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Distance Calculation of Residues Neighbouring to Lysozyme Antigenic Sites

SITE-NEIGHBOURING RESIDUES WHOSE EVOLUTIONARY SUBSTITUTION CAN MODIFY THE CHARACTERISTICS AND BINDING ENERGY OF THE SITES

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By using the antigenic structure of lysozyme determined in this laboratory and the X-ray co-ordinates we have calculated the closest-atom distances between each of the residues in the three antigenic sites and all the other amino acids of the lysozyme molecule. These calculations enabled us to identify the nearest neighbours to each of the site residues. Thus the immediate environment of each site residue is described. For the three antigenic sites there is a total of 71 neighbouring residues. The effects of evolutionary amino acid substitutions in site-neighbouring residues on the binding capacity of protein binding sites in general and on protein antigenic sites in particular are discussed. These, together with the direct replacements in site residues, will account for the major effects. However, the limitations of this treatment are stressed. The smaller effects on antigenic sites of replacements at once-removed and even at more distant locations, which, when they become cumulative, could be considerable, are brought to attention, together with any influences of conformational readjustments that can take place as a result of evolutionary amino acid replacements.

In very early reports from this laboratory (Atassi, 1968; Atassi & Thomas, 1969) and periodically in review articles (Atassi, 1972, 1975, 1977*b*) it was stressed that 'the environment of a residue influences its involvement in an antigenic site. The environment will be the immediate sequence around the residue in question as well as the approaching folds of the polypeptide chain from otherwise distant regions in the sequence'. Subsequent to our determination of the entire antigenic structures of sperm-whale myoglobin (Atassi, 1975) and of hen egg-white lysozyme (Atassi, 1978), several studies have attempted to correlate the cross-reactions of myoglobins or lysozymes from various species with the antisera to sperm-whale myoglobin or to hen lysozyme respectively. From our earlier studies (Atassi, 1970; Atassi *et al.*, 1970*a*,*b*; Habeeb & Atassi, 1971) we had shown that conformational readjustments can take place as a result of evolutionary amino acid replacements and that these readjustments could have a detrimental effect on the binding capability of an antigenic site. More recently we identified (Lee & Atassi, 1977) the first example of a detrimental electrostatic inductive effect resulting from a substitution outside of, but close to, an antigenic site. Thus environmental changes were experimentally shown to be an important factor that could have a potentially destructive effect on the binding capacity of the site.

In the present paper we have calculated from the X-ray co-ordinates of hen lysozyme the minimum distances between the closest atoms of each residue of the antigenic sites and all the residues in the lysozyme molecule. This has enabled us to identify the nearest neighbours to each site residue, which thus describe its immediate environment and which when any of them undergoes an evolutionary amino acid replacement could influence to various degrees the reactivity of the antigenic site.

Methods

The identities of the three antigenic sites of hen lysozyme have been reported (Atassi, 1978). The computations of inter-residue distances were performed on an Interdata computing system by

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employing the 0.2nm (2Å) atomic co-ordinates of lysozyme (Blake *et al.*, 1967; Imoto *et al.*, 1972; Feldman, 1976).

Interatomic distances were computed for all possible atom pairs (excluding H and peptide C, N and O) in which one member of the pair is contributed by a residue within a particular antigenic site and the other member by residues outside that site. The minimum distances occurring between residues thus compared were selected and the atoms of the respective residues contributing to these distances were identified. Therefore, for each of the residues in the three antigenic sites of lysozyme, their distances from all other residues in the molecule were calculated. The computation time per site residue for one complete iteration was about 35min. Thus for all 16 residues in the three antigenic sites the total computation time was 560min.

Results

The minimum interatomic distances between each of the site residues and the remainder of the residues in the molecule are given graphically in Figs. 1–3. These Figures will help to illustrate the complexity of the environment of each site residue and the number of residues that contribute to this environment. The Figures provide an attempt to represent a three-dimensional concept on a two-dimensional plot. Such a presentation will obviously have its shortcomings. Also, in these diagrams the minimum interatomic distances are plotted irrespective of whether any of the interactions are obstructed by intervening third-party atoms(s).

