Structural Requirements for Binding of Bradykinin to Antibody. III. The Effect of Carrier on Antibody Specificity*

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ABSTRACT: Binding of bradykinin, alanine analogs of bradykinin, and fragments of bradykinin has been examined in a radioimmunoassay employing the intrinsically labeled peptide. The antibodies used were elicited by immunization with bradykinin coupled to poly-L-lysine, bradykinin coupled to ovalbumin, or human kininogen I.

A single antiserum directed against bradykinin-polylysine appeared to be functionally homogeneous and to recognize bradykinin in a preferred conformation which required its entire nonapeptide sequence. This was indicated by the importance of the prolines in positions 2 and 3 and the glycine in position 4 and by the inability of fragments incorporating this region to bind to antibody. Two antisera against bradykinin-ovalbumin were functionally heterogeneous. They required for binding the aromatic residues in positions 5 and 8 as they exist in the proper nonapeptide sequence. An antiserum against human kininogen I was also heterogeneous and was directed primarily against the carboxyl-terminal residues of bradykinin. These results indicate that antibodies of varying specificities may be directed against a short defined amino acid sequence. The nature of the carrier of the immunogen may play a significant role in determining the homogeneity and specificity of antibody directed against bradykinin.

The availability of sequence variants of bradykinin has made possible the detailed examination of the effects of these structural alterations on binding with antibody directed against the native peptide (Spragg *et al.*, 1967). This earlier study employing antibody against bradykinin-polylysine indicated that changes in amino acid side chains which altered charge or hydrophobic character had little effect on binding to antibody. Alterations in residues which had an obligatory effect on conformation, such as changes in glycine or proline, exhibited the most profound effect. In studies utilizing fragments of bradykinin, it became apparent that removal of only one amino acid was of more profound significance for binding than alteration of any single amino acid residue, including those residues which affect conformation (Spragg *et al.*, 1968). The binding curves obtained in this study suggested that the antibody employed was relatively homogeneous.

The previous studies of structural requirements for binding of bradykinin to antibody have employed antibody elicited by bradykinin coupled *via* the amino terminus to poly-L-lysine. In the present report, data on antibody binding by bradykinin analogs and fragments are extended to antibodies elicited by two other immunogens: bradykinin coupled *via* the amino terminus to ovalbumin, and purified human kininogen I, a natural product which contains the bradykinin sequence at its carboxy terminus. The binding data indicate that in each of these three different antisera the antibodies are primarily directed against different regions of the bradykinin molecule.

Materials

Antibradykinin Antibodies. ANTIBODY DIRECTED AGAINST BRADYKININ COUPLED TO POLY-L-LYSINE (aBK-TC-PL).[1] Preparation of this immunogen, in which the α-amino group of bradykinin was coupled to ϵ-amino groups of poly-L-lysine using toluene-2,4-diisocyanate, immunization of rabbits, and demonstration of antibody specificity have been reported (Spragg *et al.*, 1966). In the present study, antiserum from a single bleeding from one rabbit was used; its titer, determined by binding to [^{14}C]bradykinin, was 7.2 μg/ml of antibody protein (Talamo *et al.*, 1968).

Antibody Directed against Bradykinin Coupled to Ovalbumin (aBK-TC-OA). Coupling of the amino terminus of bradykinin to the available side chains of ovalbumin using toluene-2,4-diisocyanate and the immunization of animals have been described (Talamo *et al.*, 1968). In the present study, antisera from a single bleeding of each of two rabbits were used. The titer was at least 22 μg ml of antibody protein for T24 (Talamo *et al.*, 1968); for T36, the titer has not been determined.

ANTIBODY DIRECTED AGAINST KININOGEN I (AKININOGEN). Human kininogen I was purified as previously described (Pierce and Webster, 1966). Antibody was elicited in sheep by immunization with kininogen emulsified in Freund's adjuvant. In this study, lyophilized antiserum from a single bleeding of one sheep was reconstituted to the original volume with distilled water and aliquots were stored at $-70°$ until use. The

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[1] Abbreviations used are: aBK-TC-PL, antibody directed against bradykinin coupled to poly-L-lysine; aBK-TC-OA, antibody directed against bradykinin coupled to ovalbumin; akininogen, antibody directed against kininogen I.

antibradykinin titer of this serum was 16.2 μg ml of antibody protein (Talamo *et al.*, 1968).

Peptides. 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 did not significantly alter the binding data.

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

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 2 μl of stock diluted to 0.3 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 remained stable for at least 1 year as assessed by radioimmunoassay.

