BIKEN JOURNAL Vol. 11, 219-223, 1968

PROOF OF INDEPENDENCY OF TWO ANTIGENIC SITES IN EGG WHITE LYSOZYME

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(Received June 26, 1968)

SUMMARY Two immunologically active peptides, one derived from the portion from Gln^{57} to Ala^{107} (peptide 7a) and the other from the N-and C-terminal region (peptide 17) of hen egg white lysozyme (HL), were tested in respect to their specificities in reactions with rabbit antibody prepared against native HL.

Both peptides inhibited the precipitin reaction between HL and HL antiserum. A mixture of the two peptides was more inhibitory than either peptide alone.

The independency of the binding speciticity of each peptide can be shown more clearly by equilibrium dialysis. One peptide was acetylated with ^{14}C-acetic anhydride and the effect of the other peptide upon its binding was tested. The binding of one peptide by anti-HL antibody was not influenced by the other peptide and *vice versa*.

INTRODUCTION

Although the high serological specificities of many kinds of proteins have been well established, the nature and specificity of each antigenic determinant within a protein molecule is not fully understood. There is much information available on the structures of proteins and seems likely that the specificity of each determinant in a globular protein consisting of a single polypeptide chain would be different. LAPRÈSLE and DURIEUX (1957) emphasized this possiblity and since then many attempts have been made to prove it. Thus, CRUMPTON and WILKINSON (1965) reported that a mixture of peptides derived from apomyoglobin showed a slightly higher degree of precipitation inhibition than any one of the peptides alone.

We also succeeded in isolating at least two kinds of immunologically active peptide (SHINKA *et al.*, 1962; SHINKA *et al.*, 1967. FUJIO *et al.*, 1968a) from HL by limited digestion with pepsin. Fortunately these two peptides have very similar association constants in the reaction with purified HL antibody. Therefore we could utilize equilibrium dialysis to test whether the binding of one affected that of the other. It was shown that the binding of each peptide with the corresponding antibody was independent of that of the other peptide.

MATERIALS AND METHODS

1. *Peptide 7a and peptide 17*

The two peptides were prepared by the methods described in our previous reports (SHINKA *et al.*,

1967; FUJIO *et al.*, 1968a).

2. *Production of antisera and purification of antibody*

Rabbits were immunized with chromatographically purified HL, as described previously (FUJIO *et al.*, 1968a). Rabbit anti-HL antibody was specifically purified from pooled sera. The antibody was precipitated at the equivalence of each serum with HL and dissociated in 0.2 N acetic acid. HL was separated from antibody on a Sephadex G-150 column in 0.2 N acetic acid. The details of this procedure were given in a previous report (FUJIO *et al.*, 1968a).

Only the 7S fraction of anti-HL antibody was used for equilibrium dialysis.

3. *Quantitative inhibition of the precipitin reaction*

Various amounts of peptide 7a or peptide 17 and a mixture of both in 200 μl of 0.15 M NaCl, 0.02 M phosphate buffer (PBS), pH 6.0, were incubated at 37°C for 30 min with 100 μl of rabbit HL antiserum (#P28). An equivalent amount of HL in 20 μl of PBS, pH 6.0, was then added. The mixture were incubated at 37°C for 1 hr and at 4°C for 2 days. The precipitates were washed three times with 1.0 ml volumes of chilled PBS, pH 6.0. The precipitates were dissolved in 3 ml of 0.1 N NaOH and left overnight at room temperature. The next morning the opitcal density of the solution was read at 280 mμ. The decrease in the optical density at 280 mμ due to inhibitor was expressed as a precentage of the optical density obtained in the absence of inhibitor. All tubes were set up in duplicate.

4. *Preparation of [1-^{14}C] acetyl peptides*

Peptide 7a and peptide 17 were acetylated with [1-^{14}C] acetic anhydride according to the method described previously (FUJIO *et al.*, 1968a). The [1-^{14}C] acetylated peptides 7a and 17 were further diluted with the corresponding cold peptides for equilibirum dialysis. The specific activity of the [1-^{14}C] acetylated peptide 7a was 265 cpm per μg and that of peptide 17 was 446 cpm per μg.