To simplify the treatment and better evaluate the major influences that effectively make up the environment of each site residue, we have identified the amino acids in which any atom of their side chains falls within 0.70nm (7.0Å) from any atom on the side chain of the site residue. Tables 1, 2 and 3 list the nearest-neighbour residues for antigenic sites 1, 2 and 3 respectively. Since many of these environmental residues influence more than one residue in a given antigenic site, we have presented the information in a cross-tabulation manner. This has the distinct advantage of identifying neighbouring residues

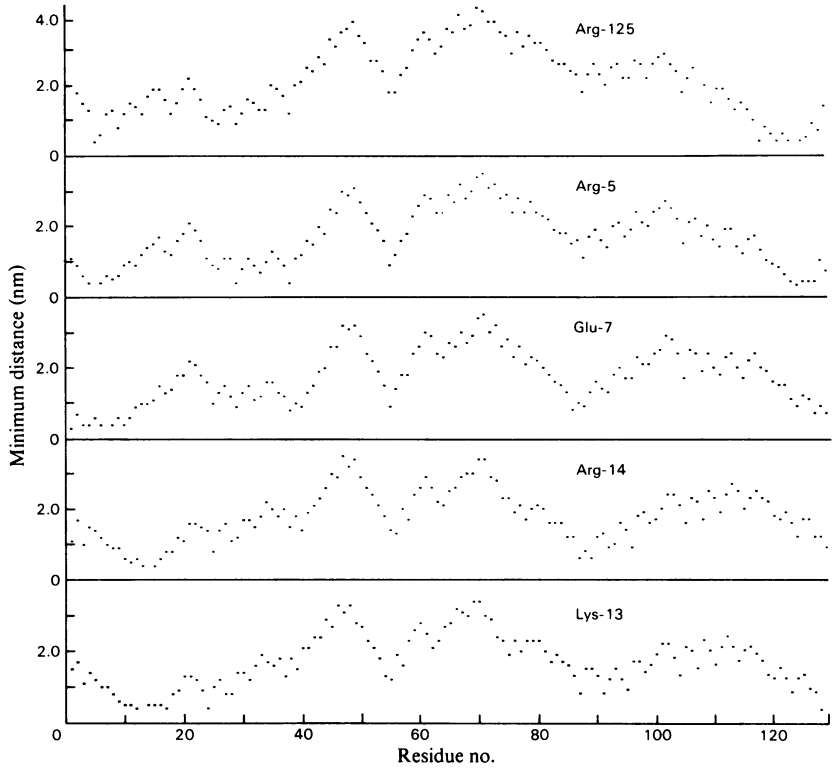

Fig. 1. *Two-dimensional graphical representation of the minimum distances between the residues of the lysozyme molecule and each of the residues in its antigenic site* 1

These are nearest atom distances and are shown without regard to obstruction by other residues. For discussion see the text.

