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Immunology

Antiviral effect on MS-2 coliphage obtained with a synthetic antigen

(vaccine/viral coat protein/peptide synthesis/peptide-carrier conjugate/phage neutralization)

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ABSTRACT **The coat protein of bacteriophage MS-2 was cleaved with cyanogen bromide to yield three fragments, possessing the sequences 1–88 (P_1), 89–108 (P_2), and 109–129 (P_3), respectively. The mixture of peptides P_2 and P_3, which could not be separated, was found capable of inhibiting the neutralization of the phage by antiserum to the whole MS-2. The peptides corresponding to P_2 and P_3 were therefore synthesized. The synthetic P_3 had no capacity to interfere with neutralization of MS-2, nor did its macromolecular conjugate with multichain poly(DL-alanine) elicit neutralizing antibodies. On the other hand, the synthetic P_2 was very efficient in inhibiting the inactivation of the phage by the antiserum against phage. Furthermore, a synthetic antigen prepared by attachment of P_2 to multichain poly(alanine) induced antiserum in rabbits that was capable of neutralizing MS-2 activity almost as efficiently as the antiserum prepared against the intact coat protein. This inactivation is specific, because it can, in turn, be totally inhibited by P_2 peptide.**

Only a fraction of the antibodies elicited by a complex macromolecular immunogen which possesses biological activity is directly involved in the neutralizing of that activity (1–3). The inhibitory antibodies are those directed against a limited number of antigenic determinants, which are relevant to the catalytic site or the active conformation. A selective fraction of antibodies directed against a given antigenic determinant(s) has been prepared either by appropriate fractionation of the total antibodies (1, 4, 5), or by immunization with an antigen containing only the relevant determinant(s) of the material involved (5), bound to a suitable carrier. Furthermore, when the structure of such a determinant is known, it is possible to synthesize it and use the synthetic peptide for elicitation of antibodies reactive with the intact protein (6–8). Antibodies provoked in this manner towards the synthetic peptide corresponding to the loop region of lysozyme (8) were shown to be directed to a conformation-dependent determinant. Their specificity is determined by the conformation of the isolated or synthetic loop fragment, which resembles the conformation this region assumes in the intact native protein.

In view of the above, it should be feasible to employ a similar approach not only for enzymes but also for components of viruses or bacteria. A relevant synthetic fragment of such a component may serve for induction of immune response towards the whole agent. Indeed, Anderer (6) used the COOH-terminal hexapeptide of tobacco mosaic virus protein for obtaining antibodies that interacted to some extent with the intact tobacco mosaic virus.

In the present study we describe a synthetic antigen that provokes in rabbits antibodies capable of inactivating the coliphage MS-2. This phage is an RNA-containing virus with icosahedral symmetry. The viral capsid consists of 180 identical coat-protein subunits with a molecular weight of 13,700 each, and a single molecule of another protein, denoted A protein (9).

Abbreviations: Boc, *t*-butoxycarbonyl; Bzl, benzyl; A--L, multi-poly(DL-alanyl)--poly(L-lysine).

The coat protein consists of a single polypeptide chain with 129 amino acid residues, which have been sequenced (10, 11). A limited immunological study on this phage indicated that antisera prepared against the total phage neutralize its activity (12–14). Because the coat protein can be readily isolated in the monomeric form (15), we previously used it for immunization, and showed that the resulting antisera were also efficient in neutralizing the intact phage (16). This system seemed, thus, a suitable model for finding out whether synthetically prepared relevant regions of the phage coat protein may lead, after appropriate conjugation, to the formation of antibodies that neutralize the phage. This paper provides evidence that antibodies raised against a totally synthetic molecule show antiviral activity, namely, they inactivate the viable coliphage.

