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Protein prosthesis: β-Peptides as reverse-turn surrogates

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Abstract: The introduction of non-natural modules could provide unprecedented control over folding/unfolding behavior, conformational stability, and biological function of proteins. Success requires the interrogation of candidate modules in natural contexts. Here, expressed protein ligation is used to replace a reverse turn in bovine pancreatic ribonuclease (RNase A) with a synthetic β-dipeptide: β^2-homoalanine–β^3-homoalanine. This segment is known to adopt an unnatural reverse-turn conformation that contains a 10-membered ring hydrogen bond, but one with a donor–acceptor pattern opposite to that in the 10-membered rings of natural reverse turns. The RNase A variant has intact enzymatic activity, but unfolds more quickly and has diminished conformational stability relative to native RNase A. These data indicate that hydrogen-bonding pattern merits careful consideration in the selection of beneficial reverse-turn surrogates.

Keywords: conformational stability; expressed protein ligation; foldamer, β-peptide; reverse turn; ribonuclease A

Introduction

A goal of modern protein science is to learn how to create new proteins with desirable properties. One tack is to remodel the scaffolds of extant proteins using non-natural modules. Oligomers of β-amino acids ("β-peptides") can have greater secondary structural stability, on a per-residue basis, than do conventional peptides and are resistant to degradation by proteases.[1–4] These attributes encourage the exploration of proteins containing β-peptide modules. A key question endures: Which β-peptide modules are truly useful?

In previous work,[5] we demonstrated that the thermostability of an enzyme can be increased by replacing a natural reverse turn with a di-β-peptide module consisting of two nipecotic acid (Nip) residues with the configuration: *R*-Nip–*S*-Nip. This module forms a reverse-turn conformation featuring a 12-membered-ring hydrogen bond between C=O of residue i and N—H of residue $i + 3$ (Fig. 1).[8,9] An analogous hydrogen-bonding pattern exists within the most common type of reverse turns in proteins, so-called β-turns, but the hydrogen-bonded ring in a β-turn has 10 atoms rather than 12, because each α-amino acid residue has three backbone atoms, whereas each β-amino acid residue has four backbone atoms.[10] Because nipecotic acid is a secondary amine and, therefore, forms tertiary amides, an *R*-Nip–*S*-Nip segment cannot access other hydrogen-bonding patterns. Moreover, the hydrogen-bonded 12-membered ring of an *R*-Nip–*S*-Nip module contains only two backbone bonds with unconstrained rotation; hence, this di-β-peptide module is highly preorganized for the reverse-turn conformation.

A different di-β-peptide has been used to generate analogs of the α-peptide hormone somatostatin and to create short β-peptides that display hairpin

Additional Supporting Information may be found in the online version of this article.

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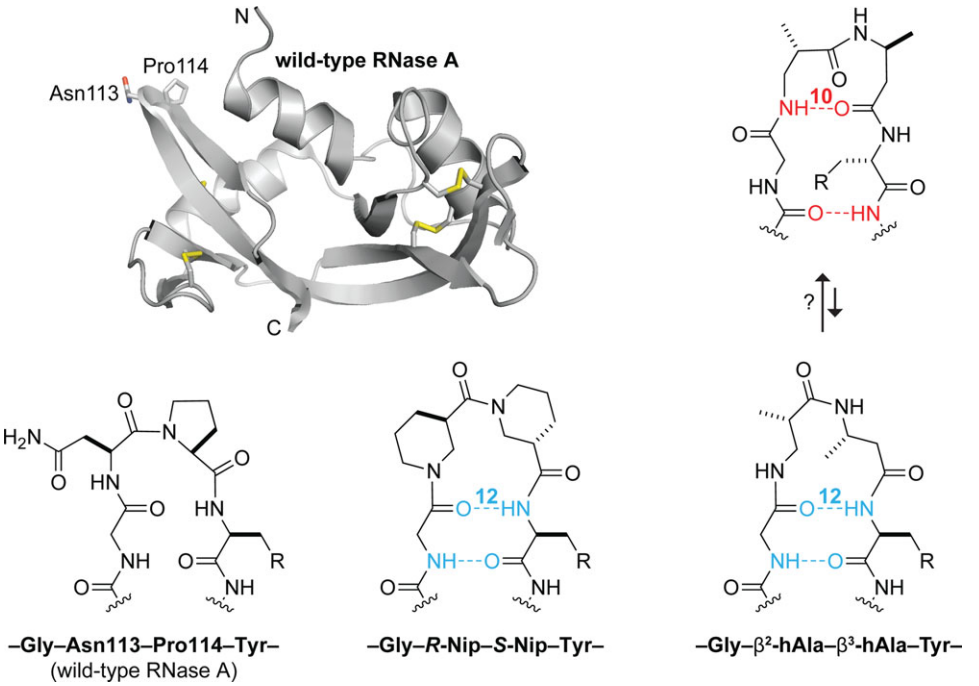

