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Enzymic and Immunochemical Properties of Lysozyme

ACCURATE DEFINITION OF THE ANTIGENIC SITE AROUND THE DISULPHIDE BRIDGE 30–115 (SITE 3) BY 'SURFACE-SIMULATION' SYNTHESIS*

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1. Previous reports from this laboratory have shown that both Lys-33 and Lys-116 are parts of an antigenic site in native lysozyme. Similar studies of tyrosine derivatives indicated that one or both of Tyr-20 and Tyr-23 are located in or very close to an antigenic site in lysozyme. The site, which was located around the disulphide bridge 30–115, was recently shown unequivocally to include the residues Tyr-20, Arg-21, Lys-116, Asn-113, Arg-114, Phe-34 and Lys-33. This was confirmed by the 'surface-simulation' synthetic approach that we have recently developed, in which the foregoing eight surface residues were directly linked via peptide bonds, with intervening spacers where appropriate, into a single peptide. The peptide does not exist in native lysozyme, but simulates a surface region of it. 2. In the present work several surface-simulation peptides were synthesized representing various parts of the region, to determine the minimum structural feature that retains full antigenic reactivity and to investigate if the spatially constructed antigenic site has a preferred direction. 3. The peptide Lys-Asn-Arg-Gly-Phe-Lys exhibited a remarkable inhibitory activity towards the immune reaction of lysozyme and accounted entirely for the maximum expected reactivity of the site in the native protein (i.e. about one-third of the total lysozyme reactivity). An immunoadsorbent of the peptide bound about one-third of the total antibody to lysozyme. 4. The residues Tyr-20 and Arg-21 are not part of the site. The previously reported immunochemical effect observed on nitration of Tyr-20 was due to a deleterious ionic effect exerted by the modified tyrosine residue on the adjacent Lys-96, which is in an entirely different antigenic site of lysozyme. Thus the modification of Tyr-20 impairs the reactivity of an adjacent antigenic site, even though the residue itself is not part of a site. The conformational and immunochemical implications of this finding are discussed. 5. The antigenic site therefore comprises the five spatially adjacent residues Lys-116, Asn-113, Arg-114, Phe-34, Lys-33. The antigenic site exhibited a preferred direction (Lys-116 to Lys-33), since the reverse surface-simulation synthetic sequence was immunochemically inefficient. The site describes a line which circumscribes part [2.1 nm in $C_{(\alpha)}$–$C_{(\alpha)}$ distance from Lys-116 to Lys-33] of the surface of the molecule.

Our previous studies on the antigenic structure of hen's-egg-white lysozyme (see the Discussion section for detail) have shown that an antigenic site [the term is used here according to the definition given by Atassi & Saplin (1968)] resides around the disulphide bond 30–115 (Atassi *et al.*, 1973) and includes both Lys-33 and Lys-116 as essential parts of the site (Lee *et al.*, 1975). One or both of Tyr-20 and Tyr-23 are located in, or very close to, an antigenic site in lysozyme (Atassi & Habeeb, 1969; Atassi *et al.*, 1971). The location of the site was deduced and confirmed by a novel surface-simulation synthetic approach (Lee & Atassi, 1976), previously developed in our laboratory (Atassi *et al.*, 1976*d*). The aforementioned lysine and tyrosine residues were found to lie in an imaginary line encircling part of the surface of the globular protein and passing through the residues Tyr-20, Arg-21, Tyr-23, Lys-116, Asn-113, Arg-114, Phe-34 and Lys-33. These spatially contiguous residues were linked via peptide bonds into a single peptide, with intervening glycine spacers where necessary (Lee & Atassi, 1976). The studies enabled us to determine that Tyr-23 is not part of the antigenic site, since its replacement by a glycine spacer had no adverse effect on the reactivity of the respective peptide, and that the antigenic site lies within (but

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may not include all of) the foregoing surface-encircling line.

In the present work, several surface-simulation peptides representing various parts of the established surface region were synthesized, purified and characterized and their immunochemistry was studied in detail. The results enabled the accurate narrowing-down of the antigenic site to its constituent conformationally (but not covalently) adjacent residues.

