Structural Requirements for Binding of Bradykinin to Antibody. II. Studies with Bradykinin Fragments*

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ABSTRACT: Antibody to bradykinin, elicited in rabbits by the injection of a branch-chain copolymer of the peptide and poly-L-lysine, has been used together with intrinsically labeled [^{14}C]bradykinin to examine the binding of singly substituted alanine analogs of bradykinin and bradykinin fragments. Earlier results concerning the identity of the antigenically important residues of bradykinin were confirmed. Alteration of charge or hydrophobic character had relatively little effect on binding to antibody, while substitution in residues which had an obligatory effect on conformation yielded the most profound change in binding.

It was apparent from the study of bradykinin fragments, particularly those incorporating the antigenically important regions of the molecule, that fragments of the sequence do not act as antigenic determinants. These observations led to the conclusion that the antibody recognized the peptide in a preferred conformation which required the entire nonapeptide sequence.

The production of antibody directed specifically against the nonapeptide, bradykinin, and the availability of many sequence variants of this peptide have permitted an examination of the capacity of the variants to bind with antibody directed against the native molecule. This has allowed for definition of the structural requirements of this peptide antigen of defined sequence. It was apparent from the first report in this series (Spragg *et al.*, 1967) that changes in amino acid side chains which altered charge or hydrophobic character had little effect on binding to antibody. Removal of one amino acid residue was of greater importance. Alterations in residues which had an obligatory effect on conformation, such as changes in glycine or proline, exhibited the most profound effect.

This previous report on the structural requirements of bradykinin analogs for binding to antibradykinin antibody left unanswered two important questions. The first of these concerned the use of an extrinsically labeled kinin, [^{3}H]acetyl-*N*-bradykinin, in the radioimmunoassay; bradykinin and its analogs were competing for antibody binding sites not with the native molecule, but with a bradykinin analog. In the present studies, intrinsically labeled bradykinin was used in the assay, permitting direct competition between native bradykinin and bradykinin analogs for antibody binding sites. The data presented employing singly substituted alanine analogs of bradykinin confirm earlier results concerning the antigenically important residues of bradykinin.

The second unanswered question concerned the size of the antigenic site. Although the Des-[Arg^{9}]-bradykinin octapeptide was poorly bound by the antibradykinin antibody, the possibility existed that smaller bradykinin fragments, particularly those incorporating the residues which had an obligatory effect on conformation, might be well recognized by the antibody. Studies herein on the capacity of bradykinin fragments to compete with intrinsically labeled bradykinin for binding to antibradykinin antibody again reveal the importance of the over-all nonapeptide conformation.

Methods

Preparation of Antibradykinin Antibody. The preparation of the immunogen used to elicit antibradykinin antibody, the immunization of rabbits, and the demonstration of specificity of the antibody have been reported (Spragg *et al.*, 1966). In the present study, antiserum from a single bleeding from one rabbit was used. This antibody was not purified because of its low serum concentration (7.2 μg/ml) determined by the method previously described (Talamo *et al.*, 1968).

Isotopically Labeled Bradykinin. [^{14}C]Bradykinin, made available by the National Heart Institute, was prepared by New England Nuclear Corp. according to the Merrifield synthesis (Merrifield, 1964) and incorporating [^{14}C]proline in positions 2 and 3. The specific activity was 250 mCi/mmole. A stock solution with a bradykinin concentration of 280 μg/ml in 0.1 N acetic acid was kept at −70°. The working solution, also stored at −70°, consisted of 10 μl of stock solution diluted to 1.5 ml with 0.1 N acetic acid; this solution had a bradykinin concentration of 18.7 mμg/10 μl, the amount used in each incubation in the radioimmunoassay. Under these storage conditions, the isotope re-