Figure 1. Ribbon diagram of wild-type RNase A (PDB entry 7rsa[6]) and structures of reverse turns relevant to this work. The number of atoms in the hydrogen-bonded rings of each artificial turn is indicated above the hydrogen bond. The Asn113–Pro114 peptide bond of wild-type RNase A is in the *cis* conformation.[7]

conformations.[11–14] In methanol, this α-branched (β^2)–β-branched (β^3) β-dipeptide forms a 10-membered hydrogen-bonded ring[13]; however, although this hydrogen-bonded ring size is comparable to that found in natural β-turns, the donor-acceptor (C=O of residue i and N—H of residue $i - 1$) pairing differs from that of natural β-turns and the *R*-Nip–*S*-Nip reverse turn (Fig. 1).[10] The location of the side chains in the β^2–β^3 segment resembles that in natural β-turns. The acyclic β-amino acids in this module have primary amines and, therefore, form secondary amides. Accordingly, more hydrogen-bonding patterns are accessible to this module than to *R*-Nip–*S*-Nip, including a hydrogen-bonding pattern that leads to the 12-membered ring observed for *R*-Nip–*S*-Nip, which has the directionality of a natural β-turn (Fig. 1). Both the 10- and 12-membered hydrogen-bonded rings available to the β^2–β^3 segment have six backbone bonds with low barriers to rotation.

In this work, we sought to evaluate β^2-homoalanine–β^3-homoalanine (β^2-hAla–β^3-hAla) as a surrogate for a reverse turn in a protein. As our target protein, we chose ribonuclease A (RNase A; EC 3.1.27.5), which is a small enzyme that has been the object of much seminal work in protein chemistry.[15–17] In folded RNase A (Fig. 1), residues 113–114 form a reverse turn that hosted the *R*-Nip–*S*-Nip di-β-peptide in our previous work.[5] Here, we report on the consequences of replacing Asn113–Pro114 of RNase A with the less constrained β^2-hAla–β^3-hAla segment having the configuration shown in Figure 1.

Results

Integrity of native $\beta^2\beta^3$hAla RNase A

$\beta^2\beta^3$hAla RNase A was produced by expressed protein ligation and purified to homogeneity according to analytical chromatography and SDS–PAGE. The average mass of the purified $\beta^2\beta^3$hAla RNase A was in accord with the expected value (Supporting Information Fig. S1). We assessed the ability of $\beta^2\beta^3$hAla RNase A to catalyze the cleavage of 6-carboxyfluorescein–dArU$(dA)_2$–6-carboxytetramethylrhodamine. The value of $k_{cat}/K_M = (1.7 \pm 0.4) \times 10^7$ M^{-1} s^{-1} for the variant is indistinguishable from that of wild-type RNase A ($k_{cat}/K_M = (1.7 \pm 0.2) \times 10^7$ M^{-1} s^{-1}). Likewise, the near and far UV CD spectra of $\beta^2\beta^3$hAla RNase A and the wild-type enzyme are comparable (Supporting Information Fig. S2).

