DETERMINATION OF THE ENTIRE ANTIGENIC STRUCTURE OF NATIVE LYSOZYME BY SURFACE-SIMULATION SYNTHESIS, A NOVEL CONCEPT IN MOLECULAR RECOGNITION

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INTRODUCTION

Hen egg white lysozyme is a "tight" protein (i.e., inaccessible to proteolytic attack and containing disulfide bonds). The author's interest in lysozyme was generated by the fact that the antigenic structures of such proteins (e.g., lysozyme, ribonuclease, serum albumin) had been extremely difficult to study and therefore appeared to hold a fascinating story that may prove to be basic to the understanding of antigen recognition and interaction of the antigen with its specific antibodies. Initially, the author's strategy was to rely on the five approaches that had been previously formulated[1,2] for attacking the antigenic structure of myoglobin (also, see preceding article). Subsequently, it was discovered that the antigenic structure of lysozyme was radically different from that of myoglobin, so that many of the approaches had to be modified, and many novel and unorthodox concepts had to be introduced into protein chemistry in order to view protein interactions in new perspectives that are amenable to direct synthetic experimentation.

The author's work on the antigenic structure of hen egg-white lysozyme commenced in 1967. At that time, the covalent structure of the protein had been determined by others.[3-7] Also, its three-dimensional structure had been elucidated.[8,9] The protein is a single polypeptide chain made up of 129 amino acid residues and is internally cross-linked by four disulfide bonds.

A large number of specific chemical derivatives of native lysozyme were prepared, purified, characterized, and their conformation and immunochemistry studied in detail. These derivatives have been extensively reviewed in a recent article.[10] Therefore, they will not be discussed here. The derivatives are summarized in Table 1, and reference to these studies will be made subsequently in the sections dealing with the precise delineation of the antigenic sites. It has already been indicated[1,2] that one of the useful approaches in the delineation of protein antigenic structures depends on the isolation

TABLE 1

Summary of Results from Some Chemical Derivatives of Lysozyme

Derivative	Residues modified	Conformational change	Immunochemical change	Conclusion
Derivatives with broken disulfides				
SCM-lysozyme[a]	The four disulfides, reduced and carboxymethylated	Large	Total	None made because of the large conformational change
SM-lysozyme[a]	The four disulfides, reduced and methylated	Large	Total	
Tyrosyl derivatives				
NT_2-lysozyme[b,c]	Tyr-20 and -23, nitrated	Present	Present	Tyr-20 and/or -23
AT_2-lysozyme[b,c]	Tyr-20 and -23, to aminotyrosine	Present	None	at or near an antigenic site
Tryptophan derivatives				
NPS_6-lysozyme[d,c]	Six tryptophans, with 2-nitrophenylsulfenyl chloride	Large	Very large	None made because of large conformational change
DISA-lysozyme[e]	Trp-123, with 2,3-dioxo-5-indolinesulfonic acid	None	None	Trp-123 not in antigenic site
Methionine derivatives				
CNBr-lysozyme[f]	Cleavage at Met-12 and -105, with CNBr	Large	Present	None made due to large conformational change
CE-lysozyme[g]	Met-12 and -105, carboxyethylated	None	None	Met-12 and -105 not in antigenic site
Arginine derivatives				
CHD-lysozyme I[h]	Ten arginines, with cyclohexanedione in 0.1*N* NaOH	Large	Very large	None made because of large conform. change
CHD-lysozyme II[h]	Ten arginines, with cyclohexanedione in 0.1*M* triethylamine	Large	Very large	
PG-lysozyme[h]	Arg-61, with phenylglyoxal	Minor	None	Arg-61 not in antigenic site
Amino group derivatives				
Gu_6-lysozyme[i]	Five amino groups, guanidinated	None	None	None, modification does not alter charge
AC_7-lysozyme[i]	Seven amino groups, acetylated	Large	Large	None made
ML_7-lysozyme[i,j]	Seven amino groups, maleylated	Large	Large	because of the large conformational change
Su_7-lysozyme[i]	Seven amino groups, succinylated	Large	Large	

TABLE 1 (continued)

Summary of Results from Some Chemical Derivatives of Lysozyme

Derivative	Residues modified	Conformational change	Immunochemical change	Conclusion
Su-lysozyme I[a]	Lys-1(α- and ε-), 13, 97, and 116; -OH at 43 (or 36 or 40), succinylated	Large	Large	
Su-lysozyme II[a]	Lys-1(α- and ε-), 13, 96, and 116, succinylated	Considerable	Large	
Su-lysozyme III[a]	Lys-1(α- and ε-), 13, 97, and 116, succinylated	Considerable	Large	
Su-lysozyme IV[a]	Lys-1(α-NH_2), 33, 96, and 116, succinylated	Minor or none	Present	One or more of Lys-33, -96, and -116 in antigenic sites
Su-lysozyme V[a]	Lys-1(α-NH_2), 33, 96, succinylated	Minor or none	Present	Lys-33 and -96 in antigenic sites
Su-lysozyme VI[a]	Lys-33 and 116, succinylated	Minor or none	Present	Lys-33 and -116 in antigenic sites
Carboxyl group derivatives				
BH_3-lysozyme[f]	Asp-119 and Leu-129, reduced by BH_3	Minor	None	Asp-119 and Leu-129 not in antigenic sites
GME_2-lysozyme[m]	Asp-119 and Leu-129, coupled to Gly-methylester	Minor	None	
HME_2-lysozyme[m]	Asp-119 and Leu-129, coupled to His-methylester	Large	Large	None made because of large conform. change

Note: For the immunochemical results with antisera to lysozyme and antisera to the derivatives, see references cited below.

[a] Lee and Atassi[69]
[b] Atassi and Habeeb[37]
[c] Atassi et al.[38]
[d] Habeeb and Atassi[70]
[e] Atassi and Zablocki[48]
[f] Johnson et al.[71]
[g] Atassi et al.[49]
[h] Atassi et al.[24]
[i] Habeeb and Atassi[72]
[j] Habeeb and Atassi[16]
[k] Lee et al.[25]
[l] Atassi et al.[20]
[m] Atassi et al.,[45] Atassi and Rosemblatt[46]

From Atassi, M. Z., *Advances in Experimental Medicine and Biology*, Vol. 86A, Plenum, New York, 1977, 89. With permission.

of a large variety of overlapping peptide fragments representing various parts of the protein molecule. The chemistry of chemical modification and chemical cleavage of proteins has recently been extensively reviewed.[11] However, because of the inaccessibility of these tight proteins, it has not been possible to prepare a variety of long and overlapping peptides without rupturing the disulfide bonds. Investigators have therefore diverted effort to study preparations with broken disulfide bonds, such as work on performic acid-oxidized ribonuclease[12,13] and reduced-carboxymethylated lysozyme.[14,15] These unfolded proteins have been found to bear no immunochemical relationship to the native protein. Correlation of antigenic sites in proteins with their native three-dimensional structure, of course, is the prime interest and has, for these tight proteins, virtually eluded investigations. Although some limited information was derived from peptic and similar fragments, these accounted only for a very small portion of the immunochemical reactivity of the intact proteins.[10]

To break the aforementioned deadlock, a reproducible cleavage procedure of high specificity was needed that could yield a variety of peptides directly from the native protein without rupturing the disulfide bonds. Based on Habeeb and Atassi's initial observation[16] that reversible masking of the amino groups by citraconylation induced in the protein conformational changes which rendered it accessible to tryptic attack at the arginyl peptide bonds, Atassi et al.[17] introduced a novel cleavage approach for obtaining fragments with intact disulfide bonds from tight proteins. The tryptic cleavage may be terminated, after scission of the arginyl bonds, by adding trypsin inhibitor before removal of the citraconyl masking groups at pH 4. If no trypsin inhibitor is added, then following the removal of the protecting groups, cleavage of the lysyl peptide bonds may be continued, if desired. By this approach, it was possible to effect the complete tryptic hydrolysis of lysozyme without rupturing the disulfide bonds.[17] The total tryptic hydrolysate showed[17] substantial inhibitory activity (85 to 89%) of the reaction of lysozyme with its antibodies. The fragments responsible for this inhibition were identified[17] mainly as the three disulfide-containing tryptic peptides: 22-33-(Cys 30-Cys 115)-115-116; 62-68-(Cys 64-Cys 80)-74-97-(Cys 76-Cys 94); and 6-13-(Cys 6-Cys 127)-126-128 (see Figure 1). This remarkably high inhibitory activity of the three peptides enabled Atassi et al.[17] to account for almost all the immune reaction of native lysozyme for the first time.

The approach is not limited to lysozyme and has proved to be of general applicability. It has been employed to obtain fragments from bovine serum albumin[18,19] and bovine ribonuclease A.[18a] Also, the cleavage reaction can be used to determine the correct disulfide pairing in proteins.[17,20]

Identification of the residues involved in binding with antibody and further narrowing down of antigenic sites in an immunochemically reactive peptide is best achieved[21] by immunochemical and conformational studies of chemical derivatives of the peptide modified at specific amino acid locations. This approach was applied extensively to immunochemically reactive peptides of lysozyme.

Following the accurate narrowing down of antigenic sites in the author's laboratories by the chemical approaches, the final delineation of the sites was accomplished by the organic synthesis and immunochemistry of peptides representing different parts of each site. The precautionary measures that must be employed in the application of this approach to problems of protein antigenic structures have been outlined elsewhere.[22] Numerous peptides have been synthesized in the author's laboratory for the delineation of the antigenic sites of lysozyme. The rationale behind the choice of these peptides is best handled in the following three sections which deal with the accurate assignment of the antigenic sites.

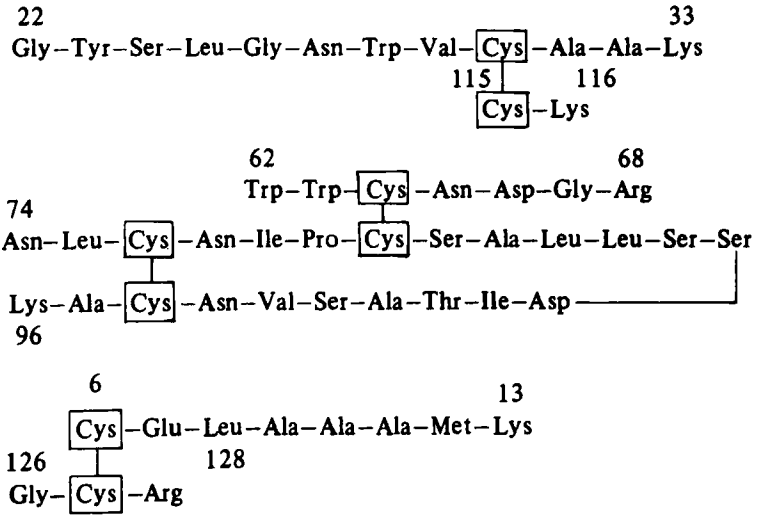

FIGURE 1. Covalent structure of the three peptides that are responsible for inhibition (85 to 89%) of the reaction of native lysozyme with its antisera. (From Atassi, M. Z., Habeeb, A.F.S.A., and Ando, K., *Biochim. Biophys. Acta*, 303, 203, 1973. With permission.)

ACCURATE DELINEATION OF THE ANTIGENIC SITE AROUND THE DISULFIDES 64-80 AND 76-94 (SITE 2)

In this and the next two sections, the information obtained from the author's chemical and synthetic approaches will be coordinated to derive the accurate location of the antigenic sites of native lysozyme. It has recently been shown[23] that lysozyme has only three major antigenic sites.