Methods

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 solution (Bray, 1960),

Quantification of Analog and Fragment Binding by Antibradykinin Antibody. Various 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. As previously documented (Spragg *et al.*, 1967), short incubation times were required because on longer incubation, enzymatic degradation of the peptides by serum proteases would occur.

The per cent inhibition of binding produced by each concentration of unlabeled bradykinin, bradykinin analogs, or bradykinin fragments was calculated using

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

Each analog was compared with native, unlabeled bradykinin in its capacity to compete with [^{14}C]bradykinin for antibody binding sites. This was expressed as

$$\text{binding activity} = \frac{50\% \text{ inhibitory concentration of bradykinin}}{50\% \text{ inhibitory concentration of inhibitor}}$$

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 are expressed as per cent inhibition of binding produced by 100 μg of each fragment.

Results

Inhibition of Binding of [^{14}C]Bradykinin by Alanine Analogs of Bradykinin. Singly substituted alanine analogs of bradykinin were examined for their ability to compete with [^{14}C]-bradykinin for binding sites on the various antibradykinin antibodies. The resulting inhibition curves are shown in Figures 1–4. In Figure 1, published previously (Spragg *et al.*, 1968), the inhibition plots were obtained using antibody directed against bradykinin coupled to poly-L-lysine. Figures 2 and 3 represent inhibition curves obtained with antibodies elicited in two rabbits by immunization with bradykinin coupled to ovalbumin. Figure 4 contains inhibition curves obtained with sheep antihuman kininogen antibody. For each of these antibodies, the binding activities of the alanine analogs of bradykinin are shown in Table I.

Inhibition of Binding of [^{14}C]Bradykinin by Fragments of Bradykinin. Bradykinin fragments of varying lengths and regions of the native peptide were tested for their ability to inhibit binding of [^{14}C]bradykinin to the different antibradykinin antibodies. The inhibition curves for the more potent inhibitors are shown in Figure 5 (with aBK-TC-PL), Figure 6 (with aBK-TC-OA), and Figure 7 (with akininogen). The per cent inhibition of binding produced by 100 μg of each fragment tested is given in Tables II–IV.

Discussion

The investigation of the specificities of antibodies directed against bradykinin takes advantage of several unique characteristics of this antigen–antibody system. Bradykinin is a small peptide of defined amino acid sequence. When incorporated into the synthetic immunogens used, bradykinin was coupled to the carriers in a highly specific fashion. Many structurally defined synthetic analogs and fragments of bradykinin as well as intrinsically labeled bradykinin are available for binding studies. The data obtainable from such studies indicate which region or regions of a particular bradykinin immunogen are antigenically important and yield information concerning the functional homogeneity or heterogeneity of the antisera employed.

Analysis of Analog and Fragment Inhibition Data from Studies Employing Antibody Directed against Bradykinin Coupled to Poly-L-lysine. The data plotted in Figure 1, which depicts inhibition curves produced following incubation of a series of singly substituted alanine analogs with antibody raised against bradykinin-poly-L-lysine, have been discussed elsewhere (Spragg *et al.*, 1968) and are presented here for comparison with the results to be discussed below. It is apparent from these curves and from an earlier study (Spragg *et al.*, 1967) that substitutions which necessarily change the over-all shape of the peptide (Ramachandran *et al.*, 1963), such as substitution of

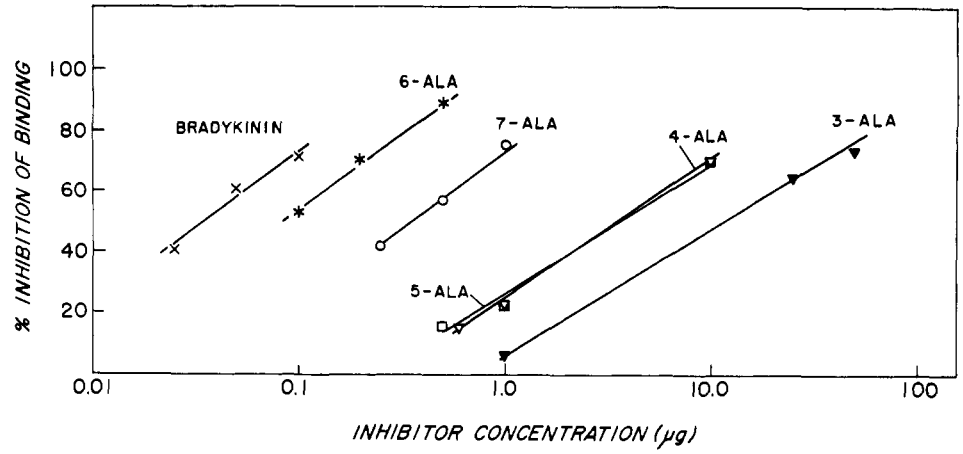

FIGURE 1: Inhibition curves resulting from the binding of a series of singly substituted alanine analogs of bradykinin to rabbit antibody directed against bradykinin-polylysine (Spragg *et al.*, 1968). The inhibition produced by native bradykinin is shown for comparison.