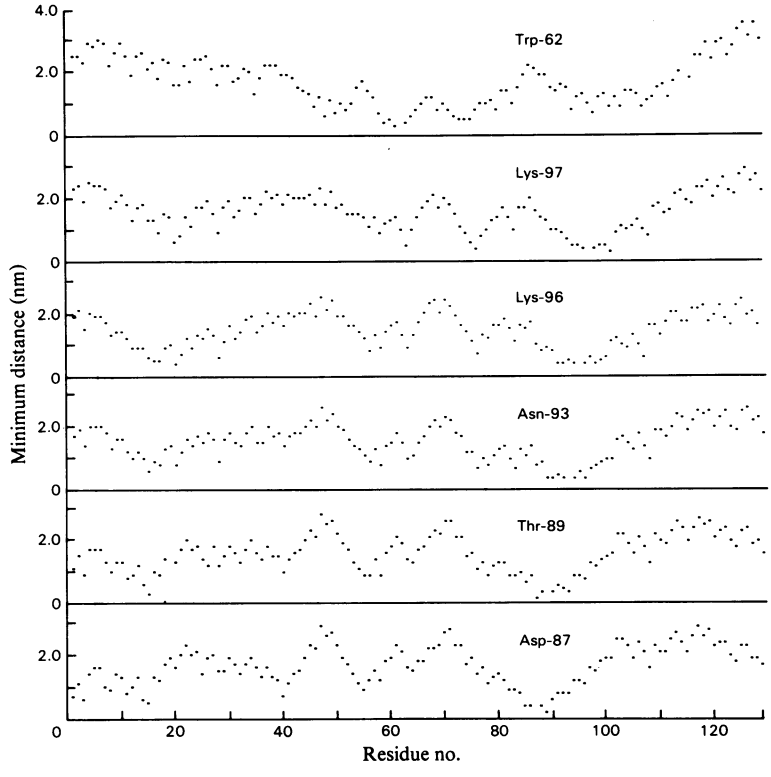

Fig. 2. *Graphical representation of the minimum distances between each of the residues in antigenic site* 2 *and all other amino acids of the lysozyme molecule*

which can exert their influence on more than one residue in a given site. Obviously the evolutionary substitution of such residues with multiple influences will have greater effects on a site than the substitution of a neighbouring residue that exerts its influence on only one residue in that site. The former substitution should weigh more heavily in the analysis of immunochemical cross-reactions. In addition, we have identified each neighbouring residue according to a subjective classification (Imoto *et al.*, 1972) that indicates their relative exposure to the surface in the lysozyme molecule.

Some neighbouring residues influence the environment of more than one antigenic site, and these are summarized in Table 4. Their substitution should therefore be expected to have a greater effect on the immunochemical cross-reactivity than substitutions that can influence only one site. Finally, we have identified in the Tables the residues whose effect on a given site residue is obstructed to any extent by third-party atom(s). Such once-removed interactions will in general have smaller effects than unobstructed interactions, when changed by substitution. However, the magnitude of the effect will depend on the site residue, on the nature and extent of the obstruction, in the neighbouring residue and on its replacement, and finally on their contribution to the network in the environmental sphere of influence (see the Discussion section).

Discussion

Of the variety of studies that have analysed the locations of the evolutionary replacements with respect to the regions of the antigenic sites, the careful analysis performed by Romero-Herrera *et al.* (1978) of a large number of myoglobins is extremely important. These authors found that the evolutionary replacements were essentially random with respect to the antigenic sites of myoglobin. In other words, there was no meaningful difference between the number of replacements within the sites and the number outside the sites when the overall size of the sites relative to the size of the myoglobin molecule is taken into account. So, clearly, mutations should not be expected to occur selectively within or outside of the sites. Analysis of immunochemical cross-reactions of various myoglobins or lysozymes have