5. *Equilibirum dialysis*

This was performed essentially according to the method of KARUSH and KARUSH (1968). The details of the experiment on binding of peptide by purified HL antibody were given in a previous paper (FUJIO *et al.*, 1968a), the molar concentrations of peptides were calculated taking the molecular weight of peptide 7a as 5,400 and that of peptide 17 as 4,000. Dialyis was carried out at 10°C for at least 36 hr.

RESULTS

1. *Precipitation inhibition by peptide 7a, peptide 17 and both*

The results of a quantitative inhibition test by peptide 7a and peptide 17 against the precipitin reaction between HL and HL antiseruum #P28 are shown in Fig. 1.

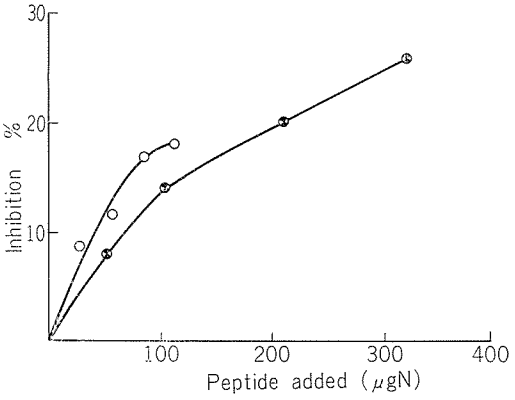

FIGURE 1 Quantitative inhibition of precipitin reaction of HL and HL antiserum #P28 by two peptides. See text for experimental details.
○——○ Inhibition by peptide 7a
⊖——⊖ Inhibition by peptide 17
HL: 3.43 μgN in 20 μl, antiserum: 100 μl of rabbit HL antiserum #P28.

With peptide 7a, a maxial inhibition of 18% was attained by addition of 114 μgN of the peptide. But with peptide 17, maximal inhibition could not be attained under our experimental conditions, although as high as 26% inhibition was attained by addition of 320μgN of the peptide. Generally more peptide 17 than peptide 7a was necessarry to attain a certain degree of inhibition.

If peptide 7a and peptide 17 represent different antigenic sites, the degree of inhibition by a mixute of the two peptides should be the sum of those attained by each peptide. The results of an experiment on this are shown in Table 1.

TABLE 1 *Additive inhibition by two peptides against the precipitin reaction between HL and HL antiserum #P28*

Peptide 7a added (μgN)	Peptide 17 added (μgN)	Inhibition (%)
114	—	18
—	320	26
114	320	38

One peptide or a mixture of the two peptides in 200 μl of PBS, pH 6.0, was incubated at 37°C for 30 min with 100 μl of undiluted rabbit HL antiserum #P28. The equivalent amount of HL (3.43 μgN) in 20 μl of PBS, pH 6.0, was added to each mixture and mixtures were incubated at 37°C for 1 hr and then at 4°C for 2 days.

In fact, the degree of inhibition by both peptides was the sum of the inhibitions by each peptide singly.

2. *Mutual effect of the two peptides upon the binding of each peptide by purified anti-HL antibody*

Anti-HL antibody #P28 was purified from HL antiserum #P28 by the method described above. A summary of the results of equilibrium dialysis experiments with peptide 7a and peptide 17 using purified anti-HL antibody #P28 are given in Table 2.

The concentration of free peptide in equilibrium dialysis was estimated both by measurement of ^{14}C and by Lowry's modification (LOWRY *et al.*, 1951) of the Folin reaction. The two methods gave essentially the same results with both peptides. As can be seen in Table 2, purified anti-HL antibody #P28 contained a fairly large amount of antibody fraction directed to each peptide. In addition, the mean association constants of binding of the two peptides with the corresponding antibody fractions were in the same order of magnitude.

Therefore the amounts of the one [1–^{14}C]-acetyl peptide bound in the presence and absence of cold peptide of the other kind were compared by equilibrium dialysis. Results are shown in Table 3 and Table 4.