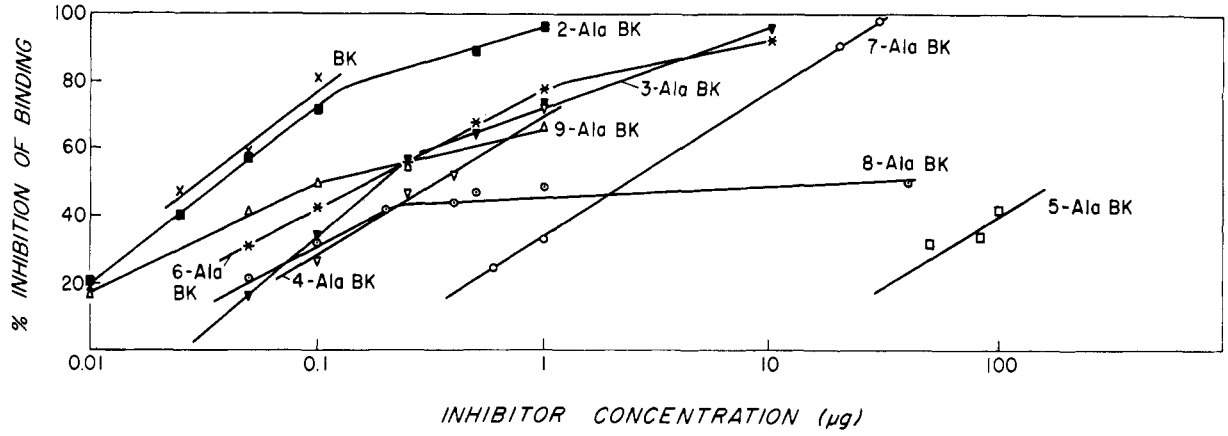

FIGURE 2: Inhibition curves resulting from the binding of a series of singly substituted alanine analogs of bradykinin to rabbit antibodies directed against bradykinin-ovalbumin (T24).

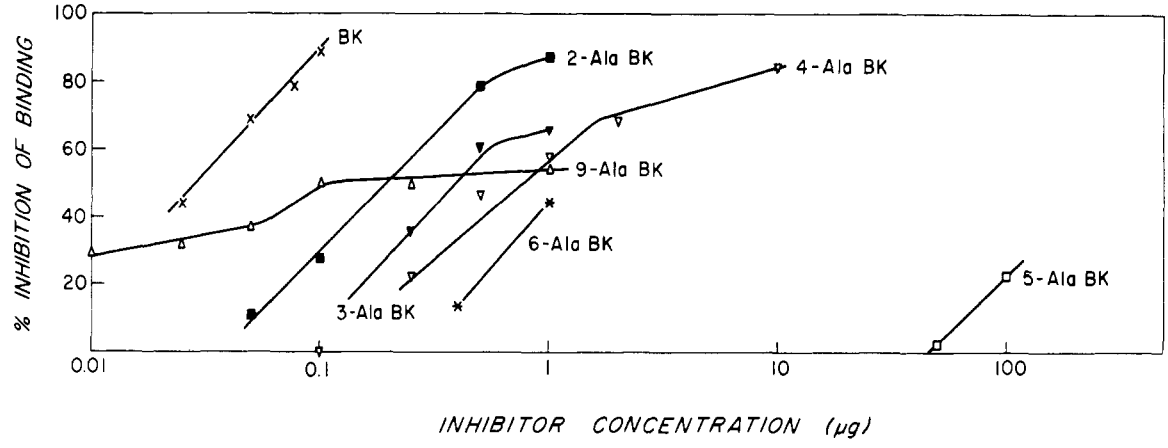

FIGURE 3: Inhibition curves resulting from the binding of a series of singly substituted alanine analogs of bradykinin to rabbit antibodies directed against bradykinin-ovalbumin (T36).

the proline in position 2 or 3 or of the glycine in position 4, have major effects on binding (Table I). Alterations of residues near the carboxy terminus of bradykinin, the region away from the backbone of the immunogen, have a far lesser effect on binding. These results suggested that conformation of the entire bradykinin nonapeptide was antigenically important. As previously reported, the curves discussed here are parallel for all analogs and may be extrapolated to full inhibiton. Addi-

