DERIVATIVES OF BACILLUS AMYLOLIQUEFACIENS RIBONUCLEASE (BARNASE) ISOLATED AFTER LIMITED DIGESTION BY CARBOXYPEPTIDASES A AND B

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SUMMARY: The carboxy-terminal sequence of barnase is -Thr-Thr-Asp-His-Tyr-Gln-Thr-Phe-Thr-Lys-Ile-Arg. Derivatives missing one, three, five and eight amino acids (CX-1, CX-3, CX-5 and CX-8)[1] have been produced by digestion with carboxypeptidases A and B, isolated and characterized. CX-1 retains full activity, CX-3 substantial activity, and CX-5 and CX-8 little or no activity. All undergo reversible thermal transitions qualitatively similar to that of native barnase, but with transition temperatures a decreasing function of the number of amino acids removed. Spectral analysis indicates some changes in the contact of aromatic residues with solvent. All react normally with anti-barnase antibody, but only CX-1 and CX-3 form normal complexes with the natural barnase inhibitor. These complexes and that of native barnase do not precipitate antibody.

Carboxypeptidases A and B have been used to obtain four derivatives, each missing a different number of amino acids from the carboxyl terminus of barnase. In the process of obtaining and characterizing the derivatives, two tryptic peptides, T8 and T5 (Barker, E. A., and Hartley, R. W., unpublished data) have been identified as the carboxyl terminus and its nearest neighbor, establishing the sequence of 12 amino acids at the carboxyl end. The inhibitor (Smeaton et al. 1965) referred to herein is a small protein which reacts one-to-one with barnase to yield a soluble complex which is devoid of ribonuclease activity. That formation of the complex does not involve covalent linkage or changes in the primary structure of either protein is suggested by the fact that they can be separated in denaturing solvents, and both activities recovered (Smeaton, J. R., and Hartley, R. W., unpublished data).

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[1] For the purpose of this report the label CX-n will refer to the derivative with n amino acids removed from the carboxyl terminus.

MATERIAL AND METHODS. The barnase preparation was the same as that used previously (Hartley, 1968, 1969). Diisopropyl phosphorofluoridate-treated carboxypeptidases A and B were obtained from Worthington Biochemical Corporation. Contaminating amino acids were removed by extraction with water and by chromatography on Sephadex G-25 for carboxypeptidases A and B, respectively.

For the initial digestion, 0.5 ml barnase stock solution (3.5 mg/ml in 0.03 M NH_4HCO_3) was diluted and aliquots of carboxypeptidases A and/or B were added. After incubation, one sixth of each was lyophilized (after addition of 0.05 ml 1.0 M $NaHCO_3$) and analyzed directly on a Beckman Model 120C Analyzer, using the regular short column procedure for basic amino acid and the lithium citrate-long column system for acidics and neutrals, in order to resolve the amides. The remainder of each incubation sample was diluted to 200 ml, titrated to pH 5.5 - 6.0 with acetic acid, and applied to a small (1.0 cm x 8.0 cm) column of CM-cellulose(Whatman CM 32) equilibrated with 0.005 M sodium acetate, pH 6.0. The column was re-equilibrated with several hundred ml of the same buffer and eluted with a linear gradient of the same salt. Several larger scale preparations were done in a similar manner, except that the portions used for isolating derivatives were lyophilized to remove the NH_4HCO_3 instead of being simply diluted. In order to recover all of the material in solution, they were dissolved first in a small amount of 50% acetic acid and then diluted and titrated to pH 5.0 for application to the column. Amino acid analysis of carboxypeptidase digests of the isolated derivatives was done in the same manner as those on the initial digestions. Hydrazinolysis was performed essentially as described by Fraenkel-Conrat (1967). Enzyme assays were as reported previously (Rushizky et al., 1963), involving production of acid-soluble oligoribonucleotides from RNA, except that incubations were for 15 minutes at 40° or 20 hours at 10°, as noted.

The thermal transitions of material from the different chromatographic peaks were observed as changes in absorbance at 286 mu (Hartley, 1968). All were adjusted to a concentration of about 0.1 mg/ml (A_{280} = 0.2) in 0.2 M sodium acetate (pH 6.0) by appropriate dilutions.

