from Peninsula Labs (Belmont, CA). Cleavage of Boc groups was achieved from starting Boc-amino acyl PAM resin and from protected peptidyl resin after each coupling step by treating with 50% TFA in dichloromethane. Double coupling of each Boc amino acid to the growing α-amino deprotected amino acyl and peptidyl resin was carried out by the DCC method except by the DCC-HOBt active ester method for Asn and Gln. After coupling the final amino acid and Boc removal, part of the peptide resin was treated with acetic anhydride to acetylate the α-amino group of Cys 1. Final protected peptide resins were treated with HF at 0 °C for 1 h in the presence of 10% anisole. The HF was removed by flushing with dry nitrogen followed by vacuum aspiration. Remaining anisole was extracted from solid with ethyl acetate and the peptide

* Other abbreviations used: Tos, tosyl; Boc, t-butyloxycarbonyl; HPLC, high performance liquid chromatography; DCC, dicyclohexylcarbodiimide; HOBt, hydroxybenzotriazole; Tic, tetrahydroisoquinoline-3-carboxylic acid.

thereafter extracted from the resin with 10% acetic acid (3 × 10 ml). Pooled acetic acid extract (30 ml) in each case was added to 450 ml of deionized water and the pH adjusted to 8.5 with concentrated ammonium hydroxide. Intramolecular disulfide formation was achieved by slow addition of 20 ml of 10 mM-$K_3[Fe(CN)_6]$. After stirring for 30 min at room temperature, the pH was lowered to 5.0 with acetic acid, and excess ferricyanide was removed by adding 20 g of Bio-Rad anion exchange resin (AG3-X4A) and stirring for 30 min. The resin was then filtered and the filtrate concentrated by rotary evaporation to about 100 ml. A 10 ml aliquot was gel filtered on Sephadex G-25 (1.7 × 140 cm) in 10% acetic acid to remove salts and polymerized peptide. Fractions containing the monomer form were lyophilized and further purified by reverse phase HPLC on TSK-120T (250 × 4.6 mm) with a linear gradient from 0.1% TFA in 10% acetonitrile to 0.1% TFA in 35% acetonitrile in 35 min, using a flow rate of 0.9 ml/min. The main peak fractions were collected and lyophilized. The yields of final purified peptides based on amino acid analysis were close to 30%. Identity of synthetic peptides was confirmed by amino acid analysis after hydrolysis with constant boiling HCl, at 110 °C for 24 h in vacuo. AVP-Gly-Lys-Arg: Asp 0.8, Glu 0.8, Phe 0.7, Pro 1.0, Gly 2.0, Arg 1.9, Lys 0.9, Cys 1.4, Tyr 0.6.

N^{α},N^{ω}-Boc_2-AVP-Gly-Lys-Arg

Solid phase synthesized AVP-Gly-Lys-Arg was dissolved at a concentration of 5 mg/ml in a water–dioxane (1:1) mixture, with stirring. To 1 ml solution, one drop of N-ethylmorpholine was added and then 200 μl of Boc_2O. The extent of reaction was followed by HPLC, using the same conditions described above for purification of synthetic peptides. Protected peptide was purified by semipreparative reverse phase HPLC on TSK 120T as described above.