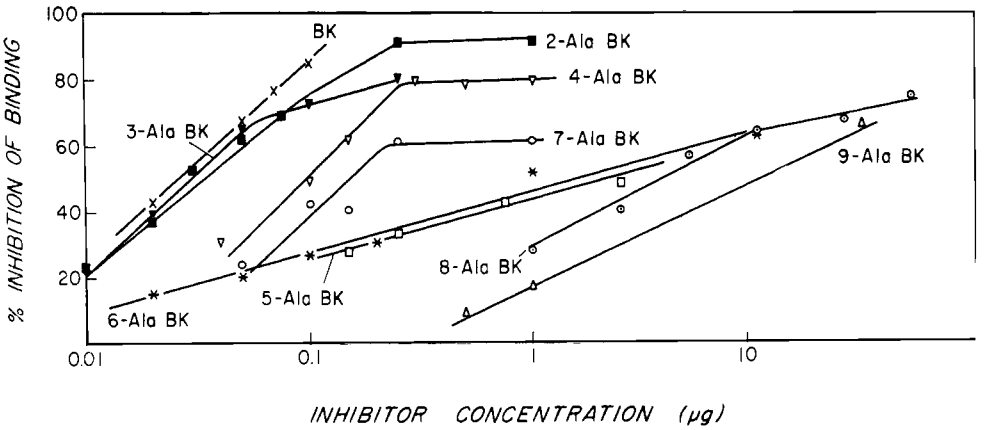

FIGURE 4: Inhibition curves resulting from the binding of a series of singly substituted alanine analogs of bradykinin to sheep antibody directed against human kininogen I.

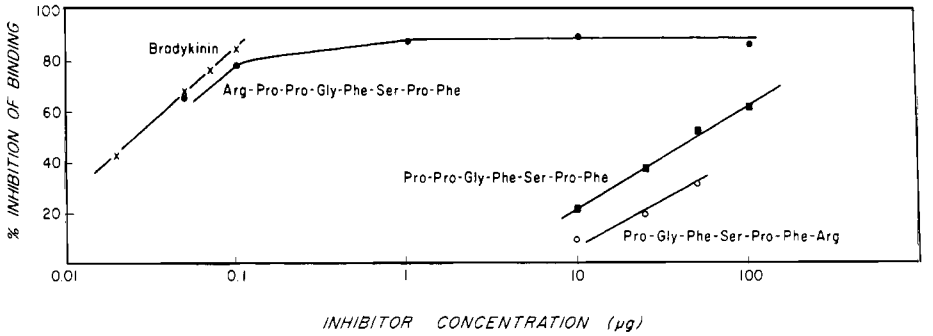

FIGURE 5: Inhibition curves resulting from the binding of bradykinin fragments to antibody directed against bradykinin-polylysine Because most fragments are not effective inhibitors in this system, inhibition plots were obtained for these fragments only.

TABLE I:

Peptide	1	2	3	4	5	6	7	8	9	aBK-TC-PL	aBK-TC-OA T24	aBK-TC-OA T36	Akininogen
Bradykinin (BK)	H-Arg	Pro	Pro	Gly	Phe	Ser	Pro	Phe	Arg-OH	1	1	1	1
Ala^{9}-BK									Ala		1/3.3	1/4.2	1/885
Ala^{8}-BK								Ala			1/666.7		1/154
Ala^{7}-BK							Ala			1/10	1/41.2		1/5.8
Ala^{6}-BK						Ala				1/2.4	1/5.7	1/41.3	1/61.5
Ala^{5}-BK					Ala					1/37	1/5833	1/8621	1/84.6
Ala^{4}-BK				Ala						1/38	1/11.3	1/26.2	1/3.6
Ala^{3}-BK			Ala							1/189	1/5.8	1/13.4	1/1.2
Ala^{2}-BK		Ala									1/1.3	1/6.7	1/1.4

tional confirmation of this point is obtained from the experimental observation that an asymptotic approach to 100% inhibition could be demonstrated without interruption of the curves.