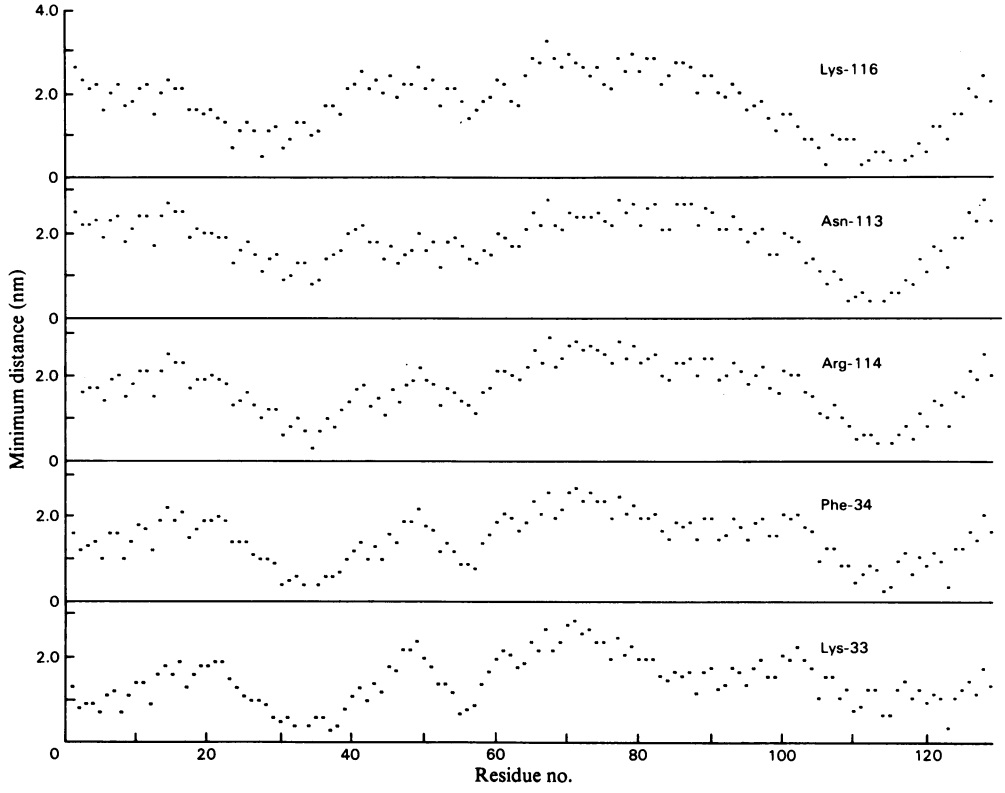

Fig. 3. *Graphical representation of the minimum distances between each of the residues in antigenic site* 3 *of lysozyme and each of the residues in the protein molecule*

Table 1. *Neighbouring residues to antigenic site* 1 *of lysozyme*

Nearest-neighbour residue	Relative exposure*	Minimum distances and closest atoms to residues in antigenic site 1 (nm)†				
		Arg-125	Arg-5	Glu-7	Arg-14	Lys-13
Lys-1	E			0.30 NZ-OE2		
Val-2	E			0.70 CA-OE1‡		
Phe-3	S		0.63 CB-CA‡	0.39 CD1-CD		
Gly-4	E		0.38 CA-CA	0.42 CA-CB		
Cys-6	S	0.61 SG-CA	0.38 CA-CA§	0.38 CA-CA§		
Leu-8	I		0.51 CB-CA‡	0.38 CA-CA§		
Ala-9	I		0.59 CB-NH1‡	0.55 CA-CA		0.58 CA-CB
Ala-10	E			0.42 CB-CA	0.60 CA-CB	0.45 CA-CB
Ala-11	S			0.59 CB-CA	0.44 CA-CB	0.52 CA-CA
Met-12	I				0.55 CA-CA	0.38 CA-CA§

Table 1—*continued*

Nearest-neighbour residue	Relative exposure*	Minimum distances and closest atoms to residues in antigenic site 1 (nm)† Arg-125	Arg-5	Glu-7	Arg-14	Lys-13
His-15	E				0.38 CA-CA§	0.51 CA-CA
Gly-16	E				0.55 CA-CA	0.51 CA-CA‡
Leu-17	I					0.53 CB-CA
Asp-18	E					0.39 CB-CG
Leu-25	S					0.37 CD1-CG
Val-29	I		0.44 CG1-CG			
Lys-33	S		0.70 CD-CB			
Phe-38	S		0.37 CE2-CA			
Asp-87	E				0.59 OD2-NH2	
Thr-89	E				0.61 OG1-NH2	
Asp-119	E	0.36 OD1-NH2				
Gln-121	E	0.56 CB-NE				
Ala-122	E	0.38 CA-NE	0.59 CA-NH1‡			
Trp-123	S	0.58 CA-CA	0.35 CA-NH1			
Ile-124	S	0.38 CA-CA§	0.30 CA-NH1‡			
Gly-126	E	0.38 CA-CA§	0.44 CA-NH2			
Cys-127	I	0.50 SG-CA	0.35 SG-NH1	0.70 SG-CA‡		
Leu-129	S	0.70 CD1-CA	0.69 CD1-NH1‡			0.27 02-NZ