TABLE I: Hydrolysis Protocols and Products.

No.	Solvent (added to 0.5 ml, 3.5 mg/ml barnase in 0.03 M NH_4HCO_3)	Carboxypeptidase (A and/or B) additions and incubations	Hydrolysis Products: Amino Acids[a]	Hydrolysis Products: Protein Derivatives
1	3.0 ml, 0.1 M NH_4HCO_3, pH 8.6	A, 0.8 mg/ml, 0.06 ml, 3 hr at 52°	None	Native barnase
2	3.0 ml, 0.1 M NH_4HCO_3, pH 8.6	B, 0.08 mg/ml, 0.03 ml, 2 hr at 52°	Arg (1.00)	CX-1
3	3.0 ml, 0.1 M NH_4HCO_3, pH 8.6	B, 0.08 mg/ml, 0.03 ml, 1 hr at 52°, followed by A, 0.8 mg/ml, 0.03 ml, 1 hr at 52°	Arg (1.00), Ile(0.96), Lys (1.04) Thr (0.41), Phe (0.37)	CX-3 and CX-5
4	12.5 ml, 0.1 M NH_4 acetate, pH 9.0	B, 1.0 mg/ml, 0.05 ml, 1 hr at 52° followed by A, 10 mg/ml, 0.025 ml, 1 hr at 52° followed by A, 10 mg/ml, 0.025 ml, 3 hr at 45°	Arg (1.00), Ile (0.91), Lys (1.09) Thr (2.06), Phe (1.09), Gln (0.88) Tyr (0.92)	CX-8

[a] Numbers in parentheses are molar amounts relative to the C-terminal amino acid Arg, which is released by carboxypeptidase B at about 1 mole per 13,000 g barnase.

Rabbit anti-barnase anti-serum was that used by Lees and Hartley (1966). Agar double-diffusion plates contained 0.1 M sodium barbital pH 8. The natural intracellular inhibitor was obtained from the cells of the bacteria used to produce the extracellular barnase. Extraction and purification procedures will be detailed in a later paper. It was by no means pure, but was free of interfering activities (e.g., protease and ribonuclease).

RESULTS. Protocols of the carboxypeptidase digestions and the products obtained are given in Table I. Several chromatograms of the digests are shown in Figure (1). CX-8, the limit product given by hydrolysis #4, Table I, chromatographs in the same position as CX-5. (Peak 1 in Figure 1). In Table II are shown the amino acids released from the various derivatives by carboxypeptidase treatment equivalent to #4 of Table I (appropriately scaled). Barnase treated only with carboxypeptidase A (#1, Table I) was carried through the entire battery and differed in no way from native barnase.

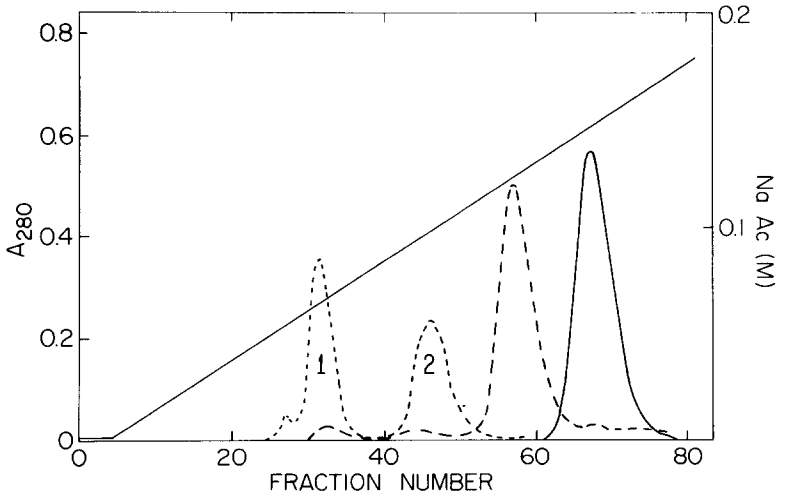

Fig. 1. Chromatograms of native and carboxypeptidase treated barnase. Solid line, native barnase: dashed line, hydrolysis #2 (CX-1), dotted line, hydrolysis #3 (peak 1 is CX-5 and peak 2 is CX-3). Column: 1 cm x 8 cm, Whatman CM-32 CM-cellulose. 100 ml linear gradient, 0.005 to 0.2 M sodium acetate, pH 6.0 (as shown). Fraction volume approximately 1.0 ml.