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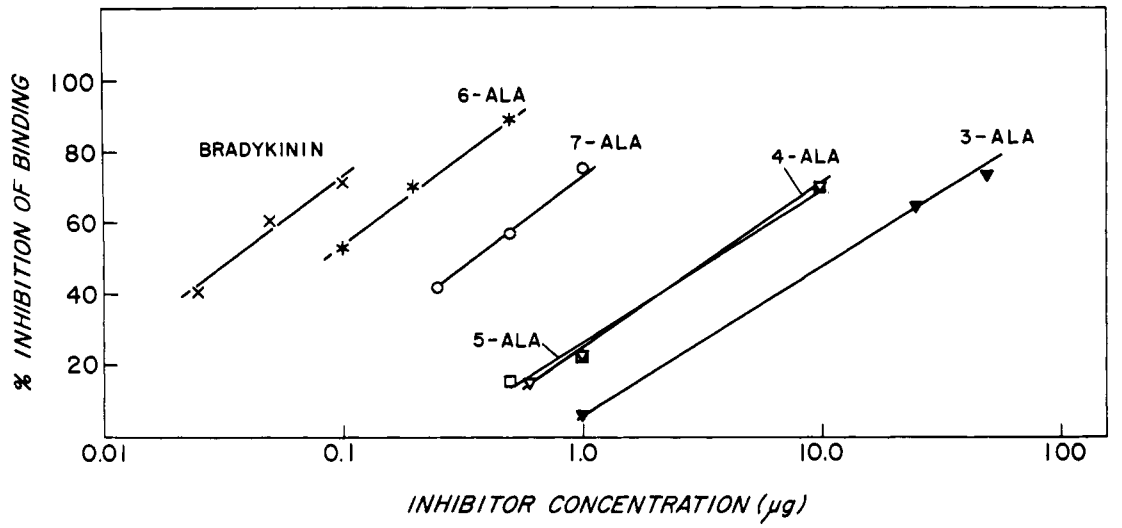

FIGURE 1: Binding inhibition produced by a series of singly substituted alanine analogs of bradykinin. The inhibition produced by native bradykinin is shown for comparison.

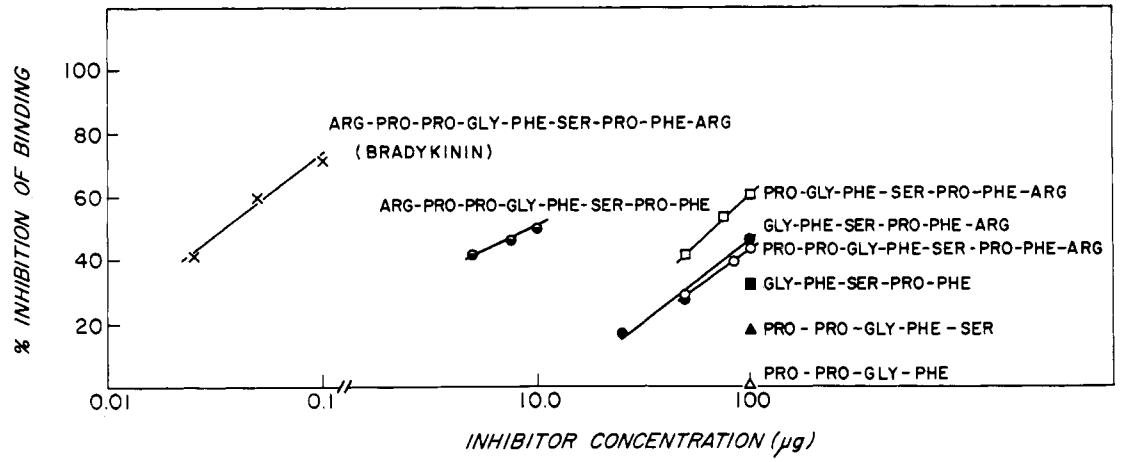

FIGURE 2: Binding inhibition produced by several synthetic fragments of bradykinin. The inhibition produced by native bradykinin is shown for comparison.

mained stable for at least 1 year as assessed by radioimmunoassay.

Bradykinin Analogs. Singly substituted alanine analogs of bradykinin were prepared by classical methods (Schröder and Lübke, 1966) and are a gift from Dr. E. Schröder. All weights given are acetate salt weights; correction to free peptide weight does not significantly alter the binding data.

Bradykinin Fragments. Bradykinin fragments were synthesized and analyzed by Suzuki *et al.* (1966) using methods identical with those previously described.

Measurement of Radioactive Binding by Antibradykinin Antibody. Working solution (10 μl) (18.7 mμg of [^{14}C]bradykinin) was incubated for 7.5 min at 4° with 50 μl of antiserum in a total volume adjusted to 160 μl by the addition of 0.1 M Tris acetate (pH 7.5). After the incubation period, the mixture was diluted to 1.0 ml with Tris acetate and applied to a Sephadex G-25 fine bead column (1.8 × 70 cm) which had been equilibrated with 0.1 M Tris acetate (pH 7.5) (Haber *et al.*, 1965). The column was developed at 4° with the same buffer at a flow rate of 1.8 ml/min. The radioactivity in the excluded volume protein peak, which contained antibody-bound material, was determined in a Nuclear-Chicago liquid scintillation system using Bray's (1960) solution.