* I, S and E refer to internal, surface and exposed residues respectively (taken from Imoto *et al.*, 1972).
† The first atom notation refers to the neighbouring residue and the second atom refers to the site residue.
‡ These interactions are obstructed by third-party atom(s).
§ These residues are linked by a peptide bond.

Table 2. *Neighbouring residues to antigenic site 2 of lysozyme*

Nearest-neighbour residue	Relative exposure*	Minimum distances and closest atoms to residues in antigenic site 2 (nm)† Trp-62	Lys-97	Lys-96	Asn-93	Thr-89	Asp-87
Phe-3	S						0.58 CZ-CA
Arg-14	E					0.61 NH2-OG1	0.59 NE2-OD2
His-15	E			0.58 CA-NZ	0.57 CD2-OD1	0.30 NE2-OG1	0.48 NE2-OD1
Gly-16	E			0.51 CA-CE			
Leu-17	I			0.46 CD2-CG			

Table 2—*continued*

Nearest-neighbour residue	Relative exposure*	Minimum distances and closest atoms to residues in antigenic site 2 (nm)†					
		Trp-62	Lys-97	Lys-96	Asn-93	Thr-89	Asp-87
Tyr-20	S		0.59 OH-CA‡	0.36 CE1-CD			
Arg-21	E			0.69 CG-CA			
Trp-28	I			0.56 CH2-CA			
Asn-48	E	0.63 ND2-NE1					
Ser-50	S	0.70 CB-CD1					
Ile-58	I	0.70 CG2-CA‡					
Asn-59	S	0.39 OD1-CD1‡					
Ser-60	I	0.53 CA-CA					
Arg-61	E	0.33 CG-NE1					
Trp-63	S	0.38 CA-CA§	0.47 CZ3-CB				
Cys-64	I	0.61 CA-CA‡					
Ser-72	S	0.56 CA-CA					
Arg-73	E	0.45 NH1-CZ3					
Asn-74	E	0.54 CA-CA					
Leu-75	S	0.46 CD2-CA	0.56 CB-CD				
Cys-76	S		0.43 SG-CB		0.68 SG-CA		
Ser-85	E						0.42 OG-CA
Ser-86	E						0.38 CA-CA§
Ile-88	S					0.38 CA-CA§	0.38 CA-CA§
Ala-90	E				0.42 CA-CB	0.38 CA-CA§	0.61 CA-OD1‡
Ser-91	I				0.54 CA-CA‡	0.55 CA-CA‡	
Val-92	S			0.40 CG1-CE‡	0.38 CA-CA§	0.46 CB-CA	
Cys-94	I		0.46 CA-CB	0.52 CA-CA‡	0.38 CA-CA§		
Ala-95	I		0.54 CA-CA	0.38 CA-CA§	0.56 CA-CA‡		
Ile-98	I		0.38 CA-CA§	0.55 CA-CA‡			
Val-99	I		0.54 CA-CA	0.39 CG1-CA			
Ser-100	S		0.46 CB-CA	0.51 OG-CA‡			
Asp-101	E		0.31 OD1-CA‡	0.60 OD1-CA‡			
Trp-108	S			0.64 CH2-CA‡			

*, †, ‡, § For footnotes see Table 1.