Experimental

Materials

Hen's-egg-white lysozyme (three times crystallized) was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and was homogeneous by starch-gel, polyacrylamide-gel and disc electrophoresis. The solid-phase peptide-synthesis resin (chloromethylated co-polystyrene with 1% divinylbenzene cross-links with 1.19 mequiv. of Cl/g) was from Vega-Fox Biochemicals (Tucson, AZ, U.S.A.). The N^{α}-*t*-butoxycarbonyl-amino acid derivatives were purchased from Beckman Instruments (Palo Alto, CA, U.S.A.). The side-chain-protecting groups were identical with those previously given (Lee & Atassi, 1976). The purity of each commercial amino acid derivative was routinely checked by t.l.c. Reagents for peptide synthesis were prepared and solvents purified as previously described (Koketsu & Atassi, 1973, 1974*a*). Carrier-free ^{125}I was purchased from New England Nuclear (Boston, MA, U.S.A.). Sepharose 4B was from Pharmacia Fine Chemicals (Piscataway, NJ, U.S.A.). CNBr was obtained from Eastman Organic Chemicals (Rochester, NY, U.S.A.) and *p*-benzoquinone was from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.).

Synthesis and purification of peptides

Solid-phase peptide synthesis was carried out as described elsewhere in detail (Koketsu & Atassi, 1973, 1974*a*). After cleavage from the resin, the crude synthetic peptides were subjected to ion-exchange chromatography on columns (2.5cm×75cm) of DEAE-Sephadex A-50 at a constant pH of 4.8 and room temperature (22°C) by using a linear concentration gradient. The mixing chamber contained 0.1M-acetic acid/pyridine buffer (1 litre) and the reservoir contained 1M-acetic acid/pyridine buffer (1 litre). The columns were eluted at 30–40ml/h. Samples (0.5ml) of the effluent fractions (5.2ml) were freeze-dried, redissolved in water (1.5ml) and their A_{230} values determined.

Immunochemical methods

Early-course antisera against native lysozyme were prepared in goats and rabbits by the procedure previously described for myoglobin (Atassi, 1967*a*). In the present work, G9 and G10 are two goat antisera and L7 and L21 are two rabbit antisera, each against native lysozyme. Antisera from individual animals were not mixed, but were studied separately. They were stored in 5ml portions at −40°C. The quantitative precipitin and inhibition experiments were carried out as previously described (Atassi & Saplin, 1968). The value of peptide molar excess relative to lysozyme at 50% of the peptide's maximum inhibition was determined from double-reciprocal plots of 1/(molar excess) against 1/(percentage inhibition).

Preparation of immunoadsorbents

Immunoadsorbents were prepared by coupling lysozyme or peptide to CNBr-activated Sepharose 4B by a procedure similar to that described by March *et al.* (1974). Also another set of immunoadsorbents was prepared by coupling to benzoquinone-activated Sepharose 4B, which was made by the procedure of Brandt *et al.* (1975). The amounts of protein or peptide attached to Sepharose were determined from the difference in concentration of the respective solutions before and after the coupling reaction, and were subsequently checked by nitrogen analysis of a portion of the immunoadsorbent. Lysozyme–Sepharose had 3.5–3.8mg/ml, whereas peptide–Sepharose had 2.7–3mg/ml of packed volume.

Supporting media, carrying glycine, histidine or sperm-whale myoglobin coupled by the aforementioned two procedures, were prepared. These were used as controls to determine the extent, if any, of non-specific background binding of labelled antibody.

Preparation of IgG fraction of the antisera*

A portion (5ml) of antiserum was mixed with half its volume of saturated $(NH_4)_2SO_4$ at 0°C. The precipitate containing IgG was washed by centrifugation (2000rev./min, 0°C, 30min) with 3×10ml of 33%-satd. $(NH_4)_2SO_4$. The pellet was dissolved in cold (0°C) 0.0175M-sodium phosphate buffer, pH6.3, and dialysed at 0°C against 6×1litre of the same buffer. The solution was then applied to a DEAE-cellulose column (1cm×25cm) which had been pre-equilibrated with the same buffer. The IgG fraction of the antiserum eluted in the first peak was freeze-dried and kept at −20°C. Before use, it was reconstituted with 0.15M-NaCl (5ml), and when necessary, unused solutions were stored at −20°C.