The delineation of antigenic site 2 will be described first because it was the first such site that was defined precisely. Also, historically, it was in the process of the delineation of this site that major novel and basic concepts were introduced into protein chemistry, most profound of which has been the ability to study protein interactions by "surface-simulation" synthesis.

Assignment of the Antigenic Site

Of the author's immunochemical studies on specific chemical derivatives of native lysozyme that are of direct relevance here are the findings that arginine 61 is not part of an antigenic site,[24] whereas lysine 96 is located within an antigenic site in native lysozyme.[25] Subsequently, it was shown[17,20] that the 2-disulfide peptide 62-68-(Cys 64-Cys 80)-74-97-(Cys 76-Cys 94) [i.e., $(SS)_2$-peptide] accounted for about a third of the total immunochemical reactivity of native lysozyme. Immunochemical studies of chemical derivatives of the $(SS)_2$-peptide[26] showed that Arg-68 and Asp-66 are not part of the antigenic site, but that either one (or both) of tryptophans 62 and 63 and one (or both) of lysines 96 and 97 are located in the antigenic site, in agreement with the aforementioned results[25] on derivatives of the intact protein. It was demonstrated[26] that the tryptophan(s) and the lysine(s) are parts of the same antigenic site, which requires the intactness of the disulfide bonds to effect its three-dimensional construction. Furthermore, it was shown that the region around Asp-87 was essential for the full reactivity of the site.[23] After modification of Asp-87, the antigenic site, however,

retained about half of its reactivity.[23] It became clear, therefore, that this site begins (or ends) at Trp-62 or Trp-63, requires Lys-96 or Lys-97 (or both), and some or all of the region 84—93.[23,26] Since the $(SS)_2$-peptide carries a single antigenic site, and the total of its reactivity together with the two smaller single-disulfide peptides accounts for 90% of the entire antigenic reaction of native lysozyme,[17] it became clear at this stage that lysozyme has only three major antigenic sites.[23]

Novel Synthetic Peptides with Diglycyl Bridges Instead of Disulfides

No further delineation of this antigenic site could be derived from chemical modification and cleavage studies and the author then focused his effort on the organic synthesis and immunochemical studies of peptides related to the $(SS)_2$-peptide. In order to determine the independent roles of the two tryptophans (residues 62 and 63) and the two lysines (residues 96 and 97), a new approach was devised[27] by synthesis of peptides in which the disulfides were substituted by diglycyl segments. In such peptides, the central four peptide bonds will be in the opposite direction to those in the natural peptide (see Figure 2). However, we reasoned[27] that only amino acid side chains will most likely participate in binding with antibody, and that appropriate fit onto the antibody combining site may be possible[2] through rotation about certain peptide bonds. The outcome of this unorthodox reasoning was not entirely certain because no precedents of such an undertaking had been reported, but the results proved it to be sound.

Several peptides were synthesized[27] and are shown in Figure 2. Peptide III exhibited the highest inhibitory activity that approximated the expected value, which is that of the $(SS)_2$-peptide after modification of Asp-87.[23] From comparison of the inhibitory activities of the peptides, we succeeded in demonstrating unambiguously that both Lys-96 and Lys-97, but only Trp-62 (and not Trp-63) were essential parts of the antigenic site.[27] Furthermore, phenylalanine substituted for tryptophan with equal immunochemical efficiency, indicating that the hydrophobic interaction of the indole nucleus in this case can be fully satisfied by the phenyl group of phenylalanine.[27] Therefore,

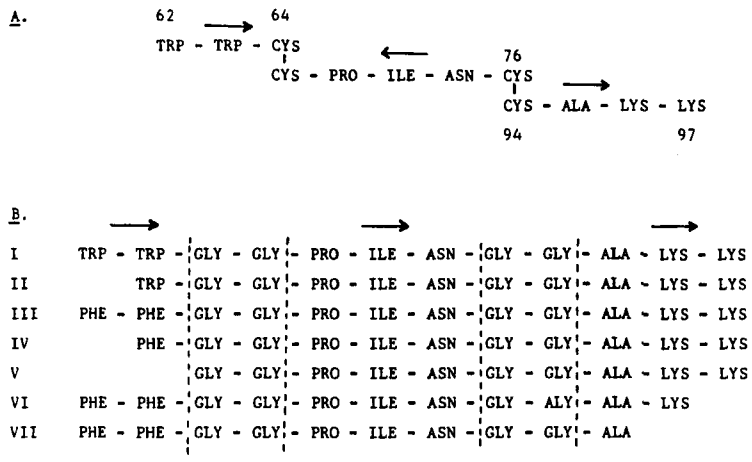

FIGURE 2. Amino acid sequence of: (A) The disulfide-linked sequences (62-64) (76-80) (94-97) of native lysozyme; (B) the peptides synthesized in our laboratory. The arrows indicate the direction (N to C) of the peptide chains. The vertical dashed lines outline the diglycyl segments which were used to substitute for the disulfides in peptide A. (From Lee, C.-L., Pai, R. C., and Atassi, M. Z., *Immunochemistry*, 13, 681, 1976.)

the residues Asp-87, Lys-96, Lys-97, and Trp-62 are essential parts of the antigenic site in this part of the molecule.[27]

The remarkable effectiveness of the substitution by diglycyl segments for the disulfide bonds may be applicable to the solution of other similar problems in proteins and should merit consideration in certain studies.

"Surface-Simulation" Synthesis: A Novel Concept Directly Linking the Spatially Adjacent Residues Forming the Site

Examination of the three-dimensional structure of lysozyme enabled Atassi et al. to explain the manner in which the aforementioned four residues construct the antigenic site, and they proposed[23] that it comprised the residues: Asp-87, Thr-89, Asn-93, Lys-96, Lys-97, and Trp-62. From the foregoing description, it can be seen that residues 87, 96, 97, and 62 were directly implicated in the active interaction with the antibody. The possible involvement of the residues Thr-89 and Asn-93 in the site was only concluded[23] from examination of the three-dimensional structure. In the three-dimensional structure of lysozyme, Thr-89 and Asn-93 lie reasonably well in an imaginary plane (or line) bearing the other residues.[23] However, since the two end and two middle residues of the antigenic site were unambiguously assigned, the boundaries of the site were therefore clearly defined.[23]

Although the existence of protein antigenic sites (i.e., spanning residues that are spatially adjacent in the three-dimensional structure but distant in sequence) had been anticipated relatively early,[28] this was, in fact, the first such site ever to be described[23] at the time these studies were completed. The antigenic structure of only one protein (i.e., sperm whale myoglobin) had been precisely elucidated[2] and did not reveal the existence of such sites.[22,29] The structural features conferring immunogenicity on certain parts of a protein molecule were (and continue to be) unclear.[2] Accordingly, very conclusive proof for the structure of the site was needed which had to be independent of the above findings. For this, Atassi et al. devised[30] a novel and entirely unorthodox approach which linked the relevant spatially adjacent surface residues constructing the site into a single peptide. From examination of the three-dimensional structure of lysozyme, the distances between the contiguous residues of the antigenic site were measured (see Figure 3). The residues Trp-62, Lys-97, Lys-96, Asn-93, Thr-89, and Asp-87 described an imaginary line circumscribing part of the surface topography of the native protein molecule.[30] Accordingly, a peptide was initially synthesized,[30] carrying phenylalanine instead of tryptophan and having a glycine spacer between phenylalanine and lysine (peptide II in Figure 3), in order to obtain the correct separation between their side chains. These studies established[30] for the first time and most conclusively and accurately the existence of an antigenic site which clearly comprised spatially adjacent residues that are distant in the sequence, reacting as if in direct peptide linkage. Since this approach attempts to mimic part of the surface topography of a protein molecule, Lee and Atassi subsequently defined it[31] by the appropriately descriptive term, "surface-simulation" synthesis.

The surface-simulation synthetic peptides in Figure 3 were designed[32] to study the conformational restrictions of site 2 and to investigate if the site has a preferred direction in order to achieve a more precise description of this site.

Accurate Definition and Conformational Restrictions of the Site

The immunochemical studies with the surface-stimulation synthetic peptides revealed[32] that peptide II (in Figure 3) had the highest immunochemical reactivity with each of the antisera studied. Omission of the glycine spacer between phenylalanine and lysine (peptide I) resulted[32] in a large detrimental effect on the immunochemical reactivity. This demonstrated the limitations on the conformational alterations that can be

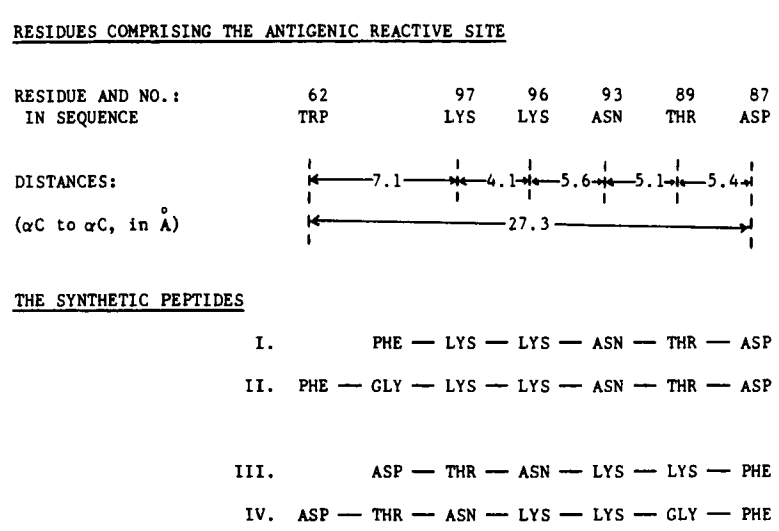

FIGURE 3. A diagram showing the spatially contiguous surface residues constituting antigenic site 2 and their numerical position in the primary structure of lysozyme. The distances (in A) separating the consecutive residues of the site are given as C^{α}-to-C^{α} distances together with the overall extended dimension of the site. Below, the primary structures of the surface-simulation synthetic peptides studied here are given. Previously it was shown[27] that tryptophan can be replaced by phenylalanine with equal efficiency. (From Lee, C.-L. and Atassi, M. Z., *Biochim. Biophys. Acta*, 495, 354, 1977. With permission.)

tolerated by such antigenic sites composed of spatially adjacent residues that are distant in sequence. These restrictions of the conformational degrees of freedom were more stringent than had been expected. The results explain the sensitivity of the antigenic structure of lysozyme to conformational changes in several chemically modified analogues, that have previously been reported by the author, and in evolutionarily substituted homologous proteins.[10] The electrostatic inductive effect that we recently observed,[33] exerted by modifications or substitutions on a neighboring antigenic site, is also further rationalized.