Table 3. *Neighbouring residues to antigenic site 3 of lysozyme*

Nearest-neighbour residue	Relative exposure*	Minimum distances and closest atoms to residues in antigenic site 3 (nm)†				
		Lys-116	Asn-113	Arg-114	Phe-34	Lys-33
Leu-8	I					0.65 CD2-CA
Asn-27	S	0.53 OD1-CA				
Val-29	I					0.57 CG1-CB‡
Cys-30	I			0.58 SG-CB‡	0.41 SG-CD1	0.45 CA-CB
Ala-31	I				0.54 CA-CB	0.56 CA-CA
Ala-32	I				0.56 CA-CA‡	0.38 CA-CA§
Glu-35	S				0.38 CA-CA§	0.56 CA-CA‡
Ser-36	I				0.57 CA-CA‡	0.29 CA-CA‡
Asn-37	E				0.57 CA-CA	0.56 OD1-NZ
Phe-38	S					0.36 CE1-CA
Ile-55	I					0.70 CG1-CA‡
Asn-106	E	0.29 OD1-NZ				
Val-109	E		0.36 CG2-ND2			
Ala-110	S		0.49 CA-CB	0.45 CA-CG	0.46 CB-CB	
Trp-111	S	0.32 CH2-CD	0.57 CA-CA‡	0.55 CA-CA‡	0.65 CA-CB	
Arg-112	E	0.40 CA-CB	0.38 CA-CA§	0.59 CA-CA‡		
Cys-115	I	0.38 CA-CA§	0.61 CA-CA‡	0.38 CA-CA§	0.42 SG-CD1	0.66 SG-CB‡
Gly-117	E	0.38 CA-CA§				
Thr-118	S	0.47 CG2-CA		0.54 CG2-CA		
Val-120	S	0.57 CG1-CA‡				
Trp-123	S				0.36 CZ2-CE1	0.37 CH2-CG

*, †, ‡, § For footnotes see Table 1.

Table 4. *Residues neighbouring more than one antigenic site*

Nearest-neighbour residue	Closest residues in antigenic sites*		
	Site 1	Site 2	Site 3
Phe-3	Arg-5, Glu-7	Asp-87	
Leu-8	Arg-5, Glu-7		Lys-33
His-15	Lys-13, Arg-14	Asp-87, Thr-89, Asn-93, Lys-96	
Gly-16	Lys-13, Arg-14	Lys-96	
Leu-17	Lys-13	Lys-96	
Val-29	Arg-5	Lys-33	
Phe-38	Arg-5	Lys-33	
Trp-123	Arg-5, Arg-125		Lys-33, Phe-34

* For distances between and identities of nearest atoms see Tables 1–3.

shown that the cross-reactions are not always explained in terms of drastic replacements within the sites (Atassi, 1977*b*; Atassi & Habeeb, 1977). We have atrributed this (Atassi, 1970, 1972, 1975, 1977*b*; Atassi *et al.*, 1970*a*,*b*) to destructive conformational and environmental influences of substitutions outside the sites. Other studies (Hurrell *et al.*, 1977) have also suggested that environmental changes resulting from amino acid substitutions outside the sites can alter the binding capacity of residues(s) in the sites. On the other hand, other workers (Ibrahimi *et al.*, 1979) have explained the influences of substitutions outside the antigenic sites by alternatively considering the possibility of the existence of antigenic sites other than those that we have determined in myoglobin and lysozyme. This latter explanation is very unlikely in view of the fact that with several antisera the activity of the antigenic sites or the sum of the amounts of antibodies bound by the synthetic antigenic sites of myoglobin accounts for 98–99% of all the antibodies bound by myoglobin (Atassi, 1977*b*; Twining & Atassi, 1979). Similarly the three surface-stimulation synthetic antigenic sites of lysozyme account for 98–100% of the total immunochemical reactivity of lysozyme (Atassi, 1978; Atassi & Lee, 1978).