Preparation of specific lysozyme antibodies

The IgG fraction (252mg/1.5ml) was mixed with lysozyme–Sepharose (bed vol. 4ml). The suspension, which was maintained by gentle rotation, was incubated at 37°C for 1h and then at 0°C for 24h. The unbound protein was run into a fraction collector and the column was washed with 0.15M-NaCl/

* Abbreviation: IgG, immunoglobulin G.

0.1M-sodium phosphate buffer, pH7.2, until the eluate was free of protein. Specific antibody was eluted from the immunoadsorbent at 0°C by 5M-guanidine hydrochloride, pH8.5, into tubes already containing 0.0175M-phosphate buffer, pH7.2, so that the guanidine hydrochloride solution was immediately diluted to one-third or one-quarter of its original concentration. This precaution avoided precipitation of the antibody in subsequent steps. The solution was immediately dialysed extensively against the same buffer and then freeze-dried.

Radioiodination of specific antibody

The carrier-free ^{125}I (1mCi) was diluted with 0.1M-sodium phosphate buffer, pH7.8 (0.2ml), followed by addition of 0.02ml of a 0.05M solution of iodine in 0.1M-KI. The resulting iodine solution was added slowly to the cold (4°C) antibody solution (2–3mg in 0.1ml) of 0.1M-sodium phosphate buffer, pH7.8. The mixture was stirred magnetically at room temperature until the colour of iodine disappeared (about 30min). The labelled antibody solution was subjected to gel filtration on a column (1cm×30cm) of Sephadex G-25 which was eluted with 0.012M-sodium phosphate buffer, pH7.0. The labelled antibody was dialysed exhaustively against 0.15M-NaCl/0.1M-phosphate buffer, pH7.0, and used for subsequent binding studies.

Binding of ^{125}I-labelled antibody by immunoadsorbents

Peptide or lysozyme immunoadsorbents (1ml packed bed vol.) were first treated with bovine serum albumin (10mg in 1ml) followed by normal IgG (2mg in 1ml) and excess protein was washed out thoroughly with 0.15M-NaCl/0.1M-phosphate buffer (pH7.0) after each treatment. This precaution was found quite useful in decreasing non-specific background binding of label in subsequent steps. The operations were carried out in glass columns (1cm×6cm) fitted with sintered-glass discs. ^{125}I-labelled antibody was added as a solution (0.4–0.5ml) in the same buffer. Usually 70000–80000c.p.m. was used per experiment. The immunoadsorbents were incubated as described above, followed by removal of unbound label (which was measured) and then exhaustive washing with the same solvent until 1ml of the effluent contained less than 100c.p.m. Elution of specific bound antibody was effected by 5M-guanidine hydrochloride, pH8.5, followed by 0.5M-glycine/HCl, pH1, to ensure that no more antibody remained on the immunoadsorbent.

In all these determinations, control experiments were carried out in an identical manner but with glycine–Sepharose and myoglobin–Sepharose to monitor the existence and extent of any non-specific binding of label by the supporting matrix. Another control was also done in which non-immune goat ^{125}I-labelled IgG was passed on to the lysozyme or peptide immunoadsorbents to determine the amount, if any, of non-immune label that may be bound by these media.

Analytical methods

Spectral measurements, peptide 'mapping' and amino acid analysis of triplicate acid hydrolysates (22, 48 and 72h each) were performed by the procedures previously described (Atassi & Saplin, 1968). Labelled samples in Aquasol (New England Nuclear) were counted for radioactivity in an Isocap 300 liquid-scintillation counter (Searle Analytic, Fort Lee, NJ, U.S.A.). Concentrations of protein and peptide solutions were based on their nitrogen contents, which were determined as described by Atassi *et al.* (1971). The theoretical nitrogen contents were calculated from the amino acid sequence and are shown in Table 1. The calculated molecular weight and nitrogen content of lysozyme were 14306 and 18.80% respectively.

Table 1. *Amino acid compositions, molecular weights and nitrogen contents of the pure synthetic peptides*
Results are expressed in mol of amino acid per mol of peptide and represent the average of six acid hydrolyses on each peptide (two each at 22, 48 and 72h). Molecular weights and nitrogen contents were calculated from amino acid compositions. The structures of peptides I–VI are shown in Fig. 1.