H-Cys(Et)-Tyr-Phe-NH_2

To the solution of Phe-OMe.HCl (2.16 g) in dimethylformamide (30 ml) alkalized by triethylamine (1.39 ml) and cooled to 0 °C, Boc-Tyr (2.81 g), HOBt (1.35 g) and DCC (2.2 g) were added. After stirring at 0 °C for 1 h and at room temperature overnight the mixture was cooled to 0 °C (2 h), filtered and the filtrate poured into water (400 ml). The precipitate was washed sequentially with 0.05 M-H_2SO_4, water, 5% Na_2CO_3, and water, and then dried by repeated evaporation from solution in methanol–benzene. The product (3.84 g, 89.8%), homogeneous according to TLC (R_F: 0.81 (S1), 0.63 (S2), 0.73 (S3), 0.76 (S4)), was dissolved in methanol (30 ml) and cooled. After addition of methanol–ammonia (30 + 30 ml) mixture, the solution was left aside in a tightly closed flask for 90 h at room temperature. After evaporation of solvents and reevaporation from methanol, the product, homogeneous according to TLC (R_F: 0.76 (S1), 0.60 (S2), 0.69 (S3), 0.70 (S4)), was dissolved in trifluoroacetic acid. After standing for 70 min at room temperature and repeated evaporation with toluene, the obtained trifluoroacetate of Tyr-Phe-NH_2 was divided into two portions and one half was used for the synthesis of Met-Tyr-Phe-NH_2. The other half was dissolved in dimethylformamide (20 ml), cooled to 0°C, alkalized by triethylamine (0.6 ml), and Z-Cys(Bzl) (1.55 g), HOBt (0.6 g) and DCC (0.92 g) were added. After mixing for 2 h at 0°C and overnight at room temperature, the product was worked up as described above for the previous product of DCC/HOBt coupling. The product (Z-Cys(Bzl)-Tyr-Phe-NH_2, 1.5 g) crystallized from 2-propanol, was homogeneous according to HPLC ($k' = 9.6$, 65% of methanol in 0.05% trifluoroacetic acid). Part of this product (650 mg) was dissolved in liquid ammonia (100 ml) and reduced by metallic sodium until the blue color persisted for 1 min. The solution was decolorized by the addition of NH_4Cl and after addition of ethyl bromide (0.4 ml) the stirring continued for 30 min. After evaporation to dryness in vacuo, the residue was dissolved in water (25 ml) and applied to a column of Dowex 50 (30 ml). The column was washed with water, and the product was eluted by 5%

ammonia and lyophilized. Part (30 mg) of the lyophilizate (490 mg) was purified by HPLC (Separon SIC_{18}, 250 × 10 mm, 44% methanol in 0.05% trifluoroacetic acid) and 22 mg of Cys(Et)-Tyr-Phe-NH_2 was obtained. R_F: 0.45 (S1), 0.64 (S2), 0.29 (S3), 0.75 (S4); $k' = 6.52$ (Separon SIC_{18}, 150 × 4 mm, 40% methanol in 0.05% trifluoroacetic acid); $E_{2,4}^{Gly} = 0.89$, $E_{5,7}^{His} = 0.47$. For $C_{23}H_{30}N_4O_4S.CF_3COOH$ (572.6) calculated: 52.44% C, 5.46% H, 9.78% N; found: 52.72% C, 5.76% H, 10.02% N; FAB MS (*m/z*): 459 (M + H); ^{1}H NMR (CD_3SOCD_3): 1.11 t, 3 H (J = 7.2, CH_3); 2.48 q, 2 H (CH_2–CH_3); 2.66, 2.70, 2.82, 2.84, 2.88, 3.03, 6 H (β-CH_2); 3.79 q, 1 H (α-CH); 4.47 m, 2 H (α-CH); 6.62 + 7.00 d, 4 H (Tyr); 7.23 m, 5 H (Phe); 7.10 + 7.33 bs, 2 H ($CONH_2$); 7.69 bs, 1 H (OH); 8.18 + 8.54 d, 2 H (CONH).

H-Met-Tyr-Phe-NH_2 (refs[5–6]): Half of the dipeptide trifluoroacetate described above was coupled with Boc-Met using DCC/HOBt procedure. Product was deprotected by trifluoroacetic acid and purified by HPLC.

Analytical HPLAC

Peptide binding to BNP II and effects of hormone modification on neurophysin recognition were evaluated by analytical HPLAC[3,7] using columns of BNP II immobilized on Accell SL78 prepared as described before[2]. Affinity support was slurry-packed into HPLC glass columns 100 × 6.6 mm. Zones (20 μl) containing different amounts of peptide or protein were eluted isocratically on the column equilibrated with 0.4 M-$AcONH_4$, pH 5.7. Variation of the elution volume V with the concentration of mobile peptide [P] was evaluated using equation (*1*) (refs[3,7])

$$1/(V - V_0) = ((K_{M/P})/M_T) + [P]/M_T, \qquad (1)$$

where V_0 is unretarded elution volume, M_T is total amount of functionally active immobilized neurophysin (M) and $K_{M/P}$ is the dissociation constant of matrix-bound neurophysin and mobile peptide (P). For zonal elution, [P] is not easily definable since it changes continuously during the elution. However, the extent of dependence of $1/(V-V_0)$ on [P] can be approximated by the dependence of $1/(V-V_0)$ on $[P]_0$, the initial concentration of P in the zone applied.