From these data alone, the C-terminal sequence can be written:

-(Tyr,Gln,Thr) - (Phe,Thr) - (Lys-Ile) - Arg

TABLE II: Limit Carboxypeptidase A and B Digests of Derivatives.

Substrate	Amino Acids Released[a] (Hydrolyses as #4, Table I)						
	Arg	Ile	Lys	Thr	Phe	Gln	Tyr
Barnase	1.09	0.99	1.18	2.24	1.18	0.96	1.00
CX-0, presumably native barnase, but reisolated after hydrolyses #1, Table I	1.06	1.07	1.09	2.18	1.06	0.94	1.00
CX-1	0	0.91	1.00	1.97	1.08	0.98	1.00
CX-3 (peak 2, Fig. 1)	0	0	0	2.20	1.02	0.96	1.00
CX-5 (peak 1, Fig. 1)	0	0	0	0.91	0	0.93	1.00
CX-8	0	0	0	0	0	0	0

[a] Molar amounts relative to Tyr, one residue of which is released per mole of barnase and of all derivatives except CX-8

Actually a range of carboxypeptidase A digestions has been carried out which clearly place the Ile between the Lys and Arg, and the Tyr and Gln to the left of the second Thr. Furthermore, brief carboxypeptidase A digestion of CX-1 yields only isoleucine and a trace of lysine. Thus we have

-(Tyr,Gln) - Thr - (Phe,Thr) - Lys-Ile-Arg.

In the course of concurrent work on the sequencing of barnase (Barker, E. A., and Hartley, unpublished data), a number of tryptic peptides have been fully sequenced, including two which can clearly be identified as contributing to this C-terminal sequence:

T5, Thr-Thr-Asp-His-Tyr-Gln-Thr-Phe-Thr-Lys and

T8, Ile-Arg.

The C-terminal sequence data is summarized in Fig. 2.

Hydrazinolysis of the derivative CX-8 has confirmed the new C-terminal histidine, but this residue has in no case been observed after carboxypeptidase digestions.

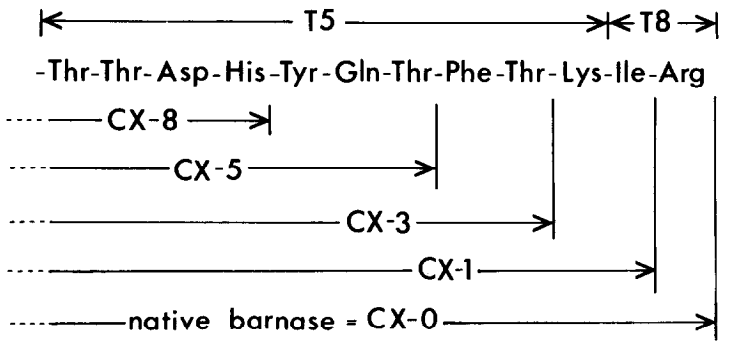

Fig. 2. The carboxy-terminal dodecapeptide of barnase. T5 and T8 are tryptic peptides. Also illustrated is the nomenclature of the four well-defined carboxypeptidase derivatives.