	Peptide I		Peptide II		Peptide III		Peptide IV		Peptide V		Peptide VI	
Amino acid	Calc.	Found	Calc.	Found	Calc.	Found	Calc.	Found	Calc.	Found	Calc.	Found
Asp	1	1.02	1	1.03	1	1.02	1	0.95	1	0.97	1	0.90
Gly	2	2.01	2	2.07	3	3.02	3	3.10	3	3.12	1	1.04
Tyr	2	2.00	2	1.83	1	0.90	—	—	1	0.94	—	—
Phe	1	0.99	1	1.03	1	1.02	1	0.96	1	0.99	1	0.98
Lys	2	1.85	2	2.03	2	1.91	2	2.15	1	1.14	2	2.13
Arg	2	2.00	2	2.01	2	2.11	2	1.94	2	2.18	1	1.06
Mol.wt.	1288.6		1288.6		1182.5		1002.3		1037.3		731.9	
Nitrogen content (%)	20.64		20.64		22.50		23.75		21.59		21.04	

Results

Purification and characterization of the peptides

The structures of the peptides synthesized here are shown in Fig. 1. The synthetic products on cleavage from the resin were appreciably heterogeneous, as revealed by peptide 'mapping'. Each 'crude' peptide comprised a very strong major spot and three to six minor spots. Gel filtration on Sephadex G-10 columns (2cm×60cm) did not give any purification. Purification of the peptides was necessary, since such impurities are unsuitable for immunochemical studies. Purification was achieved by chromatography on DEAE-Sephadex A-50. Each synthetic product yielded several components (Figs. 2 and 3). The major peaks indicated in Figs. 2 and 3 were subjected to two or three chromatography experiments on similar columns. During chromatography, the effluent fractions (every other tube) were checked by high-voltage electrophoresis (Atassi & Saplin, 1968) to ensure an optimum pooling of tubes containing the major component. The major chromatographic components thus isolated gave single spots by peptide 'mapping'. After elution of the ninhydrin-positive spots from heavily loaded 'maps', the colour intensity indicated that the major component in each case was 99% pure or better. Amino acid analysis of each major peak showed it to have the correct amino acid composition, which was in very good agreement with that expected from its sequence (Fig. 1).

Immunochemical reactivity of the pure peptides

The immunochemical interaction of each of the peptides was tested with four different antisera to native lysozyme. As expected, none of the peptides gave an immune precipitate with any of the antisera. However, they each exhibited an inhibitory activity towards the precipitin reaction of lysozyme with its antisera. Examples of the inhibitory behaviours of the six peptides with one antiserum (G9) are shown in Fig. 4. Table 2 summarizes the inhibitory activities of the peptides with the four antisera. The results with peptides I and II showed that peptide II was immunochemically more efficient than peptide I. Peptides I and II have exactly reverse sequences. Accordingly peptide II presented a more favourable antigenic site to the antibody. Peptide III, which differed from peptide II only by the replacement of Tyr-23 by a glycine spacer, was even more reactive than peptide II. Peptide IV, which does not contain