When the sequence of the surface-simulation synthetic site was reversed (i.e., peptide IV in Figure 3), the immunochemical reactivity with goat antisera to native lysozyme decreased drastically, but the reaction with rabbit antisera was unaffected.[32] This indicated that the antigenic site has a preferred direction towards goat antisera.[32] The other two antigenic sites of lysozyme behaved similarly, in that they exhibited a preferred directionality (see subsequent Sections describing sites 1 and 3). From the findings with antigenic site 3, where longer surface-simulation peptides were synthesized in the two opposite directions,[33] it was quite evident that the preferred direction of the site was not due to an adverse effect of a free α-NH_2 group or a terminal-COOH group on the first and last amino acid residues, Obviously, towards goat antisera, antigenic site 2 has a preferred direction on the surface of the globular protein.[32] The existence of a preferred direction even with goat antisera is significant, in that the antigenic site may be accepted by the antibody combining site only if it is presented in one way.[32] This is indeed striking, in view of the previous proposal[27] that only the side chains should be involved in antigen-antibody interaction. However, the orientation of the side chains will differ in the two peptides and may alter the free energy of binding.[32] Directionality is therefore a function of side-chain orientations.[32] The indifference of rabbit antisera to the change in the direction of this site may reflect a lower conformational specificity by these antisera relative to goat antisera. Since only two rabbits and two goats were studied here, it is unwise to generalize at the pr[illegible]

the species dependency of this site. It may now be cautiously stated that, with the antisera so far studied, the extent of sensitivity to the direction of the surface-simulation synthetic site appears to depend on the antigenic site and, for a given site, may be dependent on the immunized species.[32] Attention should be paid to this in application of the surface-simulation synthesis of antigenic sites (and also other binding sites) in proteins.[32] The synthetic site made in both directions should be examined.[32]

To ascertain that the inhibitory activity of the surface-simulation synthetic site (Table 2) is a true representative of its immunochemical reactivity, we determined[32] the ability of an immunoabsorbent of the site (peptide II in Figure 3) to bind with lysozyme antibodies. The amounts of antibody (from antisera G9 to G10) that was directly bound by the peptide affinity column (see Table 3) accounted for 35.9% and 37.6%, respectively, of the total antibody bound by lysozyme, which was in excellent agreement with the maximum inhibition values of 35.7% and 34.5% exhibited by the free peptide (see Table 2) and with the maximum expected reactivity of the site. It is thus obvious that the maximum inhibition value affords a faithful measure of the immunochemical reactivity of the peptide.[32] This agreement between inhibitory activity and direct antibody binding has also been observed for the surface-simulation synthetic antigenic sites 1 and 3 of lysozyme, for the synthetic antigenic sites of myoglobin,[29,34] and for two large inhibitory fragments of bovine serum albumin.[19,35]

In conclusion, the findings from this laboratory[30,32] clearly show that antigenic site 2 of lysozyme (Figure 3) is constructed from the alignment of the six surface residues:

TABLE 2

Quantitative Accounting of the Three Surface-Simulation Synthetic Sites for the Total Antigenic Reactivity of Lysozyme

	Maximum percentage inhibition			
	Goat antisera		Rabbit antisera	
Site	G9	G10	L7	L21
(A) Reactions with whole antisera				
Site 1	32.5	33.3	22.2	21.3
Site 2	35.7	32.3	12.7	18.2
Site 3	33.3	28.8	19.2	22.2
TOTAL	101.5	94.4	54.1	61.7
(B) Reactions with the IgG fractions of the antisera				
Site 1		32.3	33.4	31.4
Site 2		34.5	28.6	28.9
Site 3		30.3	33.3	35.2
TOTAL		97.1	95.3	95.5

Note: The values are given in maximum percentage inhibition of the quantitative precipitin reaction of lysozyme by each of the synthetic sites independently. The sites were immunochemically independent (see text). The identities of sites 1,2, and 3 are shown in Figure 9. The IgG fractions accounted for 99 to 100% of the total immune reaction of the respective parent antisera.

From Atassi, M. Z. and Lee, C.-L., *Biochem. J.*, 171, 429, 1978. With permission.

Trp-62, Lys-97, Lys-96, Asn-93, Thr-89, and Asp-87. These residues describe an imaginary line which circumscribes part (27.3 Å) of the surface topography of the globular protein. Upon binding with antibody, these residues behave functionally as if in direct peptide bond linkage.[30] In fact, the immunochemical reactivity is fully expressed by a surface-simulation synthetic peptide (peptide II, Figure 3), in which these residues are directly linked via peptide bonds. The site is sensitive to conformational restrictions and has a preferred direction with goat antisera but, surprisingly, not with the rabbit antisera so far tested.[32] Tryptophan 62 is present at the hexasaccharide binding site of the enzyme[36] and, therefore, antigenic site 2 overlaps with the enzymic binding site.[23]

THE PRECISE DELINEATION OF THE ANTIGENIC SITE AROUND THE DISULFIDE 30-115 (SITE 3)

Assignment of the Antigenic Site

From the immunochemical and conformational studies of specific chemical derivatives of lysozyme, it was shown that one (or both) of tyrosines 20 and 23 is located in, or very close to, an antigenic site in lysozyme.[37,38] Also, from three homogeneous succinylated derivatives of lysozyme, which showed no conformational changes, but had a decreased antigenic reactivity with antisera to native lysozyme, it was concluded[25] that both Lys-33 and Lys-116 are parts of an antigenic site. The loss in antigenic reactivity that could be attributed to succinylation of Lys-33 alone (19.9 and 10.9% with antisera G9 and G10, respectively) was lower than observed upon modification of both

TABLE 3

Binding of Radioiodinated Antibodies to Lysozyme by Immunoadsorbents Carrying the Three Surface-Simulation Synthetic Sites[a]

	Antibody from G9		Antibody from G10[b]	
Immunoadsorbent	Amount Ab bound (c.p.m.)	% Ab bound	Amount Ab bound (c.p.m.)	% Ab bound
Lysozyme[c]	50,180	100	73,330	100
Site 1[a]	15,760	31.4	20,335	27.7
Site 2[a]	18,015	35.9	27,600	37.6
Site 3[a]	14,950	29.8	22,300	30.0
Total of independent binding by three sites	48,425	97.1	70,235	95.8
Binding by passage[c] through sites serially	49,880	99.4	71,670	97.7

Note: The specific ^{125}I-labeled antibody fractions from antisera G9 and G10 were isolated on a lysozyme immunoadsorbent prior to use in these studies. The amounts of antibody applied were: G9, 5.21 × 10^4; G10, 7.56 × 10^4 c.p.m. Each value represents the average of four replicate analyses which varied ± 1.3% or less. Results have been corrected for the amount of antibody bound in control experiments using glycine-Sepharose®, histidine-Sepharose® and myoglobin-Sepharose®. Also another set of controls was employed using nonimmune goat ^{125}I-labeled IgG. The amount of nonspecific background binding in the various controls ranged 1 to 3% of the total label applied.

[a] Results for independent binding were obtained by passage of an aliquot of the antibody solution on only one of the immunoadsorbents indicated.

[b] Results from Atassi and Lee.[32]

[c] Results obtained by serial passage of the same antibody sample on the immunoadsorbent of site 1, then site 2, then site 3.

lysines 33 and 116 (33.6 and 31.1% with antisera G9 and G10). This behavior pointed to Lys-33 being at the "end" of the antigenic site[25] and is reminiscent of results obtained with myoglobin derivatives modified at end residues of an antigenic reactive site.[39] Finally, a disulfide peptide corresponding to the sequence 22-33-(Cys 30-Cys 115)-115-116 possessed a substantial inhibitory activity toward the immune reaction of lysozyme and was specifically bound by immunoadsorbents carrying lysozyme antibodies.[17] These findings provided strong evidence that an antigenic site in native lysozyme, incorporating both Lys-33 and Lys-116 and possibly one or both of Tyr-20 and Tyr-23, was situated around the disulfide bond 30-115. Based on these findings, it was suggested[31] that Lys-33 is at one end, and the tyrosine residues are at the other end of the antigenic site.

Surface-Simulation Synthesis of the Antigenic Site

The assignment of the site and description of its location were achieved[31] by application of the surface-simulation synthetic concept which was first devised in the author's laboratory[30] for the delineation of antigenic Site 2 in native lysozyme. Since the three-dimensional structure of lysozyme was known,[8,9,36] its examination revealed that the residues Tyr-20, Try-23, Lys-116, and Lys-33 can be accommodated, with other intervening residues, in an imaginary line (or plane) circumscribing part of the surface topography of the protein.[31] The surface-encircling line passes through the following residues: Tyr-20, Arg-21, Tyr-23, Lys-116, Asn-113, Arg-114, Phe-34, and Lys-33. The distances between these contiguous residues are shown in Figure 4. Two peptides were initially synthesized[31] by direct linking of the spatially adjacent residues constructing the site and employing glycine spacers where necessary (peptides II and III in Figure 4) in order to achieve the correct separations between their side chains. These studies established for the first time that the site was indeed formed by spatially adjacent residues on the surface that are not necessarily in direct peptide bond linkage with one another.[31] The studies also showed[31] that the contribution of Tyr-23 can be fully satisfied by a glycine spacer, which is in agreement with the fact that Tyr-23 is much less exposed than Tyr-20.

More recent studies were designed[33] to obtain a precise determination of the exact boundaries of the antigenic site and its conformational and directional requirements. Several surface-simulation peptides representing various parts of the chemically-assigned surface region were synthesized (Figure 4) and their immunochemistry studied in detail.[33] With each of the antisera studied, peptide I had a substantially lower inhibitory activity than either of peptides II or III. Clearly, therefore, the antigenic site has a preferred direction on the surface of the globular protein molecule. Peptides I and II have the same representation and arrangement of side chains. However, the side chain orientations will be different in the two synthetic directions. Therefore, directionality of site 3 (like that of site 2) is a function of side-chair orientation.[32,33] Obviously, the antibody is quite discriminatory, and attention should be paid to this phenomenon in application of the surface-simulation synthetic concept to define antigenic and other binding sites of proteins.

The immunochemical results[33] showed that deletion of the residue equivalent to Tyr-20 (peptide IV in Figure 4) had no adverse effect on the immunochemical reactivity with any of the antisera. Deletion of Lys-33 while adding back Tyr-20 (peptide V), so that the sizes of peptides IV and V are equal, caused a substantial loss in the immunochemical reactivity of the peptide. It was thus concluded[33] that Tyr-20, like Tyr-23, is not part of the antigenic site, whereas Lys-33 makes an important contribution to the reactivity of the site. With this conclusion, there remained no reason why Arg-21 (which was implicated because it is an intervening residue in the imaginary line between Lys-116, Tyr-23, and Tyr-20) should be part of the site. Investigation of peptide VI

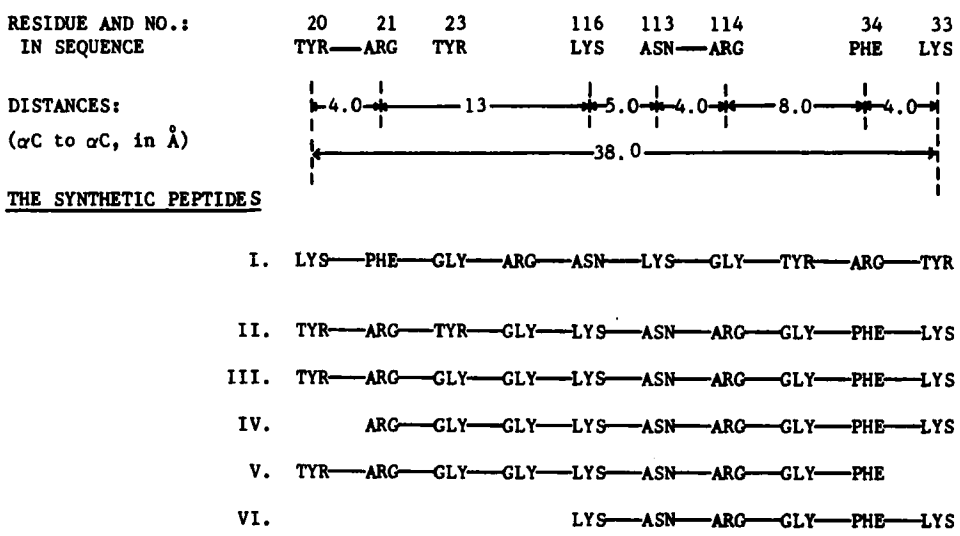

FIGURE 4. Diagram showing the spatially adjacent residues within which antigenic site 3 is located and their numerical position in the primary structure of lysozyme. The distances (in A) separating the consecutive residues of the reactive site are given as C^{α}-to-C^{α} distances, together with the overall extended dimension of the site. Below, the primary structures of the surface-simulation synthetic peptides designed to mimic the antigenic site are given. (From Lee, C.-L. and Atassi, M. Z., *Biochem. J.*, 167, 571, 1977. With permission.)