RESULTS AND DISCUSSION

Site Directed Mutants

Interactions of peptides were measured by analytical HPLAC on a matrix specifically designed in the content of immobilized NPII to allow evaluation of affinities in the range 1 μmol l^{-1} – 1 mmol l^{-1}. The data are shown in Fig. 1. From the linear variations of the extent of retardation, $1/(V-V_0)$, with the amount applied in the zone and extrapolation to zero concentration according to Eq. (*1*) it is possible to calculate equilibrium dissociation constants, $K_{M/P}$. The $K_{M/P}$ values determined for the set of peptides investigated are reported in Table I. Binding of AVP and OXT was previously characterized under the same conditions to yield a $K_{M/P}$ = 10.1 μmol l^{-1} for AVP (ref.[2]) and 10.6 μmol l^{-1} for OXT (unpublished results[8]).

Previous studies of various analogs of oxytocin, vasopressin, and the model peptides have shown the importance of particular amino acid residues for

binding to neurophysin (for a review of the binding of neurohypophysial hormone analogs to neurophysin see ref.[9]). The most important structural elements for this interaction appear to be the amino-terminal amino acid residues—especially the α-amino group and Tyr[2] side chain. The importance of the disulfide bridge has been less clear. Studies with model dipeptides[10] have shown a contribution for the side chain in position 1, but the effect of the position of sulfur itself was not evaluated. Comparison of Met and Cys(Me) is of limited validity since one can expect an overall change of hydrophobicity due to the additional methylene group. Carba analogs (i.e. analogs with CH_2—S, S—CH_2, or CH_2—CH_2 groups instead of S—S) are potentially of value, although a previous report of such analogs provides only a qualitative evaluation of effects of carba replacement in OXT and AVP[11].

The tripeptide resembling the amino-terminal part of vasopressin (Met-Tyr-Phe-NH_2) has substantial affinity to neurophysin[5,6,9,12]. We decided to modify this tripeptide by changing Met for Cys(Et), thus approaching more closely the situation in natural hormone, where sulfur is in the position of the amino terminal amino acid, and at the same time keeping the size of the side

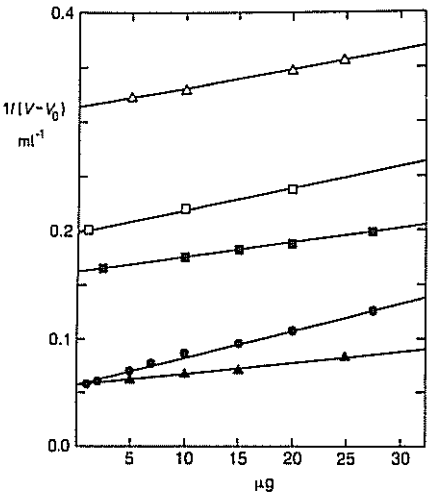

FIG. 1

Analytical HPLAC determination of synthetic peptide binding to neurophysin by zonal elution. Zones of peptides (20 μl) containing different amounts of peptide were eluted on a column (9.5 × 10 mm) of [BNP II]Accell in 0.4 M-$AcONH_4$, pH 5.7. Elution volumes are plotted as $1/(V-V_0)$ against the amount (μg) of mobile peptide. The extrapolated values are used to calculate the $K_{M/P}$ values from Eq. (*1*). These are reported in Table I. Data in the plot correspond to: ▲ Met-Tyr-Phe-NH_2; ● Cys(Et)-Tyr-Phe-NH_2; □ N^{α},N^{ω}-Boc_2-AVP-Gly-Lys-Arg; △ N^{α}acetyl-AVP-Gly-Lys-Arg; ■ [L-Tic[2]]OXT

chain unchanged. The studied peptides (Met-Tyr-Phe-NH_2 and Cys(Et)-Tyr--Phe-NH_2) differ in the same manner as carba-1 and carba-6 analogs of neurohypophyseal hormone. As shown in Table I both model peptides retained the same ability to recognize neurophysin. At least for the tripeptides, the sulfur position does not appear to be critical for BNP II binding.