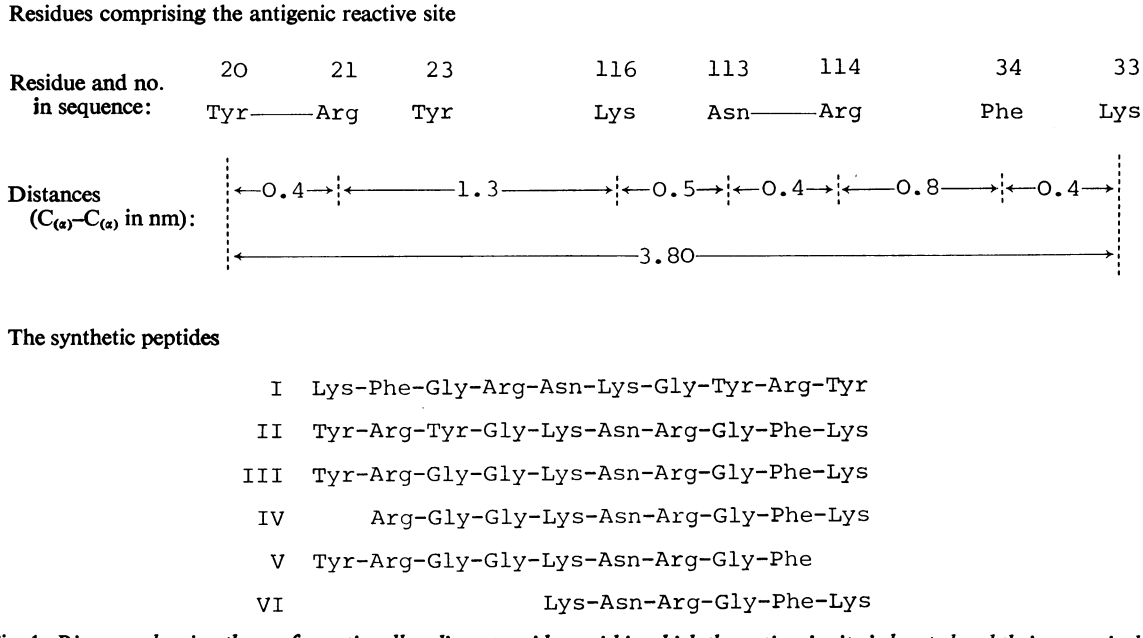

Fig. 1. *Diagram showing the conformationally adjacent residues within which the antigenic site is located and their numerical position in the primary structure of lysozyme*

The distances (in nm) separating the consecutive residues of the reactive site are given as $C_{(\alpha)}$–$C_{(\alpha)}$ distances, together with the overall dimension of the site. Below, the primary structures of the 'surface-simulation' synthetic peptides designed to mimic the antigenic site are given.

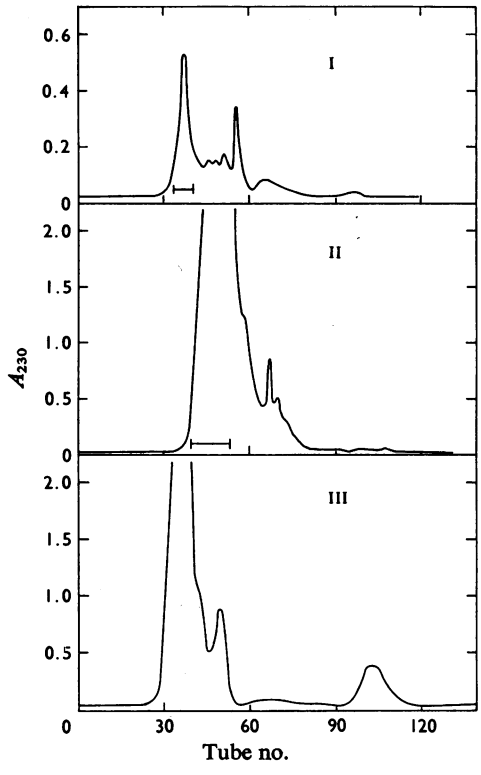

Fig. 2. *Chromatographic patterns on DEAE-Sephadex A-50 (2.5cm×75cm) of synthetic peptides I, II and III*
Chromatography was at pH4.8 by using a concentration gradient (for details see the text). Fraction volume was 5.2ml.

