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SITES OF MODIFICATION OF HUMAN ANGIOGENIN BY BROMOACETATE AT pH 5.5

Robert Shapiro, Daniel J. Strydom, Stanislawa Weremowicz, and Bert L. Vallee

Center for Biochemical and Biophysical Sciences and Medicine and Department of Pathology, Harvard Medical School, Boston, MA 02115

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Summary: Human angiogenin is inactivated by treatment with bromoacetate at pH 5.5. Use of [^{14}C]bromoacetate and tryptic peptide mapping have identified the sites of carboxymethylation as His-13 and His-114, with His-114 reacting ~1.5-fold more rapidly than His-13. At later stages in the reaction, both His-13 and -114 become modified with His-114 in part forming a bis derivative. Comparison with carboxymethylhistidine derivatives of known structure obtained from bovine pancreatic ribonuclease A indicates that the reaction order is N-1 of His-114 > N-3 of His-13 > N-3 of His-114.

Human angiogenin, a potent inducer of neovascularization, is a single-chain polypeptide of molecular mass 14,124 (1). Its amino acid sequence displays extensive similarity to that of the pancreatic RNases (2,3) and includes their three major active site residues, His-12, Lys-41, and His-119 (bovine RNase A numbering). Recognition of this homology led to the finding that angiogenin is a ribonucleolytic enzyme, albeit with a characteristic activity differing markedly from that of the digestive RNases (4,5). Chemical modification of angiogenin, based on methods previously applied to bovine pancreatic RNase A, subsequently revealed that histidine, lysine, and arginine residues are critical for enzymatic activity and suggested the involvement of histidines in angiogenic activity (4,6). Identification of essential lysines and arginines by chemical modification techniques is made difficult by the lack of

Abbreviations: RNase(s), ribonuclease(s), 3-CM-His, N-3 carboxymethylhistidine; 1-CM-His, N-1 carboxymethylhistidine; HPLC, high performance liquid chromatography; PTC, phenylthiocarbamoyl.

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specificity of these reactions; in both cases, inactivation is accompanied by modification of several residues. In contrast, carboxymethylation of only 1.5 histidines decreases ribonucleolytic activity by 95% (6). In the present report, we identify His-13 and -114 (corresponding to His-12 and -119 of RNase A) as the residues modified and N-3 carboxymethylhistidine (3-CM-His) and N-1 carboxymethylhistidine (1-CM-His), respectively, as the primary derivatives formed. Although the sites of modification and the adducts generated are the same as with RNase A (7,8), the relative reaction rates differ significantly, and the preference for His-114 over His-13 in angiogenin is much smaller than that found for the corresponding residues in RNase.

MATERIALS AND METHODS

Angiogenin was isolated from human plasma (9). Bovine pancreatic RNase A was obtained from Cooper Biomedical. Carboxymethylation of angiogenin and RNase A (20-30 μM) was performed in the dark using 30 mM bromoacetate in 100 mM sodium acetate at pH 5.5, 27°C. Reaction mixtures (0.3-1.0 mL) contained 80-250 μCi bromo[1-^{14}C]acetic acid (Amersham). Reactions were terminated and unincorporated reagent removed by passage through a C18 HPLC column (2). Aliquots of fractions eluting from this column were taken for amino acid analysis and for ^{14}C measurements by scintillation counting. Amino acid analyses were performed by the method of Bidlingmeyer et al. (10) using a 3.9 x 150 mm PICO·TAG C18 column and a Waters Associates HPLC system. In most cases, a 10-min gradient (curve 5, convex) from 10% to 49% solvent B was employed at 39°C, where solvent A was 0.14 M sodium acetate, pH 6.4, containing 0.06% triethylamine and solvent B was 60% acetonitrile. Eluates were monitored at 254 nm. In this method, amino acids are detected as their phenylthiocarbamoyl (PTC) derivatives. In some cases, noted in the text, a modified HPLC gradient of 5% B to 52% B in 10 min was employed. For determination of radiolabelled amino acids, 0.2 min fractions were collected and counted. Digestion with trypsin, peptide mapping on a C3 HPLC column, and N-terminal amino acid sequencing were performed as described (2). For angiogenin tryptic peptide nomenclature (T-7, T-11) see Strydom et al. (2). Measurements of enzymatic activity of angiogenin and RNase A were performed using tRNA as substrate (6).