Extension of the hormone sequence with the tripeptide Gly-Lys-Arg does not alter binding properties[8,13], but substitution of the free α amino group by the acetyl or Boc groups decreases recognition drastically. This is in accordance with the above mentioned essential importance of the α-amino group for the interaction. That the N^{α}-acetyl group weakens the interaction with neurophysin more than two bulky Boc groups is intriguing and may reflect a contribution of the Boc groups themselves to binding.

Another two analogs contained the conformationally restricted phenylalanine residue, Tic (tetrahydroisoquinoline-3-carboxylic acid) in either L or D configuration. Since it is known that phenylalanine residue can replace tyrosine in position 2 of oxytocin without an apparent influence on the binding, conformational restriction of this residue may give us more detailed information about the steric requirements of neurophysin for the binding. The dramatically decreased affinity of [L-Tic^2]OXT speaks in favor of a rather strictly sterically defined hydrophobic pocket. However, it must be stressed that the requirements are not as high as in the case of the uterotonic receptor, where the activity was completely eliminated by Tic replacement. The analog containing the Tic residue in a D configuration was not bound to neurophysin at all. This is in

TABLE I
Chromatographically determined equilibrium binding constants of synthetic peptides on [BNP II]Accell

Peptide	$K_{M/P}$[a], μmol l^{-1}
OXT	10.1
AVP	10.6
AVP-Gly-Lys-Arg	8.0
Met-Tyr-Phe-NH_2	79
Cys(Et)-Tyr-Phe-NH_2	80
[L-Tic^2]OXT	250
N^{α},N^{ω}-Boc_2-AVP-Gly-Lys-Arg	315
N^{α}-Ac-AVP-Gly-Lys-Arg	750
[D-Tic^2]OXT	>1 000[b]

[a] Values determined by $V/(V-V_0)$ at zero peptide concentration, in 0.4 M-$AcONH_4$, pH 5.7; [b] limit of experimentally evaluable $K_{M/P}$.

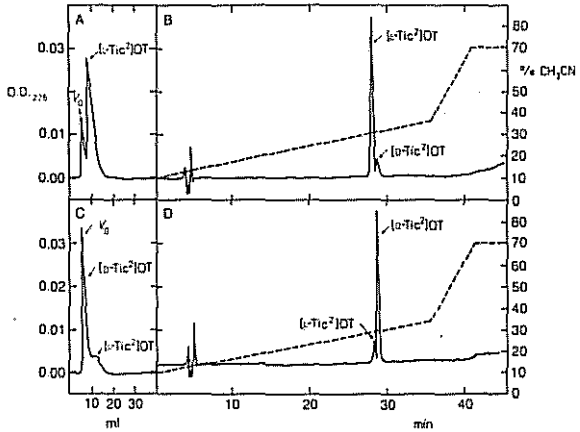

FIG. 2

HPLAC profiles of synthetic [L-Tic^2]OXT (A) and [D-Tic^2]OXT (C) preparations. Zones containing 20 μg of peptide material were eluted isocratically on the [BNP II]Accell column equilibrated with 0.4 M-$AcONH_4$, pH 5.7, at a flow rate of 1.0 ml/min. B and D: corresponding HPLC profiles (gradient of CH_3CN in 0.1% TFA, TSK 120T column, 0.9 ml/min)

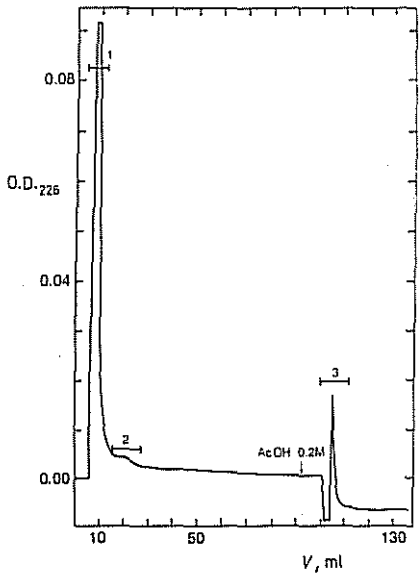

FIG. 3

HPLAC profile of synthetic AVP-Gly-Lys-Arg randomized in position 2. An aliquot containing 50 μg crude peptide was injected onto the [BNP II]Accell column equilibrated at a flow rate of 1.0 ml/min with 0.4 M-$AcONH_4$, pH 5.7. After 100 min, the eluent was changed to 0.2 M-AcOH. Fractions were collected as indicated

agreement with the results obtained with [D-Tyr^2]OXT, which also is not bound.