Thermally induced unfolding

Thermally induced unfolding of $\beta^2\beta^3$hAla RNase A monitored by CD spectroscopy (Fig. 2) revealed a decrease in the transition midpoint for $\beta^2\beta^3$hAla RNase A by $\Delta T_m = (-4.5 \pm 0.2)$°C, which corresponds to (5.6 ± 0.7) kJ/mol (Table I). Interestingly, the steepness of the transition curve is decreased considerably for $\beta^2\beta^3$hAla RNase A, and both ΔH_m and ΔS_m were found to be decreased by ~19% in comparison to RNase A (Table I). Proteolysis of RNase A and numerous variants thereof had yielded substantial information on the stability and unfolding of this enzyme.[5,18–21] Here, we exploited the high efficiency of thermolysin to degrade thermally

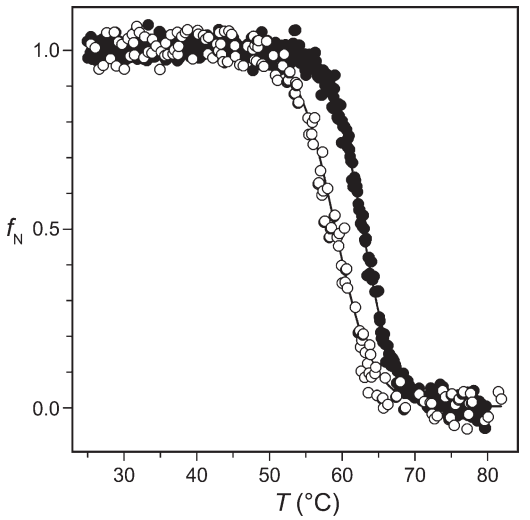

Figure 2. Thermally induced transition of $\beta^2\beta^3$hAla RNase A (open symbols) and the wild-type enzyme (closed symbols). Unfolding was followed by CD spectroscopy at 278 nm as described in the Materials and Methods section.

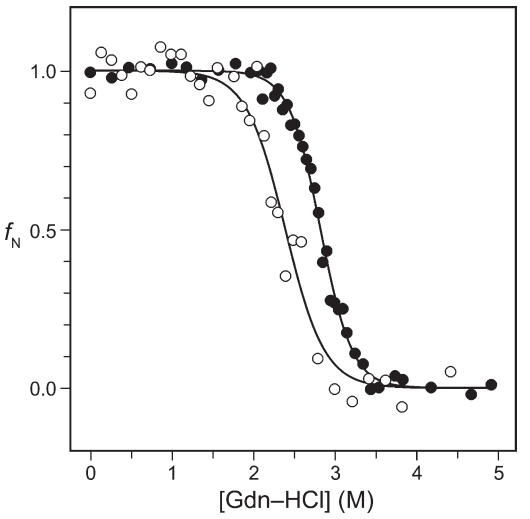

Figure 3. Gdn–HCl-induced transition of $\beta^2\beta^3$hAla RNase A (open symbols) and the wild-type enzyme (closed symbols). Unfolding was followed by CD spectroscopy at 278 nm as described in the Materials and Methods section.

unfolded RNase A in pulse proteolysis experiments. The decrease of cooperativity in the thermally induced unfolding of $\beta^2\beta^3$hAla RNase A was confirmed by the results of pulse proteolysis with thermolysin (Supporting Information Fig. S3).

Gdn–HCl-induced unfolding

Gdn–HCl-induced unfolding of $\beta^2\beta^3$hAla RNase A and the wild-type enzyme was monitored by near-UV CD spectroscopy (Fig. 3). Similar to thermally induced unfolding, the transition midpoint of $\beta^2\beta^3$hAla RNase A was found to be decreased by (0.5 ± 0.1) M, corresponding to (6.3 ± 0.7) kJ/mol (Table I). Interestingly, the steepness of the transition curve, $m\Delta G$, is again decreased by ~20% (Table I).

Unfolding rate constants

The rate of thermal unfolding was monitored by limited proteolysis with thermolysin. As can be seen in an Arrhenius plot (Fig. 4), $\beta^2\beta^3$hAla RNase A unfolds more quickly than does RNase A, yielding a value of $\Delta\Delta G_U^{\ddagger}$ = (3.9 ± 0.5) kJ/mol at the T_m of $\beta^2\beta^3$hAla RNase A (Table I). In contrast to the equilibrium measurements, the value of $\Delta H^{\ddagger}$ was nearly constant and that of $\Delta S^{\ddagger}$ increased by only ~9% for $\beta^2\beta^3$hAla RNase A.