RESULTS AND DISCUSSION

Identification of Sites of Carboxymethylation of Angiogenin. Treatment of angiogenin with [^{14}C]bromoacetate for 15.5 h at pH 5.5 decreases its enzymatic activity by 95%, and results in the incorporation of 1.6 carboxymethyl groups. C3 HPLC of a tryptic digest of the modified protein reveals four major peaks of radioactivity (Fig. 1). Material eluting in peak A (column breakthrough), containing 11% of the total radioactivity, was rechromatographed on a C18 HPLC column. Radioactivity eluting

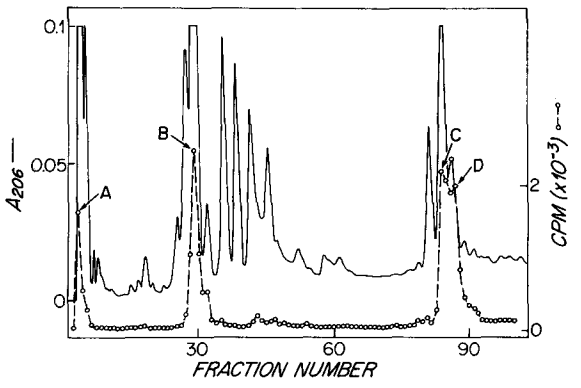

Figure 1: C3 HPLC elution pattern of radioactive tryptic peptides from angiogenin treated with [^{14}C]bromoacetate. HPLC was performed as described previously (2). Half-minute fractions were collected and 50 μL aliquots were counted.

from this column did not cochromatograph with any 206 nm absorbing material, suggesting that it is not peptide-associated.

Amino acid analysis of material in peak B from the C3 column (Table 1), containing 27% of the total counts, indicates that it derives from peptide T-7 (amino acids 6-21). The three fractions analyzed - 28, 29, and 30 - show a loss of ~0.2-0.6 histidine together with the appearance of a new peak at 2.68 min, between PTC-Glu (2.07 min) and PTC-Ser (3.78 min). Assuming that the new derivative has the same molar absorptivity as PTC-His,

Table 1. Amino Acid Compositions of Radiolabeled Tryptic Peptides of Bromoacetate-Modified Angiogenin

Amino Acid	Peak B	T-7	Peak C	T-11	Peak D
Asx	1.88	1	3.90	4	3.89
Glx	2.00	2	2.92	3	2.78
Ser	0.17		1.03	1	1.39
Gly	1.24	1	1.16	1	1.75
His	1.67	2	0.10	1	0.13
Arg	1.05	1	1.06	1	1.07
Thr	2.14	2			0.24
Ala	0.95	1	2.02	2	2.02
Pro	1.10	1	1.31	1	1.47
Tyr	2.00	2			
Val			3.03	4	2.88
Met					
Ile	0.57		2.00	2	1.81
Leu	1.01	1	2.24	2	2.29
Phe	1.25	1	1.01	1	0.99
Lys	0.98	1	0.97	1	0.96
1-CM-His			0.85		0.24
3-CM-His	0.50				
pmol analyzed	93		58		27

Peaks B, C, and D represent fractions 29, 84, and 87, respectively, from the peptide map in Fig. 1. 1-CM-His and 3-CM-His are amino acids whose PTC adducts elute at 2.36 min and 2.68 min, respectively. These identifications are based on a comparison with derivatives obtained from RNase A as discussed in the text.

Table 2. Release of ^{14}C During Edman Degradation of CM-Peptide from Tryptic Digest

Step:	1	2	3	4	5	6	7	8
^{14}C, cpm:	22	33	47	59	38	11	50	3125

One nmol of peptide from fraction 29 (Fig. 1) was sequenced.

0.25, 0.50, and 0.45 residue are present in fractions 28, 29, and 30, respectively. This is in reasonable agreement with the stoichiometries of radioactivity incorporation for the three fractions: 0.3, 0.6, and 0.6, respectively.

Angiogenin peptide T-7 contains two histidines, His-8 and His-13. In order to establish which histidine was carboxymethylated, the modified peptide (fraction 29) was subjected to Edman degradation. Table 2 shows that there was no significant release of ^{14}C until cycle 8, indicating that carboxymethylation occurred exclusively at His-13.

Radioactive peaks C and D (~25% and 21% of the total counts, respectively), elute slightly after peptide T-11 and are not well resolved. Amino acid analysis (Table 1) reveals that both peaks represent derivatives of peptide T-11, containing residues 55-60 and 102-121 in disulfide linkage. In both cases the histidine content is decreased by about 0.9 residue. Based on the 206 nm absorbance peak area of each, unmodified T-11, peak C, and peak D are present in a ratio of 1:2.3:0.9. The stoichiometry of ^{14}C incorporation is 0.8 for peak C and 1.7 for peak D. Amino acid analysis shows that both peaks yield a new absorbance peak eluting at 2.36 min, different from that seen for peak B. Assuming that this derivative has the same molar absorptivity as PTC-His, 0.8-0.9 residue is present for peak C and ~0.2 for peak D.