MATERIALS AND METHODS

Phage strain MS-2 and *Escherichia coli* C 3000 were generously supplied by Yaacov Pollak (Department of Biochemistry of this Institute). MS-2 phage was prepared according to the method of Gesteland and Spahr (17). MS-2 coat-protein was kindly provided by the deceased H. A. Sober (National Institutes of Health, Bethesda, Md.). It was obtained according to the procedure of Fraenkel-Conrat (15). Prevention of formation of aggregates was achieved by maintaining the coat protein in acid pH (18). The protein was tested for the presence of a disulfide bond by tryptic hydrolysis followed by diagonal paper electrophoresis (19). Cyanogen bromide and iodoacetamide were purchased from Fluka; Sephadex G-25 from Pharmacia; 2-mercaptoethanol from Eastman; dioxane from Merck; tryptone, yeast extract, and agar from Difco. Multichain poly(DL-alanine) was prepared as described elsewhere (20). The coupling agent used was 1-ethyl-3-(3′-dimethylaminopropyl)carbodiimide hydrochloride from Ott Chemicals. N^{α}-(*t*-Butoxycarbonyl)-L-amino acid (Boc) derivatives were either purchased from Sigma, or were a gift from I. Jacobson (Department of Biophysics of this Institute). The amino acid side chains blocked included the benzyl (Bzl) esters of aspartic acid and glutamic acid, the benzyl ethers of serine and threonine (*O*-Bzl), the benzyl thioether (*S*-Bzl) of cysteine, and the carbobenzoxy derivative of lysine. All solvents and chemicals used were of analytical grade or the best grade available.

Phage Neutralization and Inhibition Tests. The procedure was performed essentially according to the small-agar-layer method described by Adams (21). Five milliliter of 1.5% agar in L-medium (10 g of tryptone, 8 g of NaCl, 1 g of yeast extract per liter of H_2O) were poured in petri dishes (9 cm).

The top layer (L-medium and 0.65% agar) was kept at 45° as 2.5 ml samples. Before plating, 0.2 ml of *E. coli* C 3000 ($A_{490\,nm} = 5$) was added. For phage neutralization, approximately 5×10^2 plaque-forming units of MS-2 phage in 0.1 ml of P + G medium (0.05 M phosphate buffer at pH 7.6 and 0.002% gelatin) were incubated with 0.1 ml of appropriately diluted antiserum at 37°. At given time intervals, 0.1 ml of goat

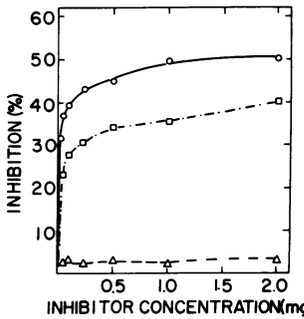

FIG. 1. Inhibition of MS-2 phage neutralization by antiserum against phage (dilution: 3×10^{-4}) with intact MS-2 coat-protein (O—O); mixture of the native peptides P_2 and P_3 (□ — · — · □); phosphate buffer (control) (Δ – – Δ).

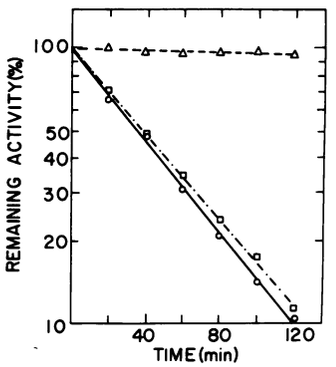

FIG. 2. Neutralization of MS-phage by antiserum against phage (dilution 3×10^{-4}) (O—O), antiserum against coat protein (dilution 10^{-3}) (□— · — · □), and normal serum (dilution 10^{-3}) (Δ – –Δ).

antiserum against rabbit immunoglobulin was added followed by 2.5 ml of top agar with bacteria, and the mixture was plated. For each time interval triplicate tests were performed. The assay plates were incubated for 16 hr at 37°. For the inhibition test, the antiserum was incubated with 0.1 ml of inhibitor for 1 hr at 37°, prior to the addition of phage, and the procedure was continued as described above.

Cleavage of the Coat-Protein. MS-2 phage coat-protein was reduced by 0.3 M 2-mercaptoethanol in 8 M urea (pH 8.2) and alkylated with 0.4 M iodoacetamide prior to cleavage. Cleavage was carried out with cyanogen bromide (22), and the resulting peptides were separated by gel filtration on a Sephadex G-25 fine column, equilibrated in 0.1 M acetic acid.