(in Figure 4) was therefore undertaken,[33] and its immunochemical behavior demonstrated unequivocally that Arg-21 is not part of the antigenic site. It is significant that the immunochemical reactivity of peptide VI accounted quantitatively with those antisera studied for the full contribution of that site.[33] The latter can be derived from the effect of modifying both Lys-33 and Lys-116 in lysozyme.[25]

To ascertain that the inhibitory activity (Table 2) of the surface-simulation synthetic site (peptide VI, Figure 4) afforded a true representation of its immunochemical reactivity, its direct binding of antilysozyme antibodies was examined[33] using an immunoadsorbent of the peptide (see Table 3). The amount of antibody from antisera G9 and G10 bound directly in this manner accounted for 29.8% and 30.0%, respectively, of total antibody bound by lysozyme, which is in excellent agreement with the values of 33.3% and 30.3% found for the maximum inhibitory activity of the peptide with the two antisera (see Tables 2 and 3). Clearly, therefore, the inhibition values (as we have seen with surface-simulation synthetic site 2) provide a faithful measure of the immunochemical reactivity of the peptide.[33]

The Inductive Effect in Modification (or Substitution) of a Residue Close to (but Outside of) an Antigenic Site

Atassi et al. had originally concluded,[37,38] from the immunochemistry of tyrosine-modified lysozyme derivatives that suffered no conformational changes (see Table 1), that one, or both, of Tyr-20 and Tyr-23 is located in, or very close to, an antigenic site in lysozyme. Accordingly, these two tyrosine residues were incorporated into the first surface-simulation synthetic scheme of this site.[31] Subsequently, the aforementioned findings[33] from a very thorough surface-simulation synthesis unequivocally showed that Tyr-20 is not part of the site. Therefore, it is pertinent to briefly present here the rationalization that was recently reported[33] for the findings.

In the three-dimensional structure of native lysozyme, the phenolic ring of Tyr-20

is extremely close (3 to 4 Å) to the hydrocarbon chain of Lys-96 (Figure 5) which, as has already been shown, is a critical residue in antigenic site 2.[23,26,30,32] Nitration of a tyrosine residue at the ortho position affords an anion which is stabilized by the electron-withdrawing mesomeric effect.[21] The increased acidity is shown by a decrease of the pKa value from 10.1 for tyrosine to 7.2 for 3-nitrotyrosine.[40] The pKa value for 3-aminotyrosine is 10.0.[40] Obviously, the presence of a newly created negatively charged group within interaction distance from Lys-96 should be expected to drastically disturb the ionic environment of this lysine residue and, consequently, its immunochemical interaction properties.[33] This effect is completely removed, as in fact was shown[37] to be the case, when the nitrotyrosine residues are reduced to aminotyrosine (see Table 1). If Tyr-20 were indeed part of antigenic site 3, this would mean that sites 2 and 3 will be untenably close. It will then be sterically impossible for two antibody molecules to occupy those two sites simultaneously on a given lysozyme molecule.[33] The fact that Tyr-20 is not part of an antigenic site makes for a more acceptable antigenic structure.[33]

A new facet, hitherto unsuspected in protein chemistry and immunochemistry, was pointed out[33] from the immunochemical effect of nitrating Tyr-20[37,38] in intact lysozyme. Thus, immunochemical changes, observed as a result of the specific modification of a residue in a protein without accompanying conformational changes, do not necessarily imply the participation of the modified residue in an antigenic (or another active) site. This face value interpretation is no longer valid unless independent data lend it additional weight.[33] Furthermore, it is not hard to envisage similar situations being generated by single amino acid evolutionary substitutions outside an antigenic site, but sufficiently close to influence its ionic and binding characteristics.[33] The immunochemical relationships of related proteins from various species are not necessarily directly proportional to sequence similarities and this had previously been consistently attributed[41-44] to considerable or even local and subtle conformational differences.[10,22] Hence, the ionic or inductive effect of a substitution on another very close residue which is a critical part of an antigenic site presented a new factor[33] which must be taken into consideration in the interpretation of the immunochemistry of protein mutants.

In summary, antigenic site 3 is made up[33] of the alignment of the side chains of the five residues Lys-116, Asn-113, Arg-114, Phe-34, and Lys-33. The line described by these residues, which encircles part of the surface topography of the native protein,

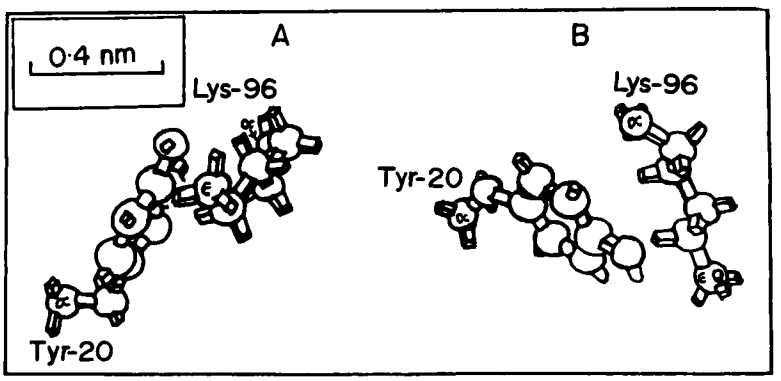

FIGURE 5. Schematic diagram showing the closeness of the phenolic side chain of Tyr-20 to the side chain of Lys-96. The diagram is taken from a constructed model of lysozyme: (a) and (b) represent two views, at right angles to one another, of the same two residues. (From Lee, C.-L. and Atassi, M. Z., *Biochem. J.*, 167 571, 1977. With permission.)

has an overall dimension of 21 Å (taking Cα-to-Cα distances). In interaction with antibody, these residues of the site function as if in direct peptide-bond linkage.[31,33] Site 3 is analogous in spatial construction to antigenic site 2. The carbonyl group of Phe-34 and the side chain of Arg-114 make contact with the hexasaccharide substrate on binding of the latter with the enzyme.[36] Therefore, antigenic site 3 overlaps with the enzymic binding site.[31]

THE PRECISE DELINEATION OF THE ANTIGENIC SITE AROUND THE DISULFIDE BOND 6-127 (SITE 1)

Assignment of the Antigenic Site Chemically and by Classical Synthesis

From the immunochemical and conformational studies on derivatives of the intact protein, it was shown that Asp-119 and Leu-129 were not parts of an antigenic site in native lysozyme.[20,45,46] Also, modification of Trp-123 by 2,3-dioxo-5-indolinesulfonic acid[47,48] or Met-12 (as well as Met-105)[49] by carboxyethylation with β-propiolactone[50] demonstrated that these residues were not located in an antigenic site. The peptide 6-13-(Cys 6-Cys 127)-126-128 carried substantial antigenic reactivity which, with two other disulfide-containing peptides (see Figure 1), jointly accounted for almost all (90%) of the antigenic reactivity of native lysozyme.[17] These results indicated the presence of an antigenic site around the disulfide bond 6-127. On one side of the disulfide, the antigenic site clearly begins after Trp-123 and ends at or before Arg-128. On the other side of the disulfide, the second part of the site must end at, or close to, Met-12.

This degree of delineation, achieved chemically, was critical in that it pointed to the appropriate regions to be synthesized for the final delineation of the site. Atassi et al., therefore, synthesized and studied[49] the immunochemistry of nine disulfide peptides comprising various overlaps of the sequences 3—14 and 125—129 around the disulfide bond 6—127 (see Figure 6). The antigenic site was found[49] to be made up of residues on the two regions (6—14) and (126—128). These studies enabled Atassi et al. to describe the covalent structure of the antigenic site (Figure 7) and from examination of the three-dimensional structure, it was proposed[49] that the residues Arg-14, Lys-13, Glu-7, Ala-10, Gly-126, and Arg-128 and the sulfur of Cys-6 may come in contact with the antibody combining site. Furthermore, Met-12 (as well as Met-105), which in the three-dimensional structure is completely buried,[9,36] is not an essential part of the antigenic site[49] and could be replaced in a synthetic peptide by a glycine without an immunochemical effect.[49]

The aforementioned delineation[49] of antigenic site 1 presented the ultimate level that could be achieved by the state of the art of protein chemistry. Although it afforded an excellent and, until then, unequalled description of an antigenic site composed of spatially contiguous residues, it came a little short of an unequivocal proof, because the residues presumed to be involved in direct binding with antibody (Figure 7) were deduced[49] from examination of the three-dimensional structure of native lysozyme. Thus, the identity of the exact residues constituting the antigenic site was, at best, hypothetical. A more precise definition of the antigenic site was therefore desirable.

Surface-Simulation Synthesis of the Antigenic Site

Rationale for the Design of the Surface-Simulation Peptides

The remarkable success of the surface-simulation synthetic concept in the precise definition of antigenic site 2[30,32] and antigenic site 3[31,33] of lysozyme made it necessary to reexamine[51] antigenic site 1 so that the entire antigenic structure of lysozyme could be defined precisely at the residue level.

The previously proposed[49] antigenic site (Figure 7) describes an imaginary line which