In order to determine whether the region of the nonapeptide which incorporated the residues having an obligatory effect on conformation could by itself inhibit binding, fragments containing the Pro^{2}-Pro^{3}-Gly^{4} region were tested. Results for a few of these peptides are depicted in Figure 5, and the data for all those examined are summarized completely in Tables II–IV. Only fragments built up from the amino terminus are capable of significantly inhibiting binding, and then only when fragments larger than a pentapeptide are employed (Figure 8a, Table II). However, this inhibition is more than 100 times less potent than inhibition by bradykinin and also less potent than the least effective nonapeptide alanine analog. Bradykinin

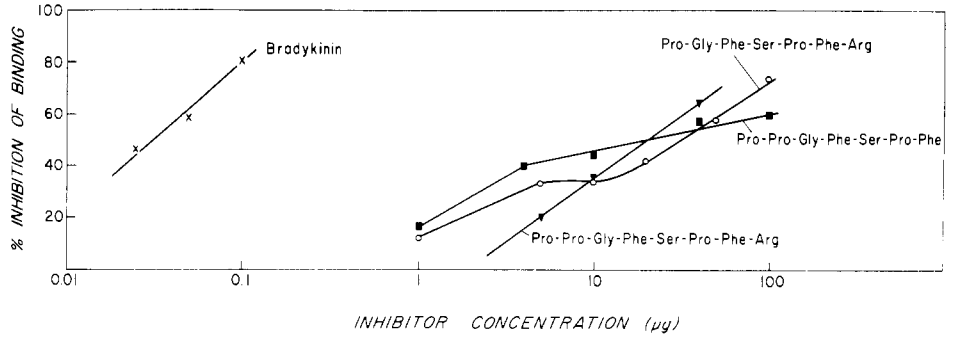

FIGURE 6: Inhibition curves resulting from the binding of bradykinin fragments to antibody directed against bradykinin-ovalbumin (T24).

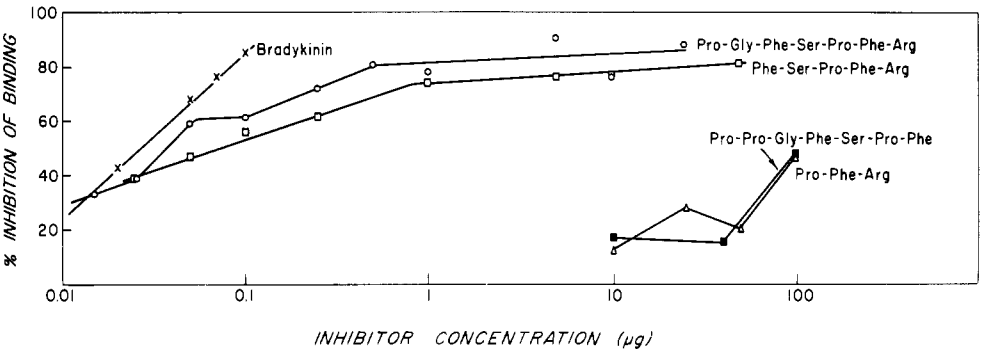

FIGURE 7: Inhibition curves resulting from the binding of bradykinin fragments to antibody directed against human kininogen I.

TABLE II: Per Cent Inhibition of Binding of [^{14}C]Bradykinin by Bradykinin Fragments from the Amino End.[a]

	aBK-TC-PL (%)	aBK-TC-OA (T24) (%)	aKininogen (%)
Bradykinin: Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg			
Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe	88.3	68	49.5
Arg-Pro-Pro-Gly-Phe-Ser-Pro	72.6	53.7	14.7
Arg-Pro-Pro-Gly-Phe-Ser	77.3	64	25.5
Arg-Pro-Pro-Gly-Phe	35.6	36.5	0
Arg-Pro-Pro-Gly	11.7	9.6	0
Arg-Pro-Pro		0	0

[a] 100 μg of each fragment was employed. Under the conditions of this experiment, 0.1 μg of bradykinin produces approximately 80% inhibition with each of the antibodies.

fragments built up from the carboxy terminus are not significantly capable of inhibiting binding, the octapeptide yielding only 26.8% inhibition of binding at a concentration of 100 μg (Figure 8a, Table III). Of the internal fragments tested, only one, the heptapeptide lacking both arginines, gave significant inhibition, possibly due to the loss of a positive guanidino charge and increased hydrophobic character (Benjamini *et al.*, 1968); the others gave inhibition below 12% (Table IV). These results confirm both the requirements for the Pro^2-Pro^3-Gly^4 region of the nonapeptide and the observation that for effective binding to aBK-TC-PL, this region must be maintained in the correct conformation, determined by the entire nonapeptide sequence. This provides a contrast to such studies as those on angiotensin (Haber *et al.*, 1967) and tobacco mosaic virus (Benjamini *et al.*, 1965) which indicate that smaller portions of an immunogenic peptide may be the immunodominant group.