Radioactivity measurements were performed on amino acid analyzer eluates from peak C (fraction 84) and peak D (fraction 87). With the former, virtually all of the radioactivity coelutes with the new absorbance peak at 2.36 min. With peak D, 19% of the radioactivity elutes at this same time, but the remainder elutes somewhat earlier together with PTC-Asp.

Angiogenin peptide T-11 contains only a single histidine, at position 114. Thus, the peptides in peaks C and D must both be modified at this site. The stoichiometries of radioactivity incorporation suggest that for peak C, His-114 has been monocarboxymethylated while for peak D both imidazole nitrogens have been carboxymethylated.

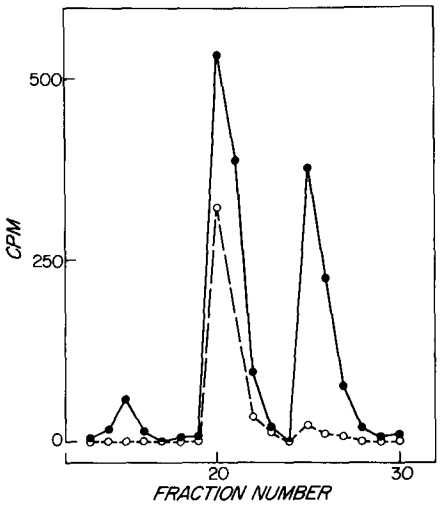

Figure 2: C18 HPLC elution patterns of radioactive PTC-amino acids from RNase A (dotted line) and angiogenin (solid line) treated with [^{14}C]bromoacetate. Modifications and chromatography were performed as described in the text. Fractions (0.2 mL) were collected and counted in their entirety.

Identification of Histidine Derivatives Formed During Reaction of Angiogenin with Bromoacetate. The above results indicate that a bis-CM derivative and two distinct mono-CM derivatives of histidine are formed during the reaction of angiogenin with bromoacetate. The structures of the two mono-CM adducts were determined by chromatographic comparison with derivatives of known structure obtained from RNase A. RNase A was modified with [^{14}C]bromoacetate under conditions (30 mM bromoacetate, 48 min., 27°C) expected to yield an ~8:1 ratio of 1-CM-His-119 to 3-CM-His-12 (8). To facilitate kinetic comparisons of the two proteins, angiogenin was treated under conditions (as with RNase, but for 4 h) producing a similar degree of inactivation (65%). After removal of free [^{14}C]bromoacetate by C18 HPLC, both proteins were hydrolyzed and subjected to amino acid analysis using a modified HPLC gradient (see Materials and Methods) that gives improved separation of the CM-His derivatives. Radioactivity measurements performed on HPLC effluents show two peaks for RNase and three for angiogenin (Fig. 2). The predominant peak from RNase A elutes in fractions 20-21 and should represent the PTC adduct of 1-CM-His. A peak containing ~7-fold less ^{14}C elutes in fractions 25-27 and should represent the PTC adduct of 3-CM-His. Both peaks are generated from the angiogenin sample, although in this case the earlier peak contains only a 1.5-fold larger amount of ^{14}C than the later

one. Based on results described in the previous section, the earlier peak derives from His-114 while the later is from His-13. The additional peak of radioactivity eluting in fractions 14-15 contains only ~4% of the total radioactivity and probably represents bis-CM-His.

In conclusion, inactivation of angiogenin by bromoacetate is associated with carboxymethylation of His-13 and His-114. At times relatively early in the reaction (4 h), modification occurs almost exclusively at N-3 of His-13 and N-1 of His-114. The ratio of the two adducts formed is 1:1.5, in contrast to the 1:8 ratio observed with RNase A. We have previously reported that angiogenin is inactivated by bromoacetate 5-fold more slowly than is RNase A (6). The present findings indicate that this decreased rate reflects largely a slower reaction at His-114 vs. His-119 of RNase A, with little difference in the reaction rates at His-13 of angiogenin vs. His-12 of RNase.

At somewhat later stages in the reaction of bromoacetate with angiogenin, His-114 becomes doubly modified; by 15.5 h, 28% of the modified His-114 residues are biscarboxymethylated, comparable with results reported for RNase A (11). Also, at this time in the reaction, it becomes apparent that both His-13 and His-114 of the same angiogenin molecule are carboxymethylated, since 45% of His-13 and 76% of His-114 have been modified. While it was originally thought that carboxymethylation at the two sites in RNase A were mutually exclusive (8), it was later shown that this is not the case (12).

Finally, it should be noted that the degree of angiogenin inactivation observed is greater than the extent of modification of either His-13 or His-114 separately. Therefore both residues must be important for activity.

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