It should be noted that under the same experimental conditions, peptides with a wide range of affinities for immobilized BNP II can be quickly screened by analytical HPLAC, providing $K_{M/P}$ values expected to be adequately descriptive of the peptide interactions fully in solution. For example, the $K_{M/P}$ value 79 μmol l^{-1} for the tripeptide Met-Tyr-Phe-NH_2 agrees reasonably well with the value of 36 μmol.l^{-1} obtained by spectrophotometric titration[12] and the value of 22 μmol l^{-1} obtained by equilibrium dialysis.

Enantiomeric Selection

The [D-Tic^2]OXT did not show any measurable recognition for immobilized BNP II. Such differences in binding properties were used to analyse synthetic preparations of [L- and [D-Tic^2]OXT (Fig. 2) to detect and quantify the presence of contaminants. Using short elutions, identification of diastereomeric contaminants in the preparation of [Tic^2]OXT can be achieved. By scaling up the amount of immobilized BNP II the column can be used for preparative purification of crude synthetic preparations.

Molecular Screening in Sequence Randomization Studies

To evaluate the applicability of analytical HPLAC as a molecular screen and to learn more about the role of Tyr^2 as a contact residue, synthetic AVP-Gly-Lys-Arg "randomized" in position 2 was prepared by coupling an equimolar mixture of all protected amino acids (except for Cys) during the residue 2 step of synthesis on the solid phase. Position 2 was chosen, since we wished to know what other residues could replace Tyr^2 as a productive contact element to bind BNP II.

TABLE II
Amino acid composition of [BNP II]Accell-bound fraction 3 (Fig. 3) of [Random^2]AVP-Gly-Lys--Arg

Residue	Cys[c]	Asp	Glu	Pro	Gly	Tyr	Phe	Lys	Arg
Expected[a]	2	1	1	1	2	-	1	1	2
Found[b]	0.8	0.9	0.9	1.0	2.2	0.6	1.3	1.0	2.1

[a] Expected for AVP-Gly-Lys-Arg, excluding Tyr^2; [b] calculated by averaging yield of Asp, Glu, Pro, Gly, Lys, and Arg; [c] determined as cysteic acid (after performic acid oxidation) and not corrected for destruction during acid hydrolysis.

After HF treatment and cyclization of the synthetic peptide, a small aliquot was eluted on the [BNP II]Accell column under conditions similar to those employed for peptide binding characterization (Fig. 3). Three main peaks are discernible in the chromatogram. A major peak of non-interacting material eluted at the void volume of the column, a second one slightly retarded, and a third one obtained upon solvent change to 0.2 M-AcOH to elute strongly interacting molecules. While identification of the components in the first and second peaks was unsuccessful due to their complexity, amino acid analysis of the peak eluted with 0.2 M-AcOH (Table II) revealed the presence of Tyr and Phe as the only major residues not accounted for (within a factor of 10%) by the other residue positions of AVP-Gly-Lys-Arg. The results strongly suggest that only two peptides, substituted in position 2 with Tyr and Phe, are selected in the neurophysin HPLAC screen. There is indirect evidence that a Trp^2 peptide may also be present in the NP-bound peak. When peak 3 (Fig. 3) was chromatographed by reverse phase HPLC with UV absorbance monitoring at both 280 and 300 nm, one peak was markedly more prominent at 300 nm relative to the other major peptide peaks. This peptide has not been characterized further. Even given the ambiguity about Trp as a replacement for Tyr^2, it is quite clear that no non-aromatic residues are fully acceptable as replacers of Tyr^2 to promote high affinity peptide–protein contact. This confirms the many findings summarized earlier from studies which used one-residue-at-time replacement.

The results with sequence randomization are reflective of the overall potential of analytical HPLAC as a molecular screen to evaluate and study recognition phenomena. Such screening can be used to examine large numbers of peptide and protein mutants for their binding properties. The ability to make large numbers of peptide mutants is increasing thanks to continuing development of chemical synthesis and recombinant DNA methods. The combination of efficient mutation generators and efficient analytical screening is likely to yield better ideas about how peptide and protein recognition occurs.

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