In an attempt to evaluate better the effects of amino acid substitutions on the environment of a given antigenic site, we have undertaken in this work the calculation of the distances between each of the site residues and all the other residues in the lysozyme molecule. This enabled us to identify the nearest neighbours within a small sphere of influence from each site residue, thus constituting its environment. In the simplest terms any given side chain can be visualized as being situated in the centre of progressively larger concentric spheres containing mutually interacting residues. The residues within a given sphere will vary in the influence they exert on the central residue. This is due to the chemical nature of the two residues and whether a direct interaction is obstructed by any intervening third-party atom(s). Nevertheless the mutually interacting residues within a sphere must be considered as a network in a certain electron and steric equilibrium. Any alteration of a residue in this sphere will be expected to perturb the interacting network to a smaller or greater extent, depending on the alteration. The outcome of this will invariably be a new electron and/or steric equilibrium. Accordingly, in this initial attempt we have considered the sphere of influence *in toto* and confined it to a 0.70nm (7.0Å) radius from the site atoms. However, it is recognized, as mentioned above, that some residues in this network will have a greater share in determining the expressed chemical characteristics of a site residue. Future studies will aim to identify better the residues within the 0.70nm (7.0Å) sphere of influence around each site residue that play the greatest role. It should be emphasized that these considerations invoke no new concepts in protein chemistry or structure.

The foregoing treatment, when it is related to protein binding sites and factors affecting them, would serve to explain that the chemical property of a side chain in a protein is not only the outcome of the chemical nature of the side chain in question (e.g. amino group, carboxy group, phenyl group etc.), but is also to a great measure determined by its environment (e.g. charge distribution, hydrogen-bonding, hydrophobic interactions, steric hindrance etc.). This is clearly evidenced from the well-known observed reactivity differences in protein chemical modification among side chains belonging to a given type of amino acid (for review see Atassi, 1977*a*). Thus, for example, not all the lysine residues in a protein have the same pK_a value for the ε-amino group nor equal reactivity with a given chemical reagent. The same statement applies equally as well to all other amino acid residues (e.g. the histidine residues, the tyrosine residues, the carboxy groups etc.). So, an amino acid side chain exists in a milieu resulting in a certain charge distribution, solvation, hydrogen-bonding and hydrophobic interactions. When a side chain constitutes an essential residue in a protein binding site, any drastic changes in its environment may alter its characteristics and consequently its contribution to the binding energy of the site. Thus an electrostatic inductive effect due to a substitution close to, but outside of, an antigenic site (e.g. replacement of a neutral or basic amino acid residue by aspartic acid near an essential-site lysine residue) can adversely affect the binding characteristics of an essential-site residue (Lee & Atassi, 1977). In addition to changes in charge distribution, binding can be weakened by influences resulting from the elimination or creation of a hydrogen bond, the disruption of a hydrophobic interaction or steric overcrowding of a site residue. Such influences can obviously be exerted when the substitutions (changes in the environment) occur within interaction distance from a site residue. However, effects may also be transmitted to a site residue when a replacement occurs not in the nearest neighbour but at a once-removed interaction. Finally, it is not unlikely that perturbations can be propagated, even though with less frequency, to one or more sites when a replacement occurs at a distant location in a protein molecule. Several such examples of effects transmitted to the active site from distant locations in the protein are well known in enzymes. The decrease in the binding energy of an antigenic site as a result of these effects would translate into exclusion of antibodies of a given affinity from binding. Therefore lower cross-reactivity will be observed.

It is well to emphasize here that conformational changes are not necessary in order for environ-

mental effects to exert themselves. In fact, quite often no detectable conformational changes are observed. This is to be expected in view of the fact that the replacements may result in electron delocalizations and charge redistribution, and changes in hydrogen-bonding and hydrophobic interactions. These are not necessarily always accompanied by a conformational change. The presence of any conformational readjustments can further complicate these analyses.