Table I. *Conformational Stability Parameters of $\beta^2\beta^3$hAla RNase A and the Wild-Type Enzyme*

	RNase A	
Parameter	$\beta^2\beta^3$hAla	wild-type
T_m (°C)	59.1 ± 0.3	63.6 ± 0.1
ΔT_m (°C)	−4.5 ± 0.2	—
ΔG at 59.1°C (kJ/mol)	0	5.6 ± 0.7
ΔH_m (kJ/mol)	356 ± 33	442 ± 21
ΔS_m (J/(mol K))	1071 ± 100	1312 ± 62
$[\text{Gdn–HCl}]_{1/2}$ (M)	2.4 ± 0.1	2.9 ± 0.1
ΔG at 2.4M Gdn–HCl (kJ/mol)	0	6.3 ± 0.7
$m_{\Delta G}$ (kJ/(mol M))	10.1 ± 3.2	12.7 ± 0.9
$\Delta\Delta G^{\ddagger}_U$ at 59.1°C (kJ/mol)	—	3.9 ± 0.5
$\Delta H^{\ddagger}_U$ (kJ/mol)	360 ± 20	342 ± 11
$\Delta S^{\ddagger}_U$ (J/(mol K))	819 ± 50	754 ± 27

Discussion

Replacing Asn113 and Pro114 of RNase A with the β^2-hAla–β^3-hAla and R-Nip–S-Nip segments accommodate a folded protein, as either surrogate yields a fully active enzyme. As enzymatic activity is an especially sensitive measure of native protein structure,[22] we conclude that both segments provide a

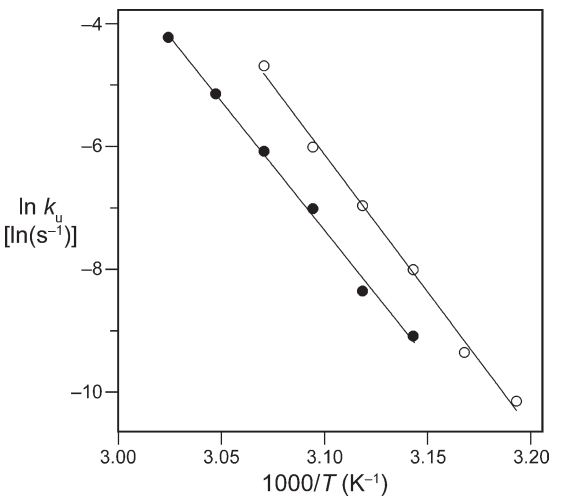

Figure 4. Arrhenius plot of the unfolding rate constants of $\beta^2\beta^3$hAla RNase A (open symbols) and the wild-type enzyme (closed symbols). Values of k_U were determined by limited proteolysis with thermolysin as described in the Materials and Methods section.

native-like tertiary structure. This finding is not rote, as replacing Asn113 and Pro114 with *R*-Nip–*R*-Nip (a diastereomer of *R*-Nip–*S*-Nip) eliminates enzymatic activity. Similarly, the transition temperatures for the P114A and P114G variants are decreased by ~10°C.[23–25] Thus, β^2-hAla–β^3-hAla and *R*-Nip–*S*-Nip are more than passive linkers[26]—both support native protein structure. This conclusion is supported by the indistinguishable CD spectra of both RNase A and $\beta^2\beta^3$hAla RNase A (Supporting Information Fig. S2).

The two surrogates differ, however, in the conformational stability that they confer upon RNase A analogs. Replacing Asn113–Pro114 with *R*-Nip–*S*-Nip increased the T_m value of RNase A by (1.2 ± 0.3)°C.[5] In marked contrast, replacing with β^2-hAla–β^3-hAla decreased the T_m value by (4.5 ± 0.2)°C (Fig. 2; Table I) and the thermodynamic stability at 2.4 M Gdn–HCl by (6.3 ± 0.7) kJ/mol (Fig. 3). Kinetic analyses indicate that this diminished stability arises in large part from the faster unfolding of the $\beta^2\beta^3$hAla variant (Fig. 4).