Analysis of Analog and Fragment Inhibition Data from Studies Employing Antibody Directed against Bradykinin Coupled to Ovalbumin. In contrast to aBK-TC-PL, aBK-TC-OA showed marked heterogeneity as indicated by the inhibition curves depicted in Figures 2 and 3, which have varying slopes (Kabat, 1966) and plateaus at different levels. Because of the heterogeneity of these antisera, the varying degree with which a single

TABLE III: Per cent Inhibition of Binding of [^{14}C]Bradykinin by Bradykinin Fragments from the Carboxyl End.[a]

	aBK-TC-PL (%)	aBK-TC-OA (T24) (%)	Akininogen (%)
Bradykinin: Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg			
Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg	26.8	80	87
Pro-Gly-Phe-Ser-Pro-Phe-Arg	32.9	73.3	74.8
Gly-Phe-Ser-Pro-Phe-Arg	12.3	24.7	69.4
Phe-Ser-Pro-Phe-Arg	6.0	21.7	49.1
Ser-Pro-Phe-Arg	22.0	22.9	52.5
Pro-Phe-Arg	10.0	6.0	44.7
Phe-Arg		9.4	23.2

[a] 100 μg of each fragment was employed. Under the conditions of this experiment, 0.1 μg of bradykinin produces approximately 80% inhibition with each of the antibodies.

TABLE IV: Per Cent Inhibition of Binding of [^{14}C]Bradykinin by Internal Bradykinin Fragments.[a]

	aBK-TC-PL (%)	aBK-TC-OA (T24) (%)	Akininogen (%)
Bradykinin: Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg			
Pro-Pro-Gly-Phe-Ser-Pro-Phe	63.5	57.2	46.1
Pro-Gly-Phe-Ser-Pro-Phe	6.3	35.6	29.0
Gly-Phe-Ser-Pro-Phe		0	11.3
Pro-Pro-Gly-Phe-Ser-Pro	11.7	17	19.2
Pro-Gly-Phe-Ser-Pro	0	0	0
Pro-Gly-Phe-Ser	0		
Pro-Pro-Gly-Phe-Ser	5.5		

[a] 100 μg of each fragment was employed. Under the conditions of this experiment, 0.1 μg of bradykinin produces approximately 80% inhibition with each of the antibodies.

antibody population recognizes the alanine analogs cannot be determined. However, the results summarized in Table I indicate that the Pro^2 or Pro^3 residues are not of importance in immune reactivity, since analogs containing alanine in these positions are effective inhibitors of [^{14}C]bradykinin binding (Table I). Of far greater importance for recognition by these antibodies are the phenylalanines in position 5 and 8 (Table I). As previously discussed (Haber *et al.*, 1967), one possible conformation of bradykinin may involve π–π bonding of the two phenylalanine rings. Of importance in this system also is the proline in position 7, the location of which between the two phenylalanines may be necessary for favorable apposition of the aromatic rings. As in the bradykinin–polylysine system, the identity of the arginine in position 9 is unimportant, but the peptide backbone length in this position must be retained for recognition by antibody (Tables I and II). It appears that the antibodies raised against bradykinin coupled to ovalbumin recognize most strongly the aromatic residues of bradykinin, with comparatively little recognition of positions 2–4, and no requirement for the side chain in position 9.

The inhibition data obtained using bradykinin fragments confirm these points, while indicating that for antigenic recognition the aromatic residues must occur within the correct nonapeptide sequence. Using fragments built up from the amino terminus, little inhibition is obtained until the pentapeptide, containing one aromatic residue, is employed; greater than 50% inhibition is achieved only with fragments composed of 6 or more amino acids (Figure 8b, Table II). Building from the opposite end there is no increase in inhibition when the phenylalanine in position 5 is added. However, an increment from 24.7 to 73.3% inhibition is observed when going from the hexa- to the heptapeptide (Figure 8b, Table III) indicating that antigenicity depends upon both amino acid identity and incorporation into the correct nonapeptide conformation. A backbone of nonapeptide length is needed for optimal recognition. This is indicated by the far better binding of Ala^9-bradykinin (Figure 2) than of des-Arg^9-bradykinin (Table II) as well as by an examination of the fragment data. As is evident from Tables II–IV, internal peptides lacking either Arg^1 or Arg^9 are considerably less effective inhibitors than the same fragments with the carboxy- or amino-terminal amino acid added. For example, Pro-Gly-Phe-Ser-Pro-Phe-Arg gives 73.3% inhibition, whereas Pro-Gly-Phe-Ser-Pro-Phe gives 35.6% inhibition.