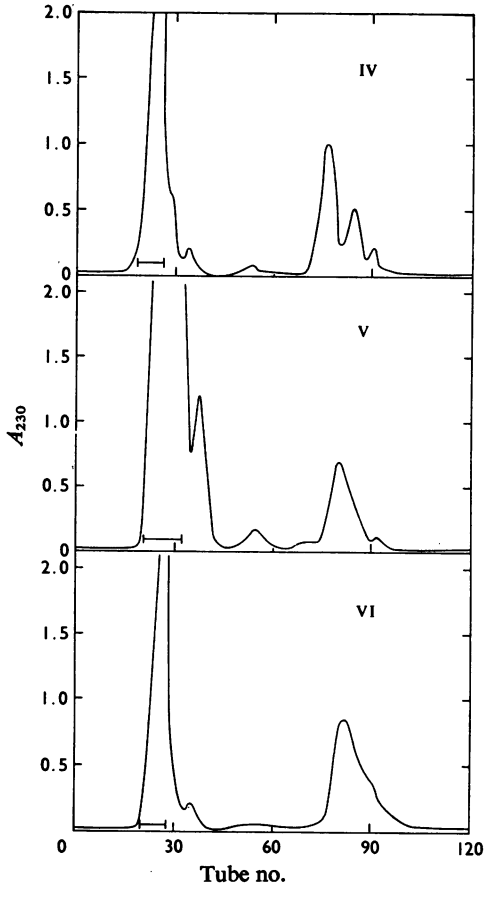

Fig. 3. *Elution patterns of chromatography on DEAE-Sephadex A-50 (2cm×75cm) of synthetic peptides IV, V and VI*
Chromatography was at pH4.8 by using a concentration gradient (see the text for details). Fraction volume was 5.2ml.

Tyr-20, has reactivity essentially equal to that of peptide III. Removal of the residue equivalent to Lys-33 in peptide V resulted in an appreciably decreased immunochemical reactivity. Finally, peptide VI, which lacks the residues equivalent to Tyr-20, Arg-21 and Tyr-23, had in fact the highest immunochemical reactivity of all the surface-simulation peptides. A control synthetic peptide corresponding to residues 15–22 of sperm-whale myoglobin (which represents an antigenic site in myoglobin; Koketsu & Atassi, 1974*a*; Atassi, 1975; Atassi & Koketsu, 1975) had no effect on the lysozyme immune reaction (Fig. 4). Conversely none of the peptides in Fig. 1 exhibited (when used in several thousand molar excess) any inhibitory effect on the myoglobin immune reaction.

With all the antisera studied a large molar excess of peptide relative to lysozyme was required to achieve maximum inhibition. This is considerably larger than would be expected from comparison with myoglobin and its synthetic antigenic sites (Koketsu & Atassi, 1973, 1974*a*,*b*; Pai & Atassi, 1975; Atassi & Pai, 1975). Previously, we had shown with lysozyme (Atassi *et al.*, 1973) and with bovine serum albumin (Habeeb *et al.*, 1974; Atassi *et al.*, 1976*b*; Habeeb & Atassi, 1976) that the inhibitory activities of peptides from the respective proteins were considerably improved when the IgG fractions, instead of the whole antisera, were used in the immunochemical studies. This was therefore attempted here to see if the binding efficiency of the most reactive peptide (peptide VI; see Table 2) would be improved.

The IgG fraction isolated from a given antiserum retained 98–100% of the total antibody activity of the antiserum. Inhibition studies with peptide VI using the IgG fraction of the antiserum are summarized in

Table 3 and are compared with the inhibitory activities of the peptide with the whole antisera. The results show that the inhibitory efficiency of the peptide is considerably improved. Not only was there a decrease in the molar excess required to achieve half the maximum inhibition, but in fact there was an appreciable improvement in the maximum inhibitory activity afforded by the peptide, especially with antiserum L7 (Table 3). This is very much like previous observations referred to above with albumin and other lysozyme peptides. The molar excesses required to achieve maximum and half-maximum inhibitions by peptide VI with the IgG fractions became quite similar to those exhibited by the synthetic antigenic sites of myoglobin. Finally, Table 3 shows that the maximum inhibitory activity of peptide VI approximated to the contribution of the intact antigenic site in native lysozyme (about one-third of the total reaction of the protein).

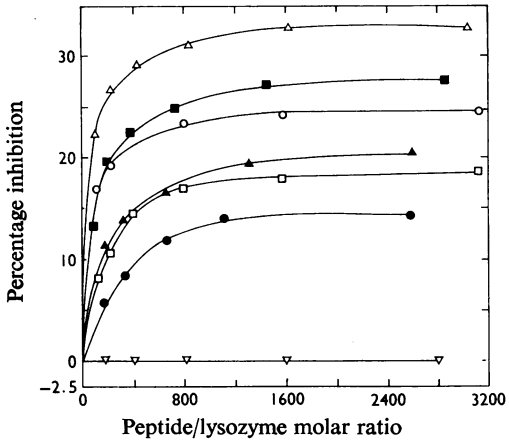

Fig. 4. *Quantitative inhibition by the pure synthetic peptides of the precipitin reaction of native lysozyme with one antiserum (G9) to native lysozyme*

●, Peptide I; ▲, peptide II; ■, peptide III; ○, peptide IV; □, peptide V; △, peptide VI; ▽ represents behaviour of a control synthetic peptide corresponding to residues 15–22 of sperm-whale myoglobin and which comprises an antigenic site in myoglobin (Atassi, 1975).