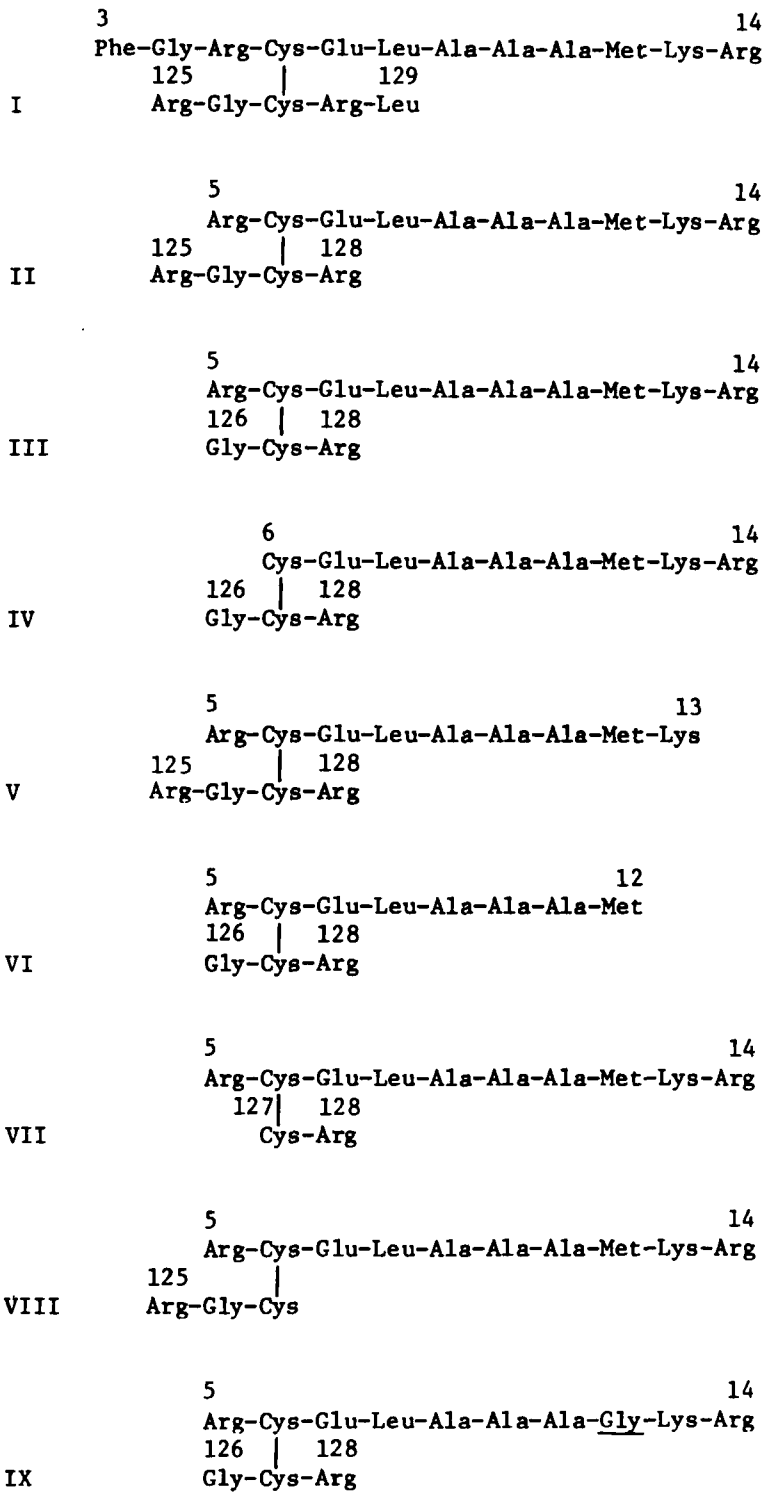

FIGURE 6. Structure of the disulfide peptides that were synthesized to correspond to various regions around the disulfide bond 6-127. Peptide IX is an analogue containing glycine at position 12 instead of methionine. (From Atassi, M. Z., Koketsu, J., and Habeeb, A.F.S.A., *Biochim. Biophys. Acta*, 420, 358, 1976. With permission.)

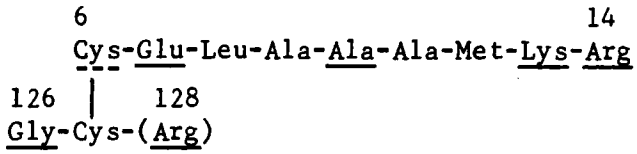

FIGURE 7. Covalent structure of antigenic site 1 that was initially delineated[49] by the classical synthesis of nine disulfide peptides around the disulfide bond 6-127 (see Figure 6.) The residues underlined by a solid line were proposed to be directly involved in the binding with antibody, while the residue underlined by a dotted line may come in contact with antibody. The classical synthetic approach left some uncertainty about the active involvement of Arg-128 in the site. See the text for details. (From Atassi, M. Z., Koketsu, J., and Habeeb, A.F.S.A., *Biochim., Biophys. Acta.* 420, 358, 1976. With permission.)

TABLE 4

Distances Separating Arg-5 from Arg-125 or Arg-128 and Design of Spacers

Separation	Distance C$^\alpha$-to-C$^\alpha$ (nm)	Required glycine spacers	Number of spacers used in synthesis
Arg-5 to Arg-125	0.93	2.57	2 and 3
Arg-5 to Gly-126 to Arg-128	1.63	4.50	4

Note: The distances (in nm) are from C$^\alpha$-to-C$^\alpha$. The number of required glycine spacers in surface-simulation synthesis is based on an ideal C$^\alpha$-to-C$^\alpha$ peptide bond distance of 0.362 nm. For details see text.

(From Atassi, M. Z. and Lee, C.-L., *Biochem. J.*, 171, 419, 1978. With permission.)

encircles part of the surface of the molecule. A careful reexamination of the lysozyme three-dimensional structure showed that Ala-10 is not very likely to be part of the antigenic site described by this imaginary line and Atassi and Lee[51] investigated whether a glycine spacer can fulfill the requirement of Ala-10 and also that of Cys-6. Finally, synthesis of the antigenic site (Figure 7) by a classical strategy[49] could not adequately differentiate whether the antigenic site required Arg-125 or Arg-128, possibly because the folding of the structure shown in Figure 7 may conceivably fulfill either requirement.[51] Accordingly, the distances between Glu-7-to-Arg-128 and Glu-7-to-Arg-125 were measured (Table 4) to determine the number of glycine spacers to be incorporated into the surface-simulation synthetic peptides if Arg-125 (or, alternatively, Arg-128) is part of the antigenic site. It was found[51] that if Arg-125 is part of the site, then approximately 2 glycine spacers are required, but if, on the other hand, Gly-126 and Arg-128 are the essential parts of the site, then approximately 4 glycine spacers are required. It had already been established[32,33] for antigenic sites 2 and 3 that the correct spacing between the residues is critical in the design of surface-simulation synthesis and, in addition, the synthetic surface-simulation sites exhibited a preferred "direction" in their immunochemical behavior. Accordingly, peptides I and II (Figure

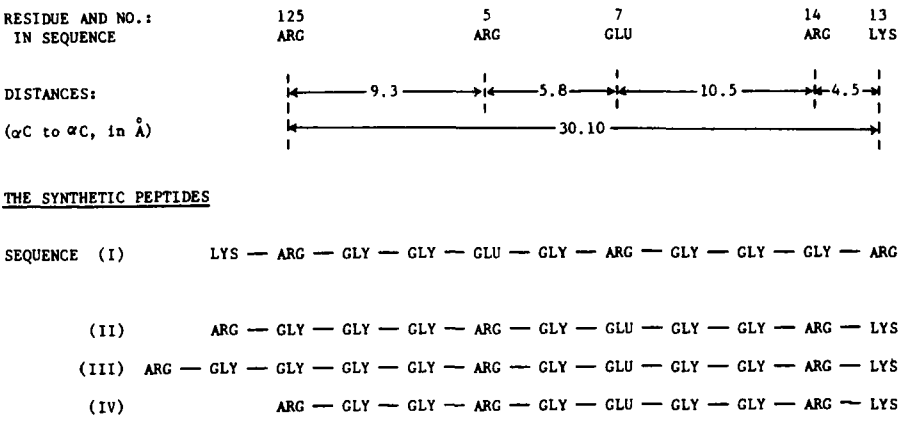

FIGURE 8. Spatially contiguous surface residues constituting antigenic site 1 and their numerical position in the primary structure of lysozyme. The distances (in A) separating the consecutive residues of the site are given as C^{α}-toC^{α} distances together with the overall extended dimension of the site. Below, the primary structures of the surface-simulation synthetic peptides that were designed to copy the site and investigate its directional and conformational requirements are given. For the rationale behind the design of the peptides, see the text. (From Atassi, M. Z. and Lee, C.-L., *Biochem. J.*, 171, 419, 1978. With permission.)

8), of exactly the reverse sequences, were synthesized using three glycine spacers[51] to represent an average distance situation for the involvement of Arg-125 or Arg-128 and to determine the most favorable synthetic direction. This was then followed by design and synthesis[51] of other peptides (Figure 8) having distances of separation corresponding to Arg-125 or Arg-128 and in the correct synthetic direction.

Accurate Definition and Conformational Restrictions of the Site

In immunochemical studies,[51] the surface-simulation synthetic peptide II (Figure 8) was more reactive with rabbit and goat antisera than the reverse sequence represented by peptide I. This established the correct synthetic direction of the surface-simulation site. The spacing between the first two arginine residues from the amino end was then varied, and it was found[51] that the structure with four glycine spacers (peptide III, Figure 8) had, with rabbit and goat antisera, a lower immunochemical reactivity than the peptide which carried two glycine spacers (peptide IV, Figure 8). Atassi and Lee were thus able to conclude unambiguously[51] that the critical residue of the site is Arg-125 and not Arg-128 and that antigenic site 1, like the other two antigenic sites of lysozyme,[32,33] exhibits stringent conformational restrictions.

That the maximum inhibitory activity of the synthetic site (peptide IV, Figure 8) expressed its true immunochemical activity, was shown[51] by the amounts of radioiodinated antibodies to lysozyme that could be bound by a peptide IV-immunoadsorbent (Table 3). The amounts of antibody, from antisera G9 and G10, bound by peptide-Sepharose® constituted 31.4% and 27.7%, respectively, of the total antibody bound by lysozyme. These values were similar to the maximum inhibitory activities (32.5% and 32.3%) of the free peptide with IgG fractions of the two antisera (see Tables 2 and 3).

In summary, antigenic site 1 of native lysozyme is constructed (Figure 9) precisely by the five surface residues:[51] Arg-125, Arg-5, Glu-7, Arg-14, Lys-13. These residues circumscribe part of the surface topography of the protein (Figure 10) and react with antibody as if in direct peptide bond linkage. The synthetic surface-simulation site

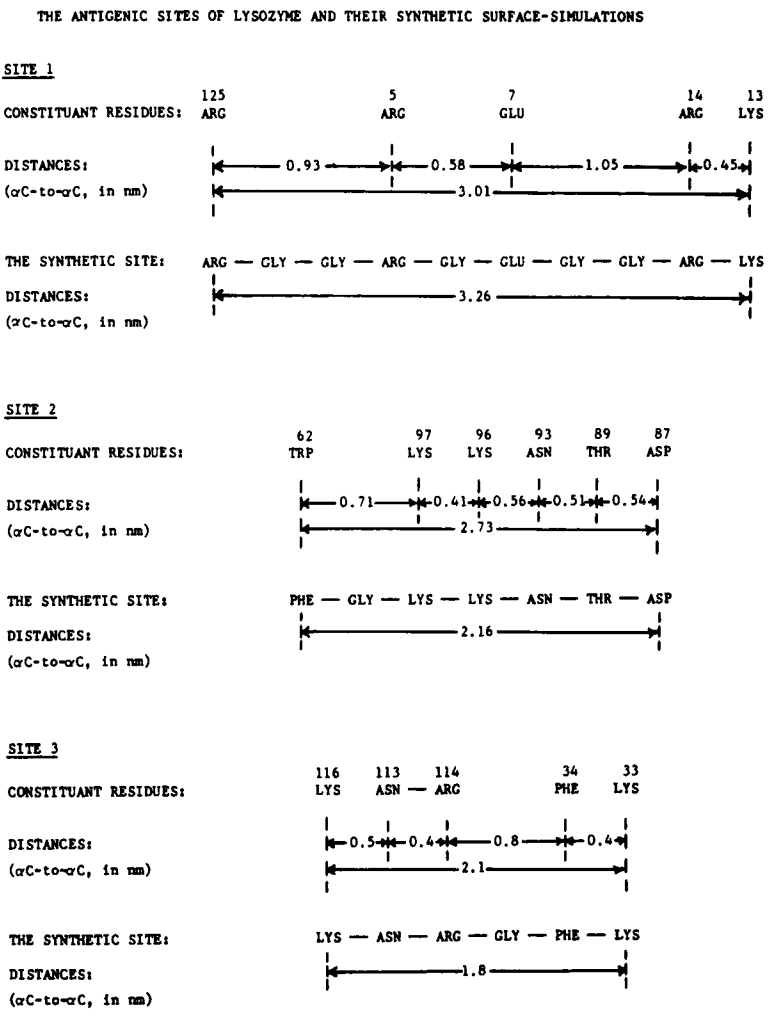

FIGURE 9. The three antigenic sites representing the entire antigenic structure of lysozyme. The diagram shows the spatially contiguous residues constituting each antigenic site and their numerical positions in the primary structure. The distances (in nm) separating the consecutive residues and the overall dimension of each site (in its extended form) are given, together with the dimension of each surface-simulation synthetic site. The latter assumes an ideal C^{α}-to-C^{α} distance of 0.362 nm. The precise boundary, conformational and directional definitions of sites 2, 3, and 1 were described earlier.[32,33,51] The three sites account quantitatively for the entire (96 to 100%) antigenic reactivity of lysozyme. (From Atassi, M. Z. and Lee, C.-L., *Biochem. J.*, 171, 429, 1978. With permission.)