Peptide Synthesis. The two synthetic peptides prepared in this study corresponded to the sequences Glu^{89}-Ala^{107} (P_2) and Gln^{109}-Tyr^{129} (P_3), respectively. Synthesis was performed according to Merrifield (23), and started either at alanine 107 or tyrosine 129. The procedure for the preparation of *t*-Boc-L-alanine resin ester was described previously (8). The resulting resin contained 0.26 mmol of alanine per g. Boc-*O*-Bzl-L-tyrosine resin ester was purchased from Sigma; it contained 0.1 mmol of Bzl-tyrosine per g. In the repeated coupling cycles, the Boc group was removed by treatment for 50 min with 66% trifluoroacetic acid, and after the compound had been washed with CH_2Cl_2 and $CHCl_3$ the peptide hydrochloride was converted to the free base by treatment for 10 min with 10% triethylamine in $CHCl_3$. After washing, a 3- to 4-fold excess of the appropriate Boc-amino acid derivative was added in CH_2Cl_2, and an equivalent amount of dicyclohexyl carbodiimide (Fluka) was added. Coupling was allowed to proceed for 2–3 hr. The coupling step with Boc-amino acid derivative was performed twice to get a higher binding rate. Asparagine and glutamine were added as *p*-nitrophenyl ester in dimethylformamide with a reaction time of 12 hr. Detailed accounts of reagent preparation and rinsing procedures have been published elsewhere (23). The progress of synthesis was monitored by removal of resin samples after the coupling of several amino acid residues, and their amino acid analysis according to Spackman, Stein, and Moore (24) in a Beckman model 121 automatic amino acid analyzer, after hydrolysis in 6M HCl under reduced pressure.

The peptides were cleaved from the resin by treatment with anhydrous HF for 2 hr at room temperature, in the presence of anisole. This treatment brought about complete removal of all the blocking groups. After removal of the excess HF under reduced pressure, the peptide-resin mixture was extracted three times with ethyl acetate to remove remaining anisole and the peptide was extracted into glacial acetic acid and lyophilized. Remaining organic material was removed by gel filtration on a Sephadex G-25 fine column (5.5 × 90 cm) equilibrated with 0.1 M acetic acid.

Attachment of the Synthetic Peptides to the Carrier A--L. The synthetic peptides P_2 and P_3 were attached to multi-poly(DL-alanyl)--poly(L-lysine): a solution of 35 mg of synthetic peptide, in 1 ml of dioxane was added to 11 ml of aqueous solution containing 125 mg of A--L and 25 mg of 1-ethyl-3-(3′-dimethylaminopropyl)carbodiimide hydrochloride. After 24 hr at room temperature, the precipitate formed was centrifuged off, and the supernatant was dialyzed against water, and lyophilized.

Immunological Procedures. Antisera were prepared by separately immunizing albino rabbits with MS-2 bacteriophage, the coat protein, the native P_1 fragment and the synthetic conjugates P_2-A--L and P_3-A--L. The immunizations were carried out by multisite intradermal injections of 1 mg antigen in complete Freund's adjuvant (Difco), except for the immunization with the coat-protein. In the latter case, 10 mg of the material was dissolved in 2 ml of a buffer solution containing 0.1 M NaCl and 0.01 M Tris at pH 7.6. The solution was centrifuged (10,000 × *g* for 10 min), and the supernatant was immediately mixed with complete Freund's adjuvant (ratio 1:2 vol/vol) and injected. Goat antiserum against rabbit IgG was obtained by biweekly booster immunization with 2 mg of purified rabbit antibodies.

RESULTS

Characterization of the Coat Protein. The purity of the coat protein was established by analytical ultracentrifugation which yielded a single peak in acetic acid solution and by amino acid analysis, the results of which corresponded with the known amino acid sequence of the protein. The lack of disulfide bond in the protein was evidenced by the diagonal technique, in which no off-diagonal peptide appeared after tryptic hydrolysis and performic acid oxidation. Further corroboration for the lack of any disulfide bond in the protein was provided by the finding that identical results were obtained independently of whether cleavage by CNBr was carried out on the native or on the reduced and alkylated protein.

Relevance of Coat Protein in Phage Neutralization. The viability of MS-2 phage is totally neutralized by antiserum prepared against the intact phage, as already reported. This neutralization can, in turn, be inhibited up to 50% by the monomeric form of the coat protein, as shown in Fig. 1. Furthermore, as shown in Fig. 2, antiserum prepared by immunization against the isolated coat protein is capable of neutralizing the phage to an extent similar to that obtained with the antiphage serum, when both sera were used in the same dilution. The