The diagrams present graphically the minimum distance of approach to each site residue of all the amino acids of lysozyme. They will help to provide a better comprehension of the number of residues forming the environment of each of the site residues and the relative magnitude of their influences on the environment as a function of the separation distance. The simplified Tables are devoted to the nearest neighbours of the site residues. The nearest neighbours are the interacting residues within a distance of 0.70nm (7.0Å) or less and which thus make up the environment of each site residue. The once-removed interactions (i.e. residues exerting their influence on the antigenic site via an intermediate residue) have not been calculated, although we recognize the potential effects of any replacements in them on the binding capacity of the site. However, we considered that these effects should be, in general, less pronounced individually than replacements in the nearest neighbours. Of course, the cumulative effect of many such smaller influences could become appreciable. At any rate, the data become extremely cumbersome as the sphere of potential influence is enlarged. Finally, there is no way to predict the effect of a distant replacement that may be propagated within the molecule to one or more antigenic sites, nor the effect of slight conformational readjustments.

The data presented here should serve as a valuable resource for analysing the immunochemical cross-reactions of lysozymes from various species. The effects of replacements in the environment of antigenic site residues can now be evaluated, together with replacements of actual site residues. These should constitute the major effects to be considered in analysing the immunochemical cross-reactions of lysozymes. However, the limitations of these analyses must be emphasized. Obviously these analyses can only be qualitative in nature. Thus a replacement can be destructive, non-destructive or somewhere in between. But it is hard to assign a numerical value to these effects. It is probable also that the influence of a residue will decay rapidly with increasing distance from the site residue. But no general rule can be put forth, since the extent of decay of the influence will vary with the residue. Moreover, the influences of substitutions at once-removed or even more distant locations cannot be ignored, especially if they become cumulative. Finally, the destructive effects of any conformational readjustments that can result from the substitution is another factor that must be taken into account.

Note Added in Proof (Received 2 January 1980)

The availability of the precise antigenic structure of hen lysozyme (Atassi, 1978) permitted an attempt (Ibrahimi *et al.*, 1979) to propose a mathematical correlation ($y = ax_1 + bx_2$) between average 'immunological distances' (y) for ten avian lysozymes with substitutions occurring within (x_1) and outside (x_2) the known hen lysozyme antigenic sites (immunological distance is inversely related to the immunological cross-reaction). The average values of the proportionality constants when only the cross-reactions with antisera to hen lysozyme (the protein for which the antigenic structure has been determined; see below) are used are $a = 7.9$ and $b = 2.0$. These suggest that the major effects are from substitutions within the antigenic sites. For the purpose of comparison, and to distinguish the 'nearest-neighbour' effects, we utilized the relationship: $y = ax_1 + bx_2 + cx_3$. In this equation y and x_1 have the same meaning as before. Substitutions outside the antigenic sites, however, were categorized into (x_2) those occurring within the group of nearest neighbours identified here and (x_3) those occurring neither within antigenic sites nor nearest neighbours. By substituting the reported immunological distances (see Ibrahimi *et al.*, 1979, and references cited therein) from hen lysozyme of the ten avian lysozymes (including hen) and the values of x_1, x_2 and x_3, ten simultaneous equations are obtained in terms of the proportionality constants a, b and c. The best values of a, b and c obtained by regression analysis of the simultaneous solution for the ten equations are: $a = 14.5$, $b = 2.6$ and $c = -4.8$. When the chemical nature of the side chains is taken into account so that the treatment is restricted to non-conservative substitutions only, these values become: $a = 46.9$, $b = -0.7$ and $c = -2.7$. The relative magnitudes of these values again suggest that the major effects are exerted by substitutions within the antigenic sites. For substitutions outside the antigenic sites, those at nearest-neighbour locations exert a more pronounced effect than those occurring elsewhere. It should be emphasized that the values of 'immunological distances' used in our calculations are the 'distances' from hen lysozyme obtained solely with antisera to hen lysozyme.

Although it is tempting from these calculations to assign greatest significance to the site residues and their nearest neighbours, it is also necessary to caution against excessive reliance on a purely mathematical interpretation of the data. It is really

unreasonable to expect that the complex network of molecular forces involved in interactions of the type discussed above can be reduced to arbitrary statistical treatments. In addition to this overriding intellectual objection to such treatments, the limited data base (i.e. ten lysozymes of known structures and cross-reactivities) is not sufficient for making statistically significant conclusions.

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