(Figure 9) shows[51] a monodirectional preference (Arg-125 → Lys-13) which appears to be species-independent since it was the same for the two rabbit and the two goat antisera studied. The directionality of the antigenic site is a function of side chain orientations.[51] The site is subject to conformational restrictions and requires the correct residue spacing in its synthetic surface-simulation.[51]

THE PRECISE AND ENTIRE ANTIGENIC STRUCTURE OF LYSOZYME

The determination of the entire antigenic structure of native lysozyme was thus

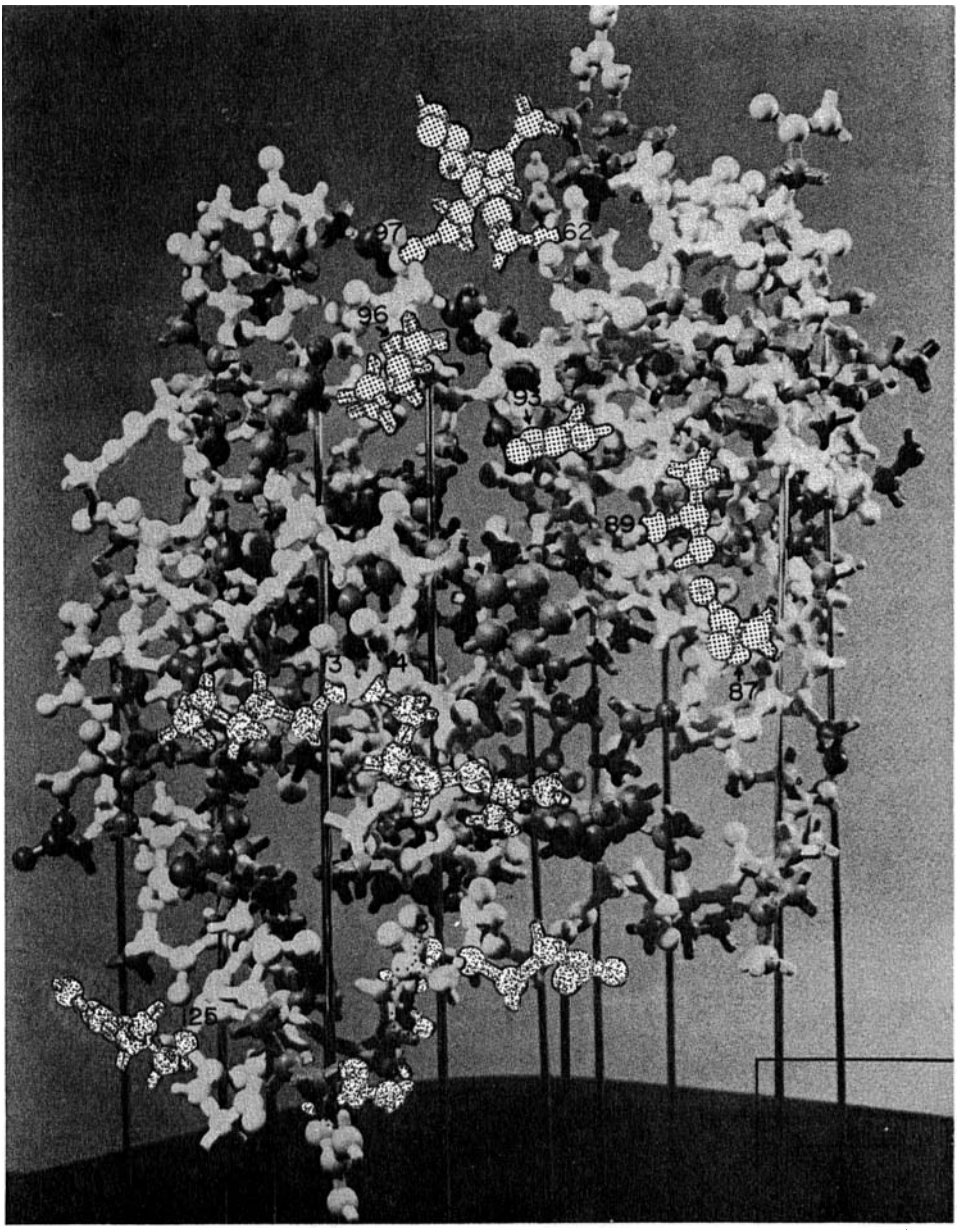

FIGURE 10. Photograph of a lysozyme model showing the relative positions of the residues constituting antigenic sites 1 and 2. The side chains of the residues in the sites are outlined and those making up site 1 are shown as speckled areas, whereas the residues constituting site 2 are shown as dotted areas to avoid confusion. The preferred direction of site 1 (at least by surface-simulation systhesis) is Arg-125 to Lys-13. Site 2 had a preferred direction only with goat antisera (Trp-62 to Asp-87), but exhibited no directional preference with rabbit antisera. (From Atassi, M. Z. and Lee, C.-L., *Biochem. J.*, 171, 429, 1978. With permission.)

achieved by the precise boundary, conformational, and directional definitions of its three antigenic sites by surface-simulation synthesis. In this section, a summary of the main features of the antigenic structure of lysozyme will be presented.

Do the Three Antigenic Sites of Lysozyme Account for its Total Immune Reaction?

To evaluate whether the entire antigenic structure of a protein ha

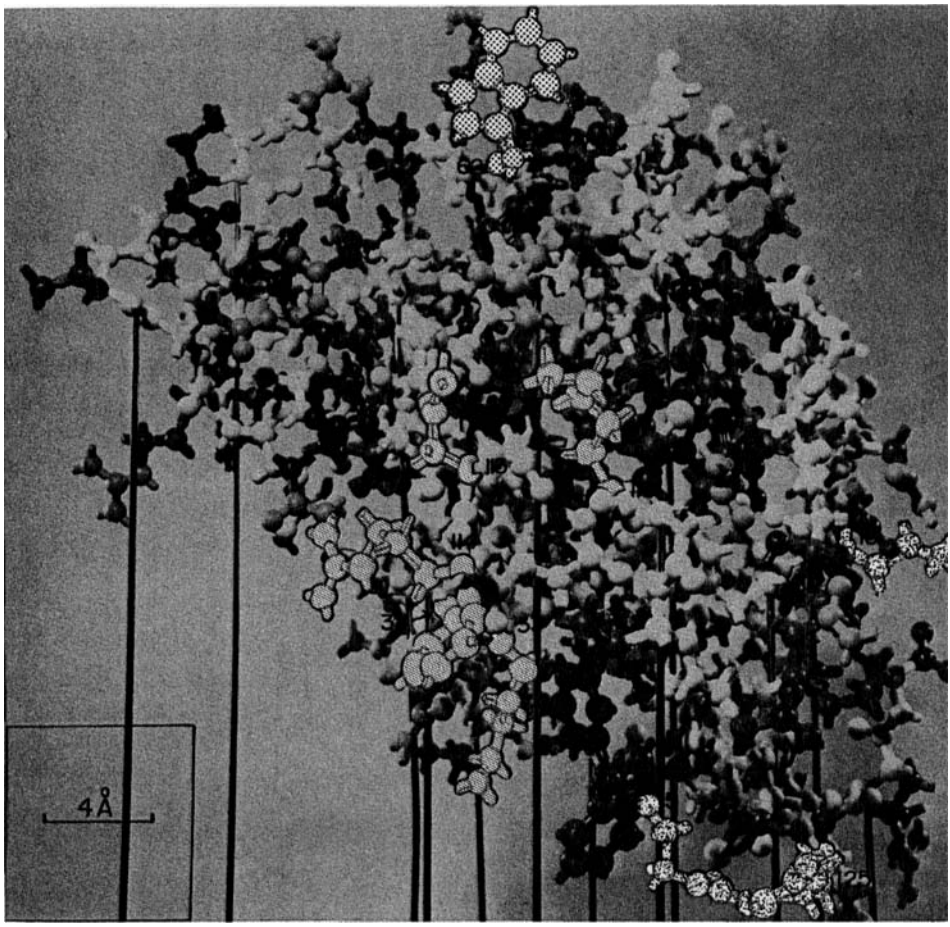

FIGURE 11. Photograph of a lysozyme model showing the position of antigenic site 3 on the molecule relative to sites 1 and 2. The side chains of the residues comprising the sites are outlined. The residues constituting site 3 are shown as striped areas. This view is taken by rotating the model 125° counerclockwise on the vertical axis relative to the view shown in Figure 10. From this perspective, only parts of site 1 can be seen which are the residues Lys-13, Arg-5, and Arg-125, shown as speckled areas. Also, of site 2 only Trp-62 can be seen and is shown as dotted areas. Site 3 showed the same directional preference (Lys-116 to Lys-33) towards rabbit and goat antisera. (From Atassi, M. Z. and Lee, C.-L., *Biochem. J.*, 171, 429, 1978. With permission.)

elucidated, it is vital to determine the extent of the lysozyme immune reaction that can be accounted for by the total reactivities of the three antigenic sites. This is most critical in view of the fact that the delineation process culminated in the identification and synthesis of antigenic sites that are unconventional in character.

A most direct way to answer this question can be derived from the total inhibitory activities of the three sites toward the lysozyme immune reaction. Table 2 summarizes the results from Atassi and Lee[52] obtained with rabbit and goat antisera. With goat antiserum G9 and the IgG fractions from goat antiserum G10 and rabbit antisera L7 and L21, the sum of the inhibitory activites of the three sites were 101.5%, 97%, 95.3%, and 95.5%, respectively. It should be noted here that the IgG fractions accounted for 99 to 100% of the immune reaction of the respective antisera.