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Ala - Ser - Asn - Phe - Thr - Gln - Phe - Val - Leu - Val - Asp - Asn - Gly - Gly - Thr - Gly -
                          5                           10                          15
Asp - Val - Thr - Val - Ala - Pro - Ser - Asn - Phe - Ala - Asn - Gly - Val - Ala - Glu - Trp -
                  20                          25                          30
Ile - Ser - Ser - Asn - Ser - Arg - Ser - Gln - Ala - Tyr - Lys - Val - Thr - Cys - Ser - Val -
            35                          40                          45
Arg - Gln - Ser - Ser - Ala - Gln - Asn - Arg - Lys - Tyr - Thr - Ile - Lys - Val - Glu - Val -
      50                          55                          60
Pro - Lys - Val - Ala - Thr - Gln - Thr - Val - Gly - Gly - Val - Glu - Leu - Pro - Val - Ala -
65                          70                          75                          80
Ala - Trp - Arg - Ser - Tyr - Leu - Asn - Met ▼ Glu - Leu - Thr - Ile - Pro - Ile - Phe - Ala -
                  85                          90                          95
Thr - Asn - Ser - Asp - Cys - Glu - Leu - Ile - Val - Lys - Ala - Met ▼ Gln - Gly - Leu - Leu -
                  100                         105                         110
Lys - Asp - Gly - Asn - Pro - Ile - Pro - Ser - Ala - Ile - Ala - Ala - Asn - Ser - Gly - Ile - Tyr
            115                         120                         125
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FIG. 3. Sequence of bacteriophage MS-2 coat-protein. The arrows indicate the points of cleavage by cyanogen bromide at methionine residues Met^{88} and Met^{108}. The sequence is taken from Lin *et al.* (ref. 10).

specificity of this neutralization was proven by the capacity of the coat protein when added in a concentration of 0.3 mg/ml, to inhibit it to an extent of 50%.

To locate the region(s) contributing to the neutralizing process, we cleaved the coat protein with CNBr, and the resulting fragments were screened for their inhibitory activity. According to the amino acid sequence of the coat protein (Fig. 3), the cleavage points are at the two methionine residues, 88 and 108, respectively. The three cleavage products are expected to be of about 10,000 daltons and two each of about 2000 daltons. The results obtained by separation of the cleavage product on Sephadex G-25 are shown in Fig. 4. The first peak corresponded to the larger peptide, denoted P_1, with amino acid analysis in agreement with the expected fragment 1-88 (Table 1). The two smaller peptides, denoted P_2 and P_3, were eluted together under the second peak of the column. The amino acid analysis of this peak corresponded to the combined composition of the two fragments (Table 1).

The relevance of these two separated fractions in the neutralization process was studied by phage inactivation experiments. In inhibition studies, difficulties arose due to the insolubility of the large P_1 fragment. For that reason, direct neutralization experiments were performed with rabbit antiserum against purified P_1. The results showed that neutralization up to 80% could be achieved with this antiserum at a dilution of 1:100.

The mixture of the two small fragments, P_2 and P_3, was capable of inhibiting the neutralization of the MS-2 phage by whole antiserum against phage, as depicted in Fig. 1. The extent of inhibition was almost 40%, close to the value obtained with the intact coat protein. Efforts to separate the fragments P_2 and P_3 from the mixture failed due to the negligible difference in their molecular size and their almost equal net electrical charge.

Synthesis of MS-2 Coat Protein Peptides. To determine which of the two small fragments described above shows the inhibitory effect on the phage neutralization, we attempted synthesis of the two peptides corresponding in sequence to fragments P_2 and P_3. For the synthesis, the Merrifield solid phase technique was used, as described under *Materials and Methods*. For both peptides the preparation was started with 1 mmol of the first Boc-amino acid bound to the resin. The yield of the crude P_2 was 500 mg (30%) and of the crude P_3 was 780 mg (40%). The amino acid composition of the two synthetic peptides is given in Table 1.

The capacity of these two synthetic peptides to inhibit the neutralization of the phage by the whole antiphage serum is depicted in Fig. 5. As demonstrated, the synthetic P_3 did not exhibit any inhibitory activity, whereas the synthetic P_2 possessed high inhibitory activity, almost identical to the inhibition effected by a similar concentration of the mixture of the P_2 and P_3 fragments obtained by cleavage of the native coat protein.