Removal of lysozyme antibody by peptide immunoadsorbent

Specific antibodies to lysozyme, prepared as described in the Experimental section, accounted for 98% of the total immune reaction of the parent antiserum. After radioiodination, the antigenic reactivity of the antibody, as determined by a micro-precipitin reaction, was entirely intact.

Table 4 shows the amount of specific anti-lysozyme antibodies bound to CNBr-prepared immunoadsorbents of lysozyme or peptide VI. Lysozyme–Sepharose

Table 3. *Inhibitory activities of peptide VI with whole antisera and with IgG fraction of the antisera: comparison with expected reaction of the site*

Results are expressed as maximum percentage inhibition by peptide VI of the precipitin reaction of native lysozyme with various antisera or their IgG fractions. Values in parentheses represent peptide/lysozyme molar ratio at 50% of the maximum inhibition. Results are the average of six replicate analyses that varied by ±0.9% or less. G9 and G10 are goat antisera and L7 and L21 are rabbit antisera, each against native lysozyme. 'Expected reaction' is calculated from the decrease in antigenic reactivity of lysozyme derivatives that can be attributed to the succinylation of both Lys-33 and Lys-116 (Lee *et al.*, 1975). n.d., Not determined.

	Maximum inhibitory activity (%)		
Antiserum	Whole antiserum	IgG fraction of antiserum	Expected reaction of the site
G9	33.3 (40)	n.d.	33.6
G10	28.8 (940)	30.3 (200)	31.1
L7	19.2 (260)	33.3 (180)	n.d.
L21	22.2 (380)	n.d.	n.d.

Table 2. *Inhibitory activity of the pure synthetic peptides*

Results are expressed in maximum percentage inhibition by a peptide of the precipitin reaction of native lysozyme with various antisera. Each value is the average of at least three or six replicate determinations, which varied by ±0.8% or less. Values in parentheses represent peptide/lysozyme molar ratio at 50% of the maximum inhibition. G9 and G10 are goat antisera, L7 and L21 are rabbit antisera. n.d., Not determined.

Peptide / Antiserum	Inhibitory activity with whole antisera (%)			
	G9	G10	L7	L21
I	14.5 (240)	16.7 (1360)	8.3 (210)	4.2 (380)
II	20.8 (160)	23.3 (640)	n.d.	13.7 (380)
III	27.8 (90)	26.3 (560)	18.2 (730)	16.7 (360)
IV	25.0 (50)	25.0 (890)	17.2 (180)	15.9 (120)
V	18.9 (150)	14.7 (340)	12.8 (80)	12.5 (100)
VI	33.3 (40)	28.8 (940)	19.2 (260)	22.2 (380)

Table 4. *Adsorption of specific goat ^{125}I-labelled antibody on lysozyme by immunoadsorbent carrying synthetic peptide VI*

The specific ^{125}I-labelled antibody fraction from antiserum G10 was isolated on a lysozyme immunoadsorbent before use in these studies (see the text for details). The amount of antibody applied to each immunoadsorbent possessed a radioactivity of 75597c.p.m. Immunoadsorbent in Expts. 1 and 2 were prepared by CNBr activation. Identical results were obtained when activation was by *p*-benzoquinone. Specific binding is corrected for the amount of label bound in the control experiment. The 'control' value is the average of three control experiments using glycine–Sepharose and myoglobin–Sepharose. In these studies 2% non-specific background binding in controls occurred. Non-specific binding of non-immune ^{125}I-labelled IgG on peptide VI or lysozyme immunoadsorbents was about 1–2% of the total label applied.