Analysis of Analog and Fragment Inhibition Data from Studies Employing Antibody Directed against Human Kininogen I.

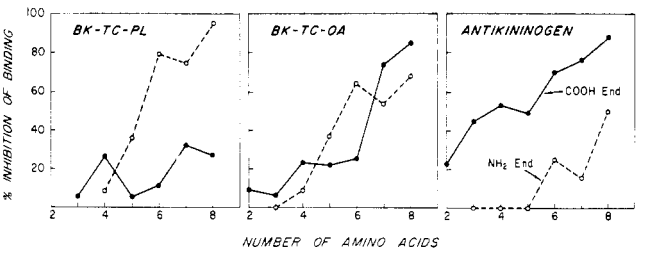

FIGURE 8: The per cent inhibition of binding produced by bradykinin fragments of increasing length in the three antibody systems. The solid lines represent inhibition as the molecule is built up from the carboxy terminus. The dotted lines represent inhibition as the molecule is built up from the amino terminus.

It is apparent from the inhibition curves obtained by binding of alanine analogs of bradykinin to antikininogen I, that this antibody population is also heterogeneous (Figure 4). The results summarized in Table I indicate the antigenic importance of the residues at the carboxyl end of the bradykinin sequence. The greatest decrease in inhibition of binding occurs when alanine is substituted for the arginine in position 9, followed by the substitution of alanine for phenylalanine in position 8. The other alanine substitutions which affect binding in this system are in positions 6 (serine) and 5 (phenylalanine). Replacement of position 4 (glycine) or positions 2 and 3 (proline) has virtually no effect on altering binding to antikininogen. It appears that this system, unlike the other two, recognizes most strongly the carboxy-terminal region of bradykinin.

This observation is supported by the data obtained using bradykinin fragments. The inhibition curves produced by a few of these fragments (Figure 7) again indicate the heterogeneity of the antikininogen antibody. The inhibition values presented in Table II, concerning fragments built up from the amino terminus, reach only 49.5% for the octapeptide. In contrast, when fragments are constructed starting from the carboxy terminus, 44.3% inhibition is obtained with the Pro-Phe-Arg tripeptide and reaches 87% inhibition for the octapeptide (Figure 8c, Table III). Of the internal fragments tested, only one, the des-Arg^1-Arg^9 heptapeptide, approaches 50% inhibition (Table IV).

Comparison of the Three Antibody Populations. The profile of binding inhibition by bradykinin analogs and fragments appears to be unique for each antiserum employed. Since a limited number of sera were examined, it is difficult to draw broad conclusions concerning the role of the carrier. One may speculate, however, on its possible role in producing three different antibody populations, all directed against bradykinin. Singer (1964) has discussed the possible effects of a carrier molecule on the antigenic specificity of a haptenic determinant. In the bradykinin–polylysine system, the nonapeptide hapten is coupled in a specific fashion to a rather featureless poorly immunogenic (Gill and Doty, 1961) and uniform carrier. Since both the hapten and the carrier have a high net positive charge, the bradykinin will probably extend away from the backbone, with much of its surface available as an antigenic determinant. Consequently, the carrier is not expected to contribute to the heterogeneity of antibody. In the single antibody studied, there is apparent homogeneity, as demonstrated by the antibody affinity and saturation levels using bradykinin analogs and fragments. This conclusion is further supported by the results indicating that the antibody directed against bradykinin-polylysine recognizes the entire structured nonapeptide.

There are several features of ovalbumin which might contribute to the production of heterogeneous antibradykinin antibody: it is itself a good immunogen in the rabbit; there are several different available sites to which bradykinin can be coupled; and at neutral pH, ovalbumin has a net negative charge. Because the carrier and the hapten in this instance have opposite net charges, it is possible that on certain sites on the surface of the molecule, distortion or masking of portions of the peptide may occur by virtue of peptide-carrier charge interactions. This would result in an antigenic determinant consisting of only a portion of the bradykinin molecule. Since these interactions will vary on different portions of the surface of the carrier, heterogeneity of the antigen is to be expected. It is not surprising then that the antibodies directed against bradykinin-ovalbumin have been shown to be heterogeneous with regard to affinity and level of saturation.