Synthetic Antigen with Antiviral Activity. The synthetic peptides P_2 and P_3, respectively, were attached to the synthetic carrier multichain poly(DL-alanine) as described above. The

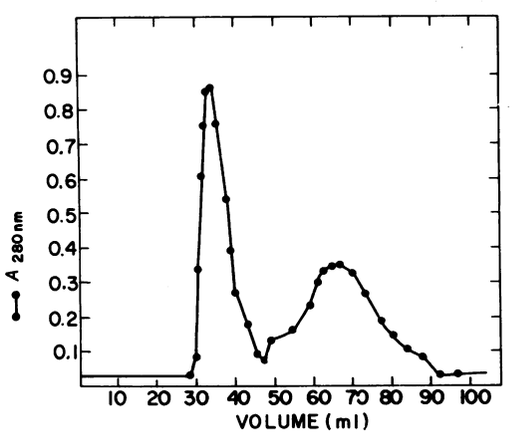

FIG. 4. Separation of the coat-protein fragments obtained by CNBr-cleavage on a Sephadex G-25 fine column (90 × 1 cm) equilibrated in 0.1 M acetic acid.

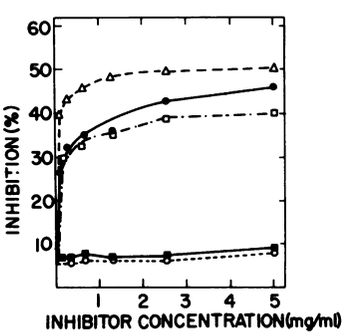

FIG. 5. Inhibition of MS-2 phage neutralization by antiserum against phage (dilution 3×10^{-4}) with synthetic peptides: intact coat-protein (△ - - △), mixture of the native peptides P_2 and P_3 (●—●), synthetic peptide P_2 (□ — · — · □), synthetic peptide P_3 (■—■), phosphate buffer control (○ - - - ○).

Table 1. Amino acid composition of the fragments obtained by CNBr cleavage of MS-2 phage, and of the synthetic peptides corresponding to the native fragments P_2 and P_3

Amino acid	Native fragment P_1	Mixture of native fragments P_2 and P_3 (assuming 1:1 ratio)	Synthetic peptide P_2	Synthetic peptide P_3
Lys	4.32 (4)	2.68 (2)	0.72 (1)	0.87 (1)
His	0 (0)	0 (0)	0 (0)	0 (0)
Arg	3.57 (4)	0.38 (0)	0 (0)	0.02 (0)
Asp, Asn	8.66 (9)	5.6 (5)	2.27 (2)	2.92 (3)
Thr	6.44 (7)	3.62 (2)	2.04 (2)	0.03 (0)
Ser	8.6 (10)	5.52 (3)	1.42 (1)	2.40 (2)
Glu, Gln	8.86 (8)	3.88 (3)	2.53 (2)	0.92 (1)
Pro	3.67 (3)	3.76 (3)	1.38 (1)	2.36 (2)
Gly	6.37 (6)	6.02 (3)	0.03 (0)	3.28 (3)
Ala	9.25 (9)	5.76 (5)	2.37 (2)	3.12 (3)
½ Cys	0.90 (1)	1.44 (1)	1.31 (1)	0 (0)
Val	12.02 (13)	1.94 (1)	0.57 (1)	0.02 (0)
Met	0.09 (0)	0.12 (0)	0 (0)	0 (0)
Ile	2.55 (2)	5.3 (6)	2.87 (3)	3.65 (3)
Leu	3.00 (3)	4.0 (4)	2.00 (2)	2.00 (2)
Tyr	2.87 (3)	1.46 (1)	0 (0)	1.10 (1)
Phe	3.11 (3)	2.48 (1)	0.99 (1)	0 (0)

The values represent number of residues per molecule, based on the known amino acid sequence of MS-2 coat protein (10), assuming that P_1 contains 3.00 residues of leucine, and P_2 and P_3 contain 2.00 leucine residues each. The numbers in parentheses represent the calculated expected values.

conjugate prepared with the P_2 peptide (P_2-A--L) contained 9.7% (wt/wt) of P_2, whereas the conjugate prepared from P_3 (P_3-A--L) contained 5.7% (wt/wt) of the peptide.

Both conjugates served for immunization of rabbits and the resultant antisera were tested for their capacity to neutralize MS-2 phage. As shown in Fig. 6, while antiserum against P_3-A--L showed essentially no neutralizing activity, the antiserum against P_2-A--L had neutralizing capacity to an extent of 85%, comparable with that obtained by the anti-coat protein. This neutralization was specific and relevant, because it could be totally inhibited by the P_2 peptide, as shown in Fig. 7.

DISCUSSION

In this study, we have demonstrated that a completely synthetic antigen can be used for induction of an immune response resulting in the phage neutralization. This may serve as an additional step in the direction of synthetic vaccines, a possibility in the future.