In summary, we have identified key attributes of a useful reverse-turn surrogate derived from β-amino acids. Comparison of β^2-hAla–β^3-hAla with *R*-Nip–*S*-Nip[5] in this role highlights the importance of preorganizing a proper hydrogen-bonding pattern—one with a hydrogen bond between C=O of residue *i* and N—H of residue *i* + 3. The recapitulation of the natural hydrogen-bonded ring size appears to be less important, and might be deleterious. These results encourage additional interrogations of natural structural elements with foldameric equivalents.[27–30]

Materials and Methods

Materials

Plasmid pTXB1, *E. coli* cells strain ER2566, and chitin beads were from New England Biolabs (Ipswich, MA). Wild-type RNase A and thermolysin were from Sigma Chemical (St. Louis, MO). All other chemicals were of commercial reagent grade or better, and were used without further purification. The protected β-dipepetide Fmoc-β^2-hAla–β^3-hAlaOH was synthesized as described previously.[8,9]

Peptide synthesis

The peptide CAYKTTQANKHIIVACEG–β^2-hAla–β^3-hAla–YVPVHFDASV (which corresponds to residues 95–124 of RNase A but with β^2-hAla–β^3-hAla replacing Asn113–Pro114) was synthesized on a 100-μmol scale with a Pioneer peptide synthesizer from Applied Biosystems (Carlsbad, CA) using standard procedures. The mass of the purified peptide determined by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry was in accord with the expected value (Supporting Information Fig. S1).

Production of RNase A(1–94)–intein fusion protein

E. coli ER2566 cells that had been transformed with a plasmid[31] that directs the expression of RNase A(1–94) fused to the *mxe*-intein were grown in Luria–Bertani medium containing ampicillin (100 μg/mL) with shaking at 37°C until O.D. = 0.5 at 600 nm. Gene expression was then induced by the addition of isopropyl-β-D-thiogalactopyranoside to 0.5 m*M*, and the cultures were grown for an additional 3–4 h at 25°C. The yield of harvested cells was ~2.3 g/L of bacterial culture. Cells were stored at −20°C until used.

Production of thioester-tagged RNase A(1–94)

Thioester-tagged RNase A(1–94) was produced as described previously.[32] It is noteworthy that the protein produced by this method possesses an additional methionine residue at its N terminus, Met(–1), which has no discernable effect on the enzymatic activity or conformational stability of RNase A.[5] Lysis and column buffer was 20 m*M* 3-(*N*-morpholino)propanesulfonic acid (MOPS)–NaOH buffer (pH 6.8) containing NaCl (0.50*M*), ethylenediaminetetraacetic acid (EDTA; 0.1 m*M*), and Triton X-100 (0.1% v/v) and cleavage buffer was 50 m*M* MOPS–NaOH buffer (pH 6.8) containing NaCl (0.50*M*), 2-mercaptoethanesulfonic acid (MESNA; 50 m*M*), and EDTA (0.1 m*M*). The cleavage product, namely RNase A(1–94) thioester, was eluted from the resin after 16 h at room temperature and then precipitated by the addition of sodium deoxycholate to 0.24 m*M* and trichloroacetic acid to 30 m*M*. The precipitate was washed with acetone, dissolved to a concentration of ~5 mg/mL in 0.20*M* sodium phosphate buffer (pH 7.7) containing guanidine–HCl (2*M*) and MESNA (50 m*M*), and used in a ligation reaction.

Ligation of thioester-tagged protein and peptide

Ligation reactions were performed in 0.20*M* sodium phosphate buffer (pH 7.7) containing Gdn–HCl (2.0*M*), the thioester-tagged protein (2 mg/mL), and the peptide (20 mg/mL; 30-fold molar excess). The pH was adjusted to ~8 with 1*M* NaOH, and the ligation reaction was allowed to proceed at room temperature for at least 16 h.

Protein folding and purification

The ligation reaction mixture was diluted with 0.10*M* Tris–HCl buffer (pH 8.0) to <0.05 mg of protein per milliliter and <0.1*M* Gdn–HCl. Reduced glutathione (GSH) and oxidized glutathione (GSSG) were added with stirring at 4°C to final concentrations of 1.0 and 0.2 m*M*, respectively. After adjusting the pH to ~8 with 1*M* NaOH, the solution was transferred to room temperature and protein folding was allowed to proceed for at least 16 h.[19]

Before application of the folding reaction to columns, any precipitate formed during folding was removed by centrifugation at 15,000*g* for 30 min. Purification used hydrophobic interaction chromatography (Phenyl Sepharose™ 6 Fast Flow, Amersham Biosciences) and cation-exchange chromatography (HiTrap SP Sepharose™ cation-exchange resin, Amersham Biosciences) as described previously.[5] Fractions (2 mL) were collected manually, and absorbance at 278 nm as well as ribonucleolytic activity were used to locate the protein.