Another approach is to determine the amount of lysozyme antibodies that can be specifically bound by the synthetic surface-simulation sites. Table 3 summarizes the binding results of ^{125}I-labelled antibodies from antisera G9 and G10 by immunoadsor-

bents of the three sites.[52] In a single passage of an antibody sample through only one of the site-Sepharose® columns, the three sites effected a calculated total binding, with antibodies G9 and G10, of 97% and 96%, respectively, relative to that bound by lysozyme. Serial passage of a single antibody sample through all three sites (site 1, then site 2, then site 3) removed, from antibodies G9 and G10, 99.4% and 97.7%, respectively, relative to the amount removed by lysozyme. It is critical to point out that the sites were immunochemically independent, since antibody eluted from one site -immunoadsorbent could not be bound by another site-immunoadsorbent, but was adsorbed quantitatively on repassage on to the site-Sepharose® that was initially used for its isolation.[52]

The ability of the surface-simulation synthetic sites (Figure 9) to account for 96 to 100% of the total immune reaction of native lysozyme provides a most powerful demonstration of the correctness of the delineation. This is the more remarkable in view of observations[1,53,54] that an intact antigenic site with no extraneous amino acids would frequently react less than when it is an integral part of a longer peptide. The extraneous residues or segments are often important for the correct folding of the site.[1]

The binding efficiency of synthetic antigenic sites has been discussed in detail earlier for the three surface-simulation sites of lysozyme[30-33,51] and for the synthetic sites of sperm whale myoglobin.[53-57] The large molar excess of peptide required can in part be attributed to the fact that, in solution, these small peptides will exist in unfolded conformational states. Antibodies to native protein antigens are directed against their native three-dimensional structure.[58,59] For proper binding with antibody, an antigenic site must be (at least reasonably approximately) in the shape it has in the native protein.[28,58,59] In the binding process, the antibody induces somewhat its own required conformation on an antigenic site,[2] and the probability for a favorable conformational state improves with increase in peptide concentration.[28] It is to be noted that the surface-simulation peptides do not even exist in native lysozyme, but merely attempt to mimic a spatial arrangement of adjacent surface residues that are mostly distant in sequence. Furthermore, it is not entirely possible to duplicate in surface-simulation synthesis the exact distances separating the residues of the antigenic site in the native protein. Therefore, the mere reactivity of these peptides and the fact that their immunochemical efficiency resembles those of the synthetic sites of myoglobin (which constitute continuous conformationally sensitive portions of the protein[2]) is most remarkable.

A large improvement was observed in the immunochemical efficiency of the peptides in reactions with the IgG fractions of the antisera (see Table 2) as compared to whole antisera. This may have indicated proteolysis and/or binding of the synthetic sites by serum proteins.[32,33,51] Similar observations have previously been made with immunochemically reactive fragments of lysozyme,[17] of bovine serum albumin,[18,19,35] and with the synthetic antigenic sites of myoglobin.[22]

Summary of the Main Features of the Antigenic Structure of Lysozyme

Native lysozyme carries three antigenic sites. The identities of the sites are summarized in Figure 9, and their locations in the three-dimensional structure are shown in Figures 10 and 11.

Site 1 — This antigenic site is formed[51] by the side chains of the spatially contiguous five surface residues which bind with antibody as if in direct peptide linkage: Arg-125, Arg-5, Glu-7, Arg-14, and Lys-13. The Cα-to-Cα dimension of the site (in its extended form) from Arg-125 to Lys-13 is 30 A. The reactivity of this site is fully expressed[51] by the surface-simulation synthetic peptide Arg-Gly-Gly-Arg-Gly-Glu-Gly-Gly-Arg-Lys, which does not exist in native lysozyme. The synthetic site exhibits a directional preference (Arg-125 to Lys-13), which appears to be independent of the species of the

immunized animal, at least with the rabbits and goats tested so far.[51] The site is subject to conformational restrictions, as demonstrated by its sensitivity to variation of the residue spacing in surface-simulation synthesis.[51] The intactness of the disulfide bond 6-127 in native lysozyme is critical for the integrity of this site.[17,49]

Site 2 — This site also consists[30,32] of spatially contiguous surface residues which are: Trp-62, Lys-97, Lys-96, Asn-93, Thr-89, and Asp-87. The overall length of the site (in its extended form) from Trp-62 to Asp-87 (Cα-to-Cα distance) is 27.3 Å. As with site 1, site 2 also forms an imaginary line circumscribing part of the surface topography of the protein.[30] The residues forming the site bind with antibody as if in direct peptide bond linkage.[30] Thus, the surface-simulation synthetic peptide Phe-Gly-Lys-Lys-Asn-Thr-Asp, which does not exist in lysozyme, carries the full reactivity of the antigenic site.[30,32] With the antisera so far studied, the synthetic site exhibits a preferred direction (Trp-62 to Asp-87) towards the goat antisera and none towards the rabbit antisera and has conformational restrictions evidenced by spacing-between-residues requirements.[32] The intactness of the disulfide bonds 64-80 and 76-94 is critical to bring together the various constituent residues of the site.[23] This antigenic site overlaps with the enzymic active site because they both share Trp-62.[26,23]

Site 3 — This site, like sites 1 and 2, is also formed of spatially adjacent surface residues. It comprises[33] the five residues Lys-116, Asn-113, Arg-114, Phe-34, and Lys-33 that describe an imaginary line which circumscribes part (21.0 Å in Cα-to-Cα distance from Lys-116 to Lys-33, in its extended form) of the surface of the molecule. The residues act functionally towards the antibody as if they are in direct peptide bond linkage.[31,33] Thus, the full immunochemical reactivity of the antigenic site is expressed by the surface-simulation synthetic peptide (which does not exist in lysozyme) having the structure Lys-Asn-Arg-Gly-Phe-Lys.[33] The antigenic site exhibited a preferred direction (Lys-116 to Lys-33) with the two rabbit and two goat antisera so far studied,[33] since the reverse surface-simulation synthetic sequence was immunochemically inefficient. The intactness of the disulfide bond 30-115 is critical for the integrity of this antigenic site in lysozyme.[17] Antigenic site 3 overlaps with the hexasaccharide binding site at the carbonyl group of Phe-34 and the side chain of Arg-114.[33]

IMPLICATIONS OF THE SURFACE-SIMULATION CONCEPT

It is quite obvious from the foregoing short treatment that the precise delineation of the entire antigenic structure of lysozyme would not have been possible without our introduction of the surface-simulation synthesis concept. It has already been pointed out[30,31,52] that the remarkable power of this unorthodox concept should not in any way be confined to determination of protein antigenic structures. Some of the potential applications of this concept have been presented[52] and will be outlined here very briefly.

In view of the fact that application of this strategy yields a report on the proximity of several surface residues simultaneously, it will be enormously more informative and precise than studying the availability of certain side chains to chemical modification or physicochemical studies on the protein solution which afford overall shape or conformational parameters. It has already been pointed out[30,31] that the results from this approach on the three antigenic sites of lysozyme afford the most powerful and convincing chemical evidence for the correctness of the three-dimensional structure of lysozyme as derived from the X-ray studies of the crystalline protein.[8,9] Thus, for example, the three antigenic sites of lysozyme (Figure 9) report on the spatial interrelationships of a total of 16 surface residues.

The utility of the immunochemical application of this concept should not be limited to exploitation of protein antigenic sites.[52] Since it has been shown[32,33,51] that the immunochemistry of surface-simulation peptides is quite sensitive to the distance separating the constituent residues as well as to the direction of synthesis (i.e., side chain orientations), this approach is eminently suited for many other investigations.[52] Other parts of the surface could be mimicked into appropriate surface-simulation synthetic peptides linking spatially contiguous residues.[52] The peptides can then be coupled to a suitable carrier and the conjugate used for immunization to enable the preparation of antibodies against the surface-simulation peptides.[52] The antibodies will recognize and react with those surface regions in the native protein, even though the regions are not antigenic sites when the native protein is used as an immunogen.[52] Several surface-simulation peptides could thus be made at will, and the antibodies to these could be employed as conformational probes to double-check the three-dimensional structure of a protein.[52] The approach could also be used to monitor the acquisition of correct residue alignments by various preselected parts of the surface of a protein molecule (or a fragment thereof) upon renaturation of a denatured protein or refolding of a derivative having previously reduced disulfide bonds.[52] This should find wide application by those interested in the mechanism of protein refolding, as it gives a unique and direct region-by-region readout of the surface.[52] Furthermore, should methods for predicting protein conformation from its sequence ever become more reliable, then the predicted three-dimensional structure could be readily double-checked by antibodies to surface-simulation synthetic peptides designed from the predicted structure.[52]

The concept should also be applicable[52] to studies on subunit interactions in oligomeric proteins, to protein-receptor interactions, and to other protein-to-protein interactions. Surface-simulation synthesis of an interacting face should interfere with and enable understanding of the molecular mechanisms of such interactions. This should pave the way to a molecular elucidation of such matters as the mode of hormone action, allergic reactions, soluble factors in immunology, and, indeed, the basis of the immune response.[52] Furthermore, the concept should be applicable in principle to other interactions involving proteins.[52] For example, surface-simulation synthesis should be applicable in certain cases to reconstructing a substrate-binding site of an enzyme and to studying the interactions of some proteins with lipids, carbohydrates, and other prosthetic groups.[52]

It should be stressed that in all these aspects, the three-dimensional structure of the protein under study must be known in detail.[31,32,52] Furthermore, surface-simulation synthesis can only be applied after all the chemical groundwork has been done implicating various residues and parts of a protein molecule in a given binding.[52]

One of the most far-reaching implications in immunology is whether an antibody combining site can be mimicked by surface-simulation synthesis. Recent findings (described in the next section) indicate that this has been achieved for the combining sites of myeloma proteins and it may have also been achieved with two peptides that were complementary to antigenic sites 2 and 3 (Figure 9) of lysozyme. Thus, by mimicking the antibody combining site, at least in terms of binding function, the surface-simulation concept has scored a climax in protein immunochemistry. Although the concept came as a by-product of the determination of the antigenic structure of lysozyme, it should present a major new asset in protein chemistry.

THE POSSIBLE SURFACE-SIMULATION SYNTHESIS OF ANTIBODY-COMBINING SITES TO LYSOZYME ANTIGENIC SITES

The remarkable success of the surface-simulation synthesis concept in reconstructing antigenic sites of spatially contiguous surface residues has suggested the feasibility of

its application in mimicking antibody-combining sites.[60] However, it is evident from the above that the proper application of surface-simulation synthesis to a protein requires the detailed knowledge of its three-dimensional structure and a full chemical identification of the residues constituting a binding site, as well as their precise conformational spacing and directional requirements. Although these requirements are obviously not known for the antibody-combining sites directed against lysozyme, the situation is not entirely hopeless. Atassi and Zablocki[60] reasoned that in all likelihood, the antibody-combining site will be expected to comprise residues (not necessarily in direct peptide bond linkage) that are complementary to those in the antigenic site with which it binds. Furthermore, the directionality and spacings between the residues in the two interacting sites must be equivalent or comparable in order for appropriate binding to take place.[60] Thus, by the precise knowledge of all the parameters of an antigenic site it should be possible to create a reasonable design of its corresponding antibody-combining site. This was done[60] for two antigenic sites in native lysozymes that were then known. Figure 12 shows the residues and spacings constituting each of antigenic sites 2 and 3 of native lysozyme and the corresponding surface-simulation synthetic antigenic sites.