The use of synthetic materials in the field of vaccination against viruses may be envisaged to have several advantages over the use of intact, attenuated, or killed viruses. First, theoretically it should be possible to attach several peptides, representing the relevant portions of different viruses, to the same carrier molecule. Thus, each adequately designed synthetic antigen will be multivalent, and capable to replace several narrowly specific vaccines. Second, this approach might eliminate immunization against many irrelevant antigenic determinants of the virus, or of irrelevant proteins which contaminate the viral preparation used for vaccination. Thus, frequently occurring and undesired side-reactions may be avoided. Third, for many vaccines, the addition of an adjuvant is required in order to initiate an immune response; some of these adjuvants are not suitable for human use. In a synthetic macromolecule this problem of adjuvanticity may find its solution by introduction of certain groups which enhance antigenicity. Antigens so designed may possess adjuvant-like properties and may prove of less hazard.

Another advantage of the synthetic approach is the possibility of designing the molecule according to the purpose desired. For example, it is known that the immune response is genetically controlled (25), a control which is partly dependent on the

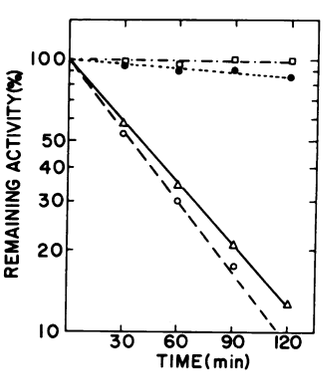

FIG. 6. Neutralization of MS-2 phage by anti-conjugate serum. Antiserum against coat-protein (dilution 10^{-3})(O- - O), antiserum against P_2-A--L (dilution 3×10^{-3}) (Δ—Δ), antiserum against P_3-A--L (dilution 3×10^{-3})(●- - -●), and normal serum (dilution 10^{-3}) (□ —·—·□).

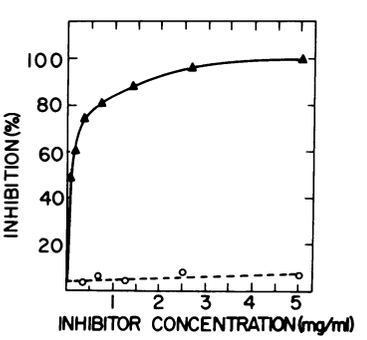

FIG. 7. Inhibition of MS-2 phage neutralization by anti P_2-A--L serum with synthetic peptide P_2. Synthetic peptide P_2 (▲—▲). Phosphate buffer control (O- - O).

structure of the macromolecular carrier (26), and in many cases is linked to the main histocompatibility of the host (25). It may thus be foreseen that the efficiency of synthetic materials for different individuals may depend on the macromolecular carrier used for its preparation. A detailed study of this correlation might enable the selection of the most efficient vaccine to be used according to tissue typing.

The prerequisite for preparation of a synthetic vaccine is the availability of a fragment which is involved in the neutralization process and hence has a chance of evoking neutralizing antibodies. To identify such a region in the MS-2 coat protein, we cleaved the material, and screened the cleavage products for their capacity to inhibit the neutralization of the phage by antiphage serum. Two such fragments were detected. However, one of them, P_1, is of a considerable molecular size (10,000 daltons) comprising about two thirds of the intact protein, and is thus unsuitable for a synthetic approach.

The second inhibitory peptide P_2 contained only 19 amino acid residues and is thus amenable for a synthetic manipulation. Indeed, the synthetic peptide corresponding to its sequence proved of biological activity (inhibition) similar to that of the native peptide. In our previous study on the loop region of lysozyme and its synthetic derivative, we also observed an identity in activity between the native and synthetic peptides, which proved to be dependent on the spatial conformation of this fragment as stabilized by an intrachain disulfide bond (8). The P_2 peptide of MS-2 coat protein does not contain a disulfide bond. The results, however, indicate that the conformation it assumes in the intact phage is conserved in the isolated protein, or might be readily assumed by it. Because the three dimensional structure of this protein is not known, and neither is the conformation which it assumes in the intact capsid, it is difficult to interpret as yet the role of the structural conformation of the peptide in its antigenic properties.

This study provides the first reported evidence that a synthetic peptide corresponding to a region which is involved in viral neutralization, can be utilized for eliciting anti-viral activity. This opens the road, conceptually, for the development of synthetic anti-viral vaccines in the future, although the prerequisite processing of all the data and details about the structure of the various pathogens and their relevant components may not be available as yet.

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