Quantification of Analog and Fragment Binding by Antibradykinin Antibody. Varying known concentrations of unlabeled bradykinin, bradykinin analogs, or bradykinin fragments were incubated with 18.7 mμg of [^{14}C]bradykinin and 50 μl of antiserum in a total volume of 160 μl. After 7.5 min, the mixture was diluted to 1.0 ml, applied to Sephadex, eluted, and counted as above.

The per cent inhibition of binding produced by each concentration of unlabeled bradykinin, bradykinin analogs, or bradykinin fragments was calculated in the following manner

$$\% \text{ inhibition of binding} = \frac{(\text{cpm bound without analog} - \text{cpm with analog}}{\text{cpm bound without analog}} \times 100$$

TABLE I: Antibody Binding Activity of Alanine Analogs of Bradykinin in an Assay Employing [^{3}H]Acetyl-*N*-bradykinin or [^{14}C]Bradykinin.

Peptide	1	2	3	4	5	6	7	8	9	Binding Activity: [^{3}H]Acetyl-*N*-bradykinin	Binding Activity: [^{14}C]-Bradykinin
Bradykinin	H-Arg	Pro	Pro	Gly	Phe	Ser	Pro	Phe	Arg-OH	1	1
[Ala^{9}]-Bradykinin									Ala	1.6	
[Ala^{8}]-Bradykinin								Ala		$^{1}/_{2}$	
[Ala^{7}]-Bradykinin							Ala			$^{1}/_{4}$	$^{1}/_{10}$
[Ala^{6}]-Bradykinin						Ala				$^{1}/_{1.4}$	$^{1}/_{2.4}$
[Ala^{5}]-Bradykinin					Ala					$^{1}/_{44}$	$^{1}/_{37}$
[Ala^{4}]-Bradykinin				Ala						$^{1}/_{57}$	$^{1}/_{38}$
[Ala^{3}]-Bradykinin			Ala							$^{1}/_{270}$	$^{1}/_{189}$
[Ala^{2}]-Bradykinin		Ala								$^{1}/_{14}$	

TABLE II: Antibody Binding Activity of Bradykinin Fragments.

1 2 3 4 5 6 7 8 9	Binding Act.
H-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-OH	1
H-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-OH	$^{1}/_{260}$
H-Pro-Pro-Gly-Phe-OH	$<^{1}/_{28,000}$
H-Pro-Pro-Gly-Phe-Ser-OH	$<^{1}/_{6,900}$
H-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-OH	$^{1}/_{3,700}$
H-Pro-Gly-Phe-Ser-Pro-Phe-Arg-OH	$^{1}/_{1,900}$
H-Gly-Phe-Ser-Pro-Phe-OH	$<^{1}/_{5,700}$
H-Gly-Phe-Ser-Pro-Phe-Arg-OH	$^{1}/_{3,400}$

The per cent inhibition of binding was plotted against the inhibitor concentration, and the 50% inhibitory concentration was determined graphically.

Each analog was compared with native, unlabeled bradykinin in its capacity to compete with [^{14}C]bradykinin for antibody binding sites. This was expressed in the following manner: binding activity = 50% inhibitory concentration of bradykinin/50% inhibitory concentration of analog.

In the case of bradykinin fragments which poorly inhibited radioactive binding by the antibody, inhibitory concentrations 5300 times the concentration of labeled native bradykinin were tested.

Results

Inhibition of Binding of [^{14}C]Bradykinin by Bradykinin Analogs. Singly substituted alanine analogs of bradykinin were examined for their ability to compete with [^{14}C]bradykinin for binding sites on the antibradykinin antibody. The resulting inhibition plots are shown in Figure 1 and are summarized in Table I.

Inhibition of Binding of [^{14}C]Bradykinin by Bradykinin Fragments. Seven synthetic fragments of bradykinin were examined for their ability to compete with [^{14}C]-bradykinin for binding sites on the antibody. These fragments included the octapeptides with either the amino- or carboxy-terminal amino acid removed, a tetrapeptide and a pentapeptide incorporating the antigenically important proline–glycine region of the molecule, a hexa- and a heptapeptide representing the carboxy-terminal region of bradykinin, and an additional internal pentapeptide. The resulting inhibition plots are shown in Figure 2. For poor inhibitors, only the per cent inhibition of binding produced by 100 μg of the fragment (a 5300-fold excess) is plotted. These results and the fragment structures are contained in Table II.

Discussion

These studies were undertaken to answer the following questions: (1) Which residues of bradykinin are antigenically important? (2) Does the molecule exist in a unique conformation in the antibody combining site? (3) What is the extent of the antigenic region of bradykinin? Binding of peptides of altered sequence as well as fragments of the native molecule was studied employing a radioimmunoassay which measured binding of intrinsically labeled bradykinin to antibody directed against the native peptide.