Samples from throughout the purification procedure were analyzed by SDS–PAGE through a 15% (w/v) acrylamide gel. After purification, 10 mg of peptide yielded 0.8 mg of a semisynthetic RNase A with β^2-hAla–β^3-hAla as residues 113–114 and termed $\beta^2\beta^3$hAla RNase A (50% yield based on thioester-tagged protein; 2% yield based on peptide). Each sample was desalted by using a μ-C18 ZipTip™ from Millipore (Billerica, MA), and MALDI mass spectra were then obtained with a Reflex™ mass spectrometry from Bruker Daltronik GmbH (Bremen, Germany).

Circular dichroism spectroscopy

Circular dichroism (CD) spectra of $\beta^2\beta^3$hAla RNase A and the wild-type enzyme were recorded at 25°C in 50 m*M* sodium phosphate buffer (pH 8.0) containing protein (1.0 or 0.5 mg/mL) and NaCl (25 mM), on a J-810 CD spectrometer from Jasco (Groß-Umstadt, Germany). Cuvettes of 1- and 0.01-cm path length were used for CD spectroscopy in the near-UV region (250–340 nm) and far-UV region (185–260 nm), respectively.

Ribonucleolytic activity

Values of k_{cat}/K_M for the enzymatic cleavage of a fluorogenic substrate, 6-carboxyfluorescein–dArU$(dA)_2$–6-carboxytetramethylrhodamine, were determined as described previously.[33]

Thermally induced transition

Values of T_m, which is the temperature at the midpoint of the thermal unfolding transition, of $\beta^2\beta^3$hAla RNase A and the wild-type enzyme were determined by CD spectroscopy at 278 nm using a heating rate of 1 K/min. Measurements were carried out in 50 m*M* sodium phosphate buffer (pH 8.0) containing protein (1 mg/mL) and NaCl (25 m*M*). The signal was fitted by nonlinear regression as described previously.[20,34] The enthalpy and the entropy at T_m (ΔH_m and ΔS_m, respectively) were determined with the van't Hoff equation.

Gdn–HCl-induced transition

Gdn–HCl-induced transition curves of $\beta^2\beta^3$hAla RNase A and the wild-type enzyme were obtained by CD spectroscopy at 278 nm (signal was averaged over 30 s). Measurements were carried out at 25°C in 50 m*M* Tris–HCl buffer (pH 8.0) containing protein (0.5 mg/mL), NaCl (100 m*M*), and Gdn–HCl (0.0–6.0*M*). To calculate values of $[D]_{50\%}$, which is the concentration of denaturant at which 50% of the protein is unfolded, the signals were fitted by nonlinear regression according to as described previously.[35,36]

Unfolding rate constants

Values of k_U for $\beta^2\beta^3$hAla RNase A and the wild-type enzyme were determined by proteolysis with thermolysin as described previously.[18,37] In a typical experiment, to 160 μL 50 m*M* Tris–HCl buffer (pH 8.0), which were preincubated in a Lauda thermostat (accuracy: ±0.1°C), was added 20 μL of thermolysin (2 mg/mL in 50 m*M* Tris–HCl buffer (pH 8.0) containing 10 m*M* $CaCl_2$). The reaction was started by the addition of 20 μL of RNase A or $\beta^2\beta^3$hAla RNase A (1 mg/mL). At known time intervals, 25-μL aliquots were removed and mixed rapidly with 8 μL of 50 m*M* EDTA. SDS–PAGE and densitometric evaluation of the RNase A bands were carried out as described previously.[18,20] The rate constants of proteolysis were determined from the decrease in the peak areas of the intact RNase A band, which followed a first-order reaction. Under the conditions applied, these values correspond to the respective unfolding rate constant, k_U. The enthalpy of activation $\Delta H^{\ddagger}$ and the entropy of activation $\Delta S^{\ddagger}$ as well as the free energy of activation for the unfolding reaction $\Delta\Delta G_U^{\ddagger}$ were determined with the Eyring equation.

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