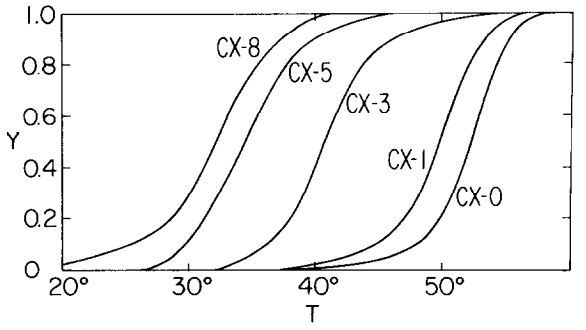

Fig. 3. Transition curves of barnase (CX-0) and derivatives. Ordinate (y) is fractional change in A_{286}. 0.1 mg/ml in 0.2 M sodium acetate pH 6.0

In Figure 3 are shown the thermal transition curves of the various derivatives. Transition termperatures, and the magnitudes of the spectral changes at 286 and 291 mu are shown in Table III. Since independent determinations of molar concentration were not possible, these changes (ΔOD_{286} and ΔOD_{291}) are shown relative to absorbance at 276 mu, which is nearly constant through the transition for all derivatives. All of the transitions were reversible, with complete recovery of the low temperature spectrum on cooling (all, of course, had been through the unfolding procedure during carboxypeptidase digestion). Also shown in Table III are the ribonuclease activities (relative to native barnase) as measured at two temperatures.

TABLE III: Other Properties of the Derivatives.

Derivatives	T_m	$\frac{\Delta A_{286}}{A_{278}}^{60°}$	$\frac{\Delta A_{291}}{A_{278}}^{60°}$	Activity(relative to barnase) 40°	10°
Barnase	52.3°	0.21	0.18	1.00	1.00
CX-1	49.6°	0.21	0.18	0.95	1.08
CX-3	40.7°	0.20	0.17	0.07	0.40
CX-5	34.5°	0.15	0.13	<0.01	<0.01
CX-8	32.5°	0.13	0.12	<0.01	<0.01

On Ouchterlony double diffusion against rabbit anti-barnase serum each of the derivatives gave a single precipitin line which fused with that of the native enzyme in a "reaction of identity". When the native enzyme and each of the derivatives was preincubated with an excess of the natural inhibitor, however, precipitin lines were obtained only with CX-5 and CX-8. Those derivatives which had ribonuclease activity (CX-1 and CX-3) were completely inhibited by excess inhibitor. Preincubation of inhibitor with an excess of CX-8 did not prevent the inhibition of subsequently added native barnase.

DISCUSSION. Barnase derivatives of the sort reported here should be of value in assessing the roles of specific amino acids in a variety of functions. Since the unfolding of this protein by heat or denaturing reagents appears to be both extensive and reversible (Hartley 1968), thermodynamic analysis of the transition for the various derivatives may yield real information as to the contribution of specific residues to the stability of the native conformation. The melting data reported here are not sufficiently precise for such treatment, nor should such be attempted until the state of purity of each derivative is better established.

It seems reasonable to assume that the C-terminal octapeptide is draped along the surface of the molecule, with its more hydrophobic side chains directed inwards and contributing towards the stability of the whole. Removal of three

amino acids does not uncover any of the buried tyrosine or tryptophan residues. This is clear from the ΔA values of Table III. Removal of the next two amino acids and to a lesser extent of the following three, does allow access of solvent to several of these buried residues. It is not established whether the tyrosine missing from CX-8 is a normal or a buried residue, but the former seems more likely.

Table III also makes it clear that the active site is intact in CX-1, reasonably so (at low temperature) in CX-3,but disassembled or missing in CX-5 and CX-8. Barnase is not significantly stabilized by substrate and is inactive above its transition temperature (Hartley, 1968), so that the effect of temperature on the activity of CX-3 was to be expected.

Under ordinary conditions the one-to-one enzyme inhibitor reaction is essentially irreversible, although covalent bonds are not involved. An immediate question is whether the inhibitor actually covers the active site or, binding elsewhere, induces an inactivating change in the conformation. The present evidence that the inhibitor reacts normally with the active CX-1 and CX-3, but not with the inactive CX-5 and CX-8 would tend to support the former alternative. The competition of the inhibitor with antibody for binding to barnase and the first two derivatives probably reflects the limited number of antigenic sites on barnase.

Whether the inactive derivatives have a greatly reduced substrate affinity or have lost key elements involved in the catalysis has not been determined, nor can it be said that these derivatives do not bind the inhibitor to some lesser extent than does the native enzyme.

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