TABLE 2 *Binding of [1-^{14}C] acetyl peptide by purified anti-HL antibody #P28*

Peptide	K_0[a] (l/mole)	Content[b] of Anti-peptide antibody (%)
[1-^{14}C]-acetyl peptide 7a	2.26×10^5	12
[1-^{14}C]-acetyl peptide 17	1.78×10^5	47

a The mean association constant (K_0) was evaluated graphically (Scatchard plot).
b The amount of antibody fraction directed to each peptide was obtained graphically by plotting the reciprocal of the amount of the peptide in the system vs. bound peptide taking the valency of antibody, as two, and the molecular weight of antibody, as 150,000.

TABLE 3 *Effect of peptide 7a on the binding of [1-^{14}C]-acetyl peptide 17 by anti-HL antibody*

Peptide 7a		[1-^{14}C]-acetyl peptide 17	
added (mμM)	bound[a] (mμM)	bound (mμM)	relative % bound
0	0	16.0	100
20.2	3.1	16.0	100
40.6	4.0	16.2	101
60.8	4.2	16.0	100

A mixture of 95 mμM of [1-^{14}C]-acetyl peptide 17 and 20 mμM of the purified anti-HL antibody #P28 (MW. of antibody taken as 150,000) in 1 ml of PBS, pH 6.0 was dialyzed against 1 ml of PBS, pH 6.0, containing various amounts (0~60.8 mμM) of cold peptide 7a at 10°C.
a Values were obtained in an independent experiment in which binding of peptide 7a by the same antibody preparation was measured.

The binding of each peptide by the corresponding antibody fraction in anti-HL is not changed by the presence of other kinds of peptide.

This binding experiment was repeated using another batch of anti-HL antibody (#P24) with essentially the same results.

TABLE 4 *Effect of peptide 17 on the binding of [1-^{14}C]-acetyl peptide 7a by anti-HL antibody*

Peptide 17		[1-^{14}C]-Acetyl peptide 7a	
added (mμM)	bound[a] (mμM)	bound (mμM)	relative % bound
0	0	4.10	100
36.0	11.5	4.06	99
72.3	15.0	4.10	100
108.3	15.9	3.88	95

A mixture of 60.2 mμM of [1-^{14}C]-acetyl peptide 7a and 20 mμM of the purified anti-HL antibody #P28 in 1 mμl of PBS, pH 6.0, was dialyzed against 1 ml of PBS, pH 6.0, containing various amounts (0~108.3 mμM) of cold peptide 17 at 10°C.
a See footnote in Table 3.

This indicates that the antibody population reacting with peptide 17 did not react with that of peptide 7a.

DISCUSSION

Lapreśle suggested long ago that each antigenic determinant in a protein might differ in specificity, but there is still no clear cut expermental support for this. One reason for this may be that physical separation of a determinant from a protein molecule may cause loss of the original affinity of the determinant. In addition, many factors may influence the precipitin reaction (MARRACK, 1961: VALENTINE *et al.*, 1967: KLINMAN *et al.*, 1967). Therefore quantitative estimation of inhibition of precipitation does not seem to be the method of choice. Equilibrium dialysis could clarify at least two points, i.e. the affinity of a fragment and the amount of antibody fraction directed to the fragment.

Actually peptide 17 inhibits the precipitin reaction only when certain antisera were used for the test, even though the presence of antibody directed to the peptide 17 in the anti-HL antibody preparations was always detected by equilibrium dialysis. These facts do not simply mean that the affinity of peptide 17 for the corresponding antibody is too low, when the precipitin reaction is not inhibited because peptide 17 still has an average association constant of 1.78×10^5 against the corresponding antibody.

Exact information on the structure of HL in solution is not available yet, but if the three dimenstional arrangement of its peptide chain is something like that of crystalline HL (PHILLIPS, 1966), then the two portions in the HL molecule corresponding to peptide 7a and peptide 17 are reasonably far apart spatially. As a matter of fact there is still a possibility that a fraction of antibody with a relatively low, if any, binding affinity for either determinant, could escape detection.

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