The peptides CS-2 and CS-3 (designed[60] to offer complementarity to antigenic sites 2 and 3, respectively, in ionic, hydrophobic, hydrophilic, and side-chain length of the

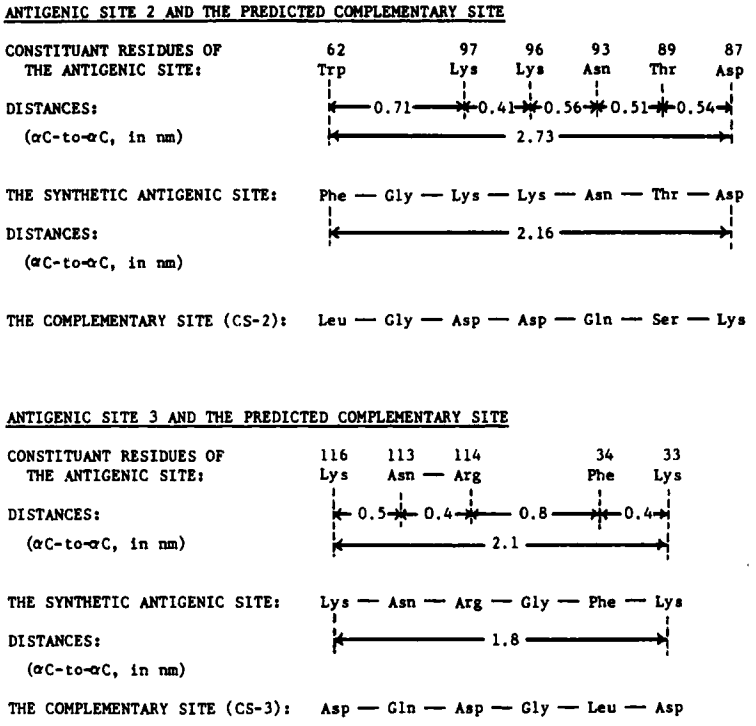

FIGURE 12. A diagram showing antigenic sites 2 and 3 of hen egg white lysozyme and their predicted complementary antibody-combining sites. The spatially contiguous surface residues constituting each antigenic site and the numerical positions of these residues in the primary structure of lysozyme are shown. The distances (in nm) separating the consecutive residues and the overall dimension of each site are given, together with the dimension of the respective surface-simulation synthetic antigenic site. Below each antigenic site is given the structure of the respective complementary surface-simulation peptide which was predicted to mimic the antibody-combining site directed against that antigenic site. (From Atassi, M. Z., and Zablocki, W., *J. Biol. Chem.*, 252, 8784, 1977. With permission.)

constituent amino acids) each exhibited[60] an appreciable inhibitory activity (Table 5) towards the reaction of lysozyme with its antisera. These activities were additive when the peptides were used in combination.[60] Peptide immunoadsorbents[60] bound only lysozyme and not antibody or myoglobin (Table 6). The two peptides had no immunochemical activity in the myoglobin or bovine serum albumin immune systems. Furthermore, three control synthetic peptides of myoglobin, of similar charge but different sequence, had no inhibitory effect on the lysozyme immune reaction.[60] Thus, the antibody-combining sites against antigenic sites 2 and 3 of native lysozyme were successfully mimicked synthetically, at least in terms of binding function.

Atassi and Zablocki have indicated[60] that the residues in peptides CS-2 and CS-3 were not implied to represent the actual residues brought together in the binding sites of the antibodies by the three-dimensional folding of the latter. This is difficult to know. Also, they have emphasized[60] that the functional success of the peptides designed here does not imply a unique antibody site to each lysozyme antigenic site. Other complementary amino acids may serve equally well in the antibody molecule. For example, the role of leucine may be satisfied by isoleucine, valine, phenylalanine, etc., but that has not been tested yet. By employing related alternatives to each residue, it will not be difficult to rationalize differences in affinity and heterogeneity of the antibody-combining site. Antibodies to these synthetic surface-simulations combining sites are now being made by immunization after coupling to appropriate carriers. These studies will reveal whether or not the antibody-combining site and the idiotypic determinants incorporate the same residues on the antibody molecule. The results will be reported in detail elsewhere.

To test whether or not the success of the present approach represents a special situation, complementary surface-simulation peptides to antigenic site 1 of lysozyme and the antigenic sites of myoglobin are now being studied. Very recently, the binding sites in two myeloma proteins, towards phosphorylcholine and a hydroxyl derivative of vitamin K_1 which are known from X-ray crystallographic studies,[2,51,61-63] were successfully mimicked in this laboratory by surface-simulation synthesis.[63a,63b]

CONCLUSIONS

Many general conclusions relating to antigenic structures of proteins were derived[1,2,22] from accurate mapping out of the antigenic structure of sperm whale Mb. The recent precise determination of the entire antigenic structure of lysozyme which is outlined briefly here has shown that all these conclusions from myoglobin are also applicable to lysozyme. These include: the small size of the antigenic sites and their sharp boundaries, their presence only in a limited number, their surface locations, and their sensitivity to conformational changes. Although the share of a given antigenic site to the total reactivity of the protein varies with the antiserum, the same antigenic sites are recognized by rabbit and goat antibodies to the native protein. For these conclusions and many other molecular features of antigenic sites and factors influencing the cross-reaction of proteins, as well as for specific recommendations concerning the chemical strategy of approach, the reader may consult earlier references.[1,2,11,22]

A fascinating difference was found to exist between the antigenic sites of myoglobin and those of lysozyme. The five antigenic sites of myoglobin are made up of residues that are in direct peptide bond linkage, whereas the three sites of lysozyme each constitutes spatially adjacent surface residues that are mostly distant in sequence, each describing a line which circumscribes part of the surface topography of the protein.[30,32,33,51,52] As previously stated,[30] "in effect, therefore, an antigenic site is like a specific short ribbon of residues poised upon the surface of the protein molecule. The residues may either be directly linked to one another like in myoglobin[2] or be so

TABLE 5

Inhibitory Activities by the Pure Synthetic Complementary Sites (Figure 12) and Comparison with Activities of the Respective Antigenic Sites

	Complementary site CS-2		Antigenic site 2		Complementary site CS-3		Antigenic site 3	
Antiserum[a]	Maximum inhibitory activity (%)[b]	Molar ratio at ½ maximum inhibition[c]	Maximum inhibitory activity (%)[b]	Molar ratio at ½ maximum inhibition[c]	Maximum inhibitory activity (%)[b]	Molar ratio at ½ maximum inhibition[c]	Maximum inhibitory activity (%)[b]	Molar ratio at ½ maximum inhibition[c]
G9	23.9	165	35.7	170	19.5	103	33.3	40
G10	19.3	70	34.5	170	26.0	73	30.3	200

[a] G9 and G10 are goat antisera, each against native lysozyme. Reactions were carried out with the IgG fractions of antisera which accounted for 99 to 100% of the immune reaction of the respective parent antiserum.
[b] Maximum percentage inhibition of the precipitin reaction of native lysozyme. Each value is the average of six replicate determinations which varied ± 0.8% or less.
[c] Indicate the peptide/lysozyme molar ratio at 50% of the maximum inhitibion.

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TABLE 6

Binding Studies by Immunoadsorbents of the Two Complementary Surface-Simulation Synthetic Sites

Immunoadsorbent	Lysozyme, antibody or myoglobin bound (c.p.m.)		
	^{125}I-Lysozyme	^{125}I-Antibody to Lsozyme	^{125}I-Myoglobin
Complement site CS-2	104,205	884	963
Complement Site CS-3	110,300	915	848
Control*	825	910	852

Note: The amount applied of each sample was 1.3×10^5 c.p.m. The specific ^{125}I-labeled antibody to lysozyme was prepared from goat antiserum G10. Each value represents the average of triplicate determinations which varied ± 1.2% or less.

* Average of triplicate determinations each on an immunoadsorbent carrying myoglobin and an immunoadsorbent carrying the synthetic peptide 15—22 of myoglobin.

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spatially arranged on the surface so that they behave functionally (towards the antibody) as if they are directly linked." Whether a given protein will carry one type or the other of antigenic sites or a mixture of both will depend on the protein.[30] The factors that determine the type of a site in a protein are not too clear, but one important factor may be dependent on the stabilization, or otherwise, of the structure by internal disulfide cross-links.[31]

Even though Atassi and Saplin had previously suggested[28] the existence of protein antigenic sites made up of spatially adjacent surface residues that are distant in sequence, their identification and precise definition in lysozyme[30-33,51] is the first such example in protein immunochemistry. A common feature to those two types of antigenic sites is that they occupy exposed regions on the surface topography of the respective protein,[30] and this will most likely be the situation with all antigenic sites in native proteins. It should be stressed that the antigenic sites both in myoglobin and in lysozyme are sensitive to conformational changes in the respective protein, with those of lysozyme showing, as expected, a much higher sensitivity. Accordingly, it is totally inadequate to identify the antigenic sites of myoglobin by the terms "linear", "sequential", or "primary", or some such terms, while identifying the antigenic sites of lysozyme by the terms "spatial", "conformational", etc. The author would like to propose that antigenic sites of the type seen in myoglobin[2] and hemoglobin[64] be named "continuous sites" which implies that they consist of conformationally distinct continuous surface portions of the polypeptide chain. For antigenic sites of the type seen in lysozyme, the term "discontinuous sites" will be appropriate. A "discontinuous site" is made up of conformationally (or spatially) contiguous surface residues that are totally or partially not in direct peptide bond linkage.

It should be relevant to comment here on the antibody-combining site that will be complementary to the antigenic sites of myoglobin and lysozyme. The dimensions of the extended antigenic sites of sperm whale myoglobin[2,22] range between 20 and 24 Å. This is quite similar to the sizes of the lysozyme antigenic sites in their

which are 30, 27, and 21 Å, respectively.[30,32,33,51] Since the antigenic sites on the protein are not in the extended form, the actual dimensions of the sites will be smaller than the values given. Nevertheless, the size of the antibody-combining sites required for the two proteins will be somewhat larger than the combining site for haptens as determined by X-ray crystallography. This is seen in the combining site of the Fab′ fragment of protein New (a human IgG) towards a hydroxyl derivative of vitamin K_1;[62,63] of the Fab′ fragment of a myeloma protein from mouse (McPc 603) towards phosphorylcholine[61] and a Bence-Jones protein (λ chain) dimer.[65,66] The latter revealed a conical cavity 10 Å deep with an opening of 15 Å connected to a pocket of 17 Å, and the part of the combining site involved in binding depended on the size and structure of the bound hapten. Therefore, the antibody-combining site can vary to provide maximum complementarity to the antigenic site with which it binds. With proteins, unlike haptens, the antigenic site will be expected to fill the entire antibody-combining site.[52] Thus, the above dimensions found for combining sites that accommodate haptens may represent an artifical situation, and the combining sites towards protein antigenic sites will be larger.[52] This should be readily achieved by the flexibility of the hypervariable region.

Examination of the antigenic sites of myoglobin and lysozyme shows that interactions with antibody are predominantly polar in nature with considerable stabilizing effects being contributed by hydrophobic interactions and some hydrogen bonding. Clearly, the arrangement of the residues in the imaginary surface-encircling line bearing the aligned residues is highly critical, but it is not obvious why certain residue arrangements are antigenic while others are not. It is wise to caution here that the sequence and three-dimensional features that confer immunogenicity on given parts or surface areas of a protein molecule are still not very clear.[2]

The three antigenic sites of lysozyme have recently been discussed in a very detailed treatment from an evolutionary viewpoint in connection with the immunochemical relationships of various bird lysozymes and of α-lactalbumin.[10] It may be mentioned here that the immunochemical relationships of various lysozymes or other protein mutants are not necessarily linearly related to sequence similarities, but are affected by conformational readjustments due to substitutions in the antigenic sites or elsewhere in the molecule,[41-44] as well as by the ionic or inductive effect of a substitution outside a site on a very close residue within an antigenic site.[33] In the latter case, conformational readjustments need not be present.[37,38,33]

For lysozyme as well as myoglobin, it has been shown that antibodies raised in rabbits and goats to a given protein recognize the same antigenic sites on that protein. In myoglobin, the antigenicity of the sites is inherent in their three-dimensional locations irrespective of any sequence differences between the injected myoglobin and the myoglobin of the immunized animal.[67] Furthermore, the successful extrapolation of the antigenic structure of sperm whale myoglobin to human hemoglobin[64] and the ability to produce in rabbits autoantibodies to rabbit myoglobin[68] strongly demonstrated that the antigenicity of certain parts of a protein molecule is primarily dependent on the uniqueness of their conformational location.

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