Analysis of Analog Inhibition Data. Five singly substituted alanine analogs of bradykinin, [Ala^3]-, [Ala^4]-, [Ala^5]-, [Ala^6]-, and [Ala^7]-bradykinin, were examined for their ability to inhibit the binding of [^{14}C]bradykinin. The data (Figure 1) confirm the results obtained in a previous experiment employing extrinsically labeled [^{3}H]acetyl-*N*-bradykinin (Spragg *et al.*, 1967). As in the earlier report, these curves are parallel for all analogs and may be extrapolated to full inhibition, suggesting that the same antibody recognizes each of the analogs.

The inhibition data for both the extrinsically labeled [^{3}H]acetyl-*N*-bradykinin and the intrinsically labeled [^{14}C]bradykinin assay systems are summarized in Table I. Although the numerical values of the binding activities differ somewhat, the rank order is the same in both systems. Results from both assays lead to several conclusions regarding the antigenic importance of various regions of the bradykinin molecule. Analogs with altered charge [Ala^9], hydrophobic character [Ala^5 or Ala^8], or hydrophilic character [Ala^6] in general compete effectively for antibody binding sites. Alterations in bradykinin producing either greater freedom or greater restriction in conformation [Ala^2, Ala^3, Ala^4, and Ala^7] are not well recognized by the antibody. Major reductions in binding to the antibody occur when either the proline in position 3 or the glycine in position 4 is replaced by alanine, indicating that the presence of these residues is necessary for maintenance of the peptide conformation recognized by the antibody.

Analysis of Bradykinin Fragment Inhibition Data. The requirement for antigenicity of the proline in position 3 and the glycine in position 4 raised the possibility that bradykinin fragments incorporating these residues might be able to bind to antibody. Accordingly, several such fragments were examined (Table II).

Two bradykinin fragments which lack the amino-terminal arginine, but incorporate the Pro^{2}Pro^{3}Gly^4 region of the molecule, are unable to compete with intrinsically labeled bradykinin for antibody binding sites. The tetrapeptide, Pro-Pro-Gly-Phe, has $^1/_{28,000}$ the binding activity of bradykinin and the pentapeptide, Pro-Pro-Gly-Phe-Ser, has $^1/_{6900}$ the binding activity of bradykinin. The heptapeptide lacking the proline in position 2 as well as the amino-terminal arginine has a binding activity which is $^1/_{1900}$ that of bradykinin. When the proline in position 3 is also removed, the resulting hexapeptide has $^1/_{3400}$ the binding activity of bradykinin. The pentapeptide lacking the carboxy-terminal arginine, the amino-terminal arginine, and the prolines in positions 2 and 3, Gly-Phe-Ser-Pro-Phe, binds to antibradykinin antibody $^1/_{5700}$ as well as bradykinin.

As is apparent from the inhibition curves produced by these fragments (Figure 2), the most effective inhibitor of this series, the octapeptide from which the carboxy-terminal arginine has been removed, has only $^1/_{260}$ the binding activity of bradykinin. This fragment is a poorer inhibitor than the least effective nonapeptide alanine analog (Table I). The octapeptide lacking the amino-terminal arginine, also a poor inhibitor, has $^1/_{3700}$ the binding activity of native bradykinin. The possibility that the inefficacy of binding by these octapeptides is due to the loss of a positively charged guanidino group is contradicted by the observation that the nonapeptide analogs, [Arg(NO$_2$)^1-Arg(NO$_2$)^9]-bradykinin and [Ala^9]-bradykinin, both bind to the antibody somewhat better than does bradykinin (Spragg *et al.*, 1967). Reduced binding of these octapeptide fragments may be ascribed to conformational changes resulting from chain shortening.

The first report in this series (Spragg *et al.*, 1967) as well as the results detailed in Table I have shown that the proline in position 3 and the glycine in position 4 are particularly important for ability to bind to antibody. The striking inability of bradykinin fragments containing the Pro^{3}Gly^4 region to bind to antibody indicates that not only are these residues necessary, but they must also be incorporated in a nonapeptide of the proper conformation for antibody recognition.

The data presented here, resulting from the radioimmunoassay with intrinsically labeled [^{14}C]bradykinin of alanine analogs and bradykinin fragments, lend further support to the hypothesis that bradykinin, bound by antibody, exists as a structured peptide, the over-all conformation of which is of primary importance in determining antigenic identity. In addition, the marked inability of bradykinin fragments to bind to antibradykinin antibody indicates that this antibody combining site is at least as large as the entire structured nonapeptide.

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