Examining in the same fashion the antikininogen antibody which recognizes bradykinin, it is apparent that much of the antibody is directed against the carboxy-terminal arginine and phenylalanine residues; unlike the other two antibodies examined, there seems to be little recognition of the amino-terminal region of bradykinin, suggesting that as it exists on kininogen I, this region of the sequence may be masked by other portions of the protein. The heterogeneity observed in this system may be explained by assuming that shifting of the "reading frame" incorporates within the antigenic site the carboxy-terminal region of bradykinin plus varying regions of the protein surface sterically adjacent to it (Singer, 1964; Haber *et al.*, 1967). Eisen and Siskind (1964) demonstrated that when small haptens are located on a unique site in a protein, heterogeneous antibodies may result, though with other immunization techniques antibodies of greater homogeneity against the hapten–protein conjugates can be demonstrated (Brenneman and Singer, 1968).

Since these observations are based on a study of four antisera, caution should be exercised in generalizing from these results. In the case of aBK-TC-OA (Figures 2 and 3, Table I), it is apparent that the same immunogen yielded very similar antibodies in two different rabbits.

The data allow the conclusion that a nonapeptide may behave as an integral antigenic determinant, that its binding characteristics to antibody cannot be explained by a simple summing of contained antigenic determinants, and that conformation is of importance. The bradykinin carriers may play a role in determining the nature of the antibody produced. It may manifest its effect by masking a portion of the determinant or possibly by altering its orientation through protein–peptide interaction. A homogeneous carrier, such as a polyamino acid, presumably has a uniform effect on a hapten (if substitution is not dense), while a protein carrier, because of its varied surface architecture, clearly has differing effects on the hapten, depending on the site of substitution. The data presented suggest that the degree of heterogeneity of the antibodies produced reflects the heterogeneity of the immunogenic stimulus.

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Dissociation-Produced Loss of Regulatory Control of Homoserine Dehydrogenase of *Rhodospirillum rubrum*. I. Factors Which Affect the Interconversion of the Regulatable and Nonregulatable States*

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ABSTRACT: Homoserine dehydrogenase of *Rhodospirillum rubrum* undergoes a reversible dissociation with a half-time of 1–2 min into a form which is insensitive to inhibition by threonine and appears not to be bound by threonine. The association–dissociation curve fits closely that for a unimolecular–bimolecular equilibrium, so it is concluded that the threonine-sensitive form is a dimer of the insensitive form. The equilibrium is shifted toward the aggregated, theonine-sensitive form by divalent cations (Mg^{+2}, Mn^{+2}, and Ca^{+2}) and monovalent anions (Cl^- and acetate), and by threonine and other allosteric ligands (isoleucine, methionine, norleucine, and β-hydroxynorvaline). Homoserine, serine, asparate, and adenosine triphosphate shift the equilibrium slightly toward the nonaggregated, theonine-insensitive form while the other substrates are without effect. Threonine and β-hydroxynorvaline, in addition, induce a slower formation of what appears to be a hyperaggregated form on the basis of the Hill coefficient for the reaction and earlier sedimentation and gel filtration data. A model consistent with the results is proposed. A method for aspartic semialdehyde purification is presented.

A level of sophistication not previously appreciated in the capabilities of enzymes has been revealed with the formulation of the concepts of allosteric modulator binding sites and the underlying structural feature of protein subunit interaction. The theory proposes that modulator binding effects the equilibrium between conformational states of peptide chains (Monod *et al.*, 1965) (or induces new conformational states (Koshland and Neet, 1968)), thus leading to shifts in their interaction with one another and modification of the catalytic site and properties. The most readily observable sort of shift in the state of interaction between protein subunits is that leading to outright dissociation or association.

Aggregation of disaggregation under the influence of modifiers or substrates has been reported for a number of enzymes subject to modulator control (glutamic dehydrogenase, Frieden, 1959a,b; Tomkins and Yielding, 1961; hemoglobin, Briehl, 1963; threonine deaminase, Changeux, 1963; acetyl-CoA carboxylase, Vagelos *et al.*, 1963; homoserine dehydrogenase, Datta *et al.*, 1964; dCMP deaminase, Maley and Maley, 1964; fructose diphosphatase, Rosen *et al.*, 1966; phosphorylase, Metzger *et al.*, 1967; formyltetrahydrofolate synthetase, Scott and Rabinowitz, 1967; aspartokinase, Wampler and Westhead, 1968; and glutaminase, Katsunuma *et al.*, 1968). In the experiments of Datta *et al.* (1964) with homoserine dehydrogenase of *Rhodospirillum rubrum*, enzyme

* From the Biology Department, State University of New York, Buffalo, New York 14214. *Received March 4, 1969*. This work was supported by a grant from the National Institutes of Health (AM-08873). Part of this work was taken from a thesis submitted by R. M. to the Graduate School of Washington University in partial fulfillment of the requirements for the degree of Doctor of Philosophy. A preliminary report has been published (Mankovitz and Segal, 1969a).

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