Expt. no.	Amount of antibody bound (c.p.m.)	Antibody bound (%)
1	21628	28.6
2	23041	30.4
Control	(2182)	
Average		29.5

adsorbed out all ^{125}I-labelled antibody applied. Peptide VI–Sepharose adsorbed out a fraction of the antibody that was quite comparable in amount with the maximum inhibitory activity exerted by the free peptide on the lysozyme immune reaction with that antiserum. In several control experiments, non-specific binding of ^{125}I-labelled antibody on glycine–Sepharose or myoglobin–Sepharose was 1.5–2.8% of the total label added. A similar extent of non-specific binding was exhibited by lysozyme–Sepharose or peptide–Sepharose on passage of ^{125}I-labelled non-immune goat IgG. The results in Table 4 have been corrected for the background of non-specific binding. Binding results identical with those shown in Table 2 were obtained when coupling to the Sepharose had been performed by benzoquinone.

Discussion

The present immunochemical findings showed that, with whole antisera, an abnormally large excess of peptide relative to lysozyme was required to obtain maximum inhibition. Indeed, in such studies a large excess is to be expected. Our results with the synthetic intact antigenic sites of sperm-whale myoglobin (Koketsu & Atassi, 1973, 1974*a*,*b*; Pai & Atassi, 1975; Atassi & Pai, 1975) showed that these sites reached maximum immunochemical reactivity at a peptide/myoglobin molar ratio of 200–300. However, in the present work, the excess of peptide relative to lysozyme was 1500–3500 when the whole antisera were used. This inordinately large excess was somewhat disturbing, and it was gratifying that the excess of peptide needed was found to be within the 'normal' range (peptide/lysozyme 200–400) when the IgG fractions of the antisera were used. Even though these studies have been performed on IgG from only two antisera, they should suffice to demonstrate the trend unambiguously. The improvement of the binding ability of the peptide when the IgG fractions were used may have indicated some proteolysis of the peptides and/or binding by serum proteins when whole antisera were used. At any rate, this observation is not unusual, and we have previously reported improved immunochemical reactivity for fragments of lysozyme (Atassi *et al.*, 1973) and of bovine serum albumin (Habeeb *et al.*, 1974; Atassi *et al.*, 1976*b*; Habeeb & Atassi, 1976) with the IgG fractions of the antisera to their respective parent proteins. Similar observations have also been made with the synthetic antigenic sites of myoglobin (Atassi, 1977).

The large molar excess required of the peptides has been attributed (Atassi & Saplin, 1968) to conformational factors. The primary immune response to native protein antigens is directed against their native three-dimensional structure (Atassi & Thomas, 1969). The short peptides studied here will be expected to exist in unfolded conformational states. For proper interaction with antibody, an antigenic site must have (at least reasonably approximately) the shape that it has in the native protein (Atassi, 1967*b*, 1970; Atassi & Saplin, 1968; Habeeb & Atassi, 1971), and antibody will be able somewhat to induce its own required conformation on an antigenic site (Atassi, 1975). The probability of finding such a favourable conformational state will improve with increase in peptide concentration (Atassi & Saplin, 1968). It is to be remembered that the peptides studied here do not even exist in native lysozyme, but merely attempt to simulate a spatial arrangement of adjacent residues, some of which are distant from each other in the sequence. Furthermore it is not entirely possible to duplicate in the synthetic peptides the exact distances separating the various side chains of the antigenic site in the native protein. Therefore the mere reactivity of these peptides is remarkable, and the fact that the immunochemical efficiency of the present synthetic site resembles those of the synthetic sites of myoglobin (which in the native protein are made up of residues directly linked to one another in the sequence; Atassi, 1975) is indeed startling.

Conclusions from previous work

A large number of specific chemical derivatives of lysozyme and immunochemically reactive fragments have been prepared in this laboratory, and their

conformation and immunochemistry studied (for a review, see Atassi & Habeeb, 1977). The data most relevant to the present report are the results from the modification of the lysine and the tyrosine residues. From a succinylated (3-carboxypropionylated) reaction product of lysozyme, three homogeneous derivatives were isolated which showed no conformational changes but had a decreased antigenic reactivity with antisera to native lysozyme (Lee *et al.*, 1975). 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 after modification of both Lys-33 and Lys-116 (33.6 and 31.1% with antisera G9 and G10). This behaviour pointed to Lys-33 being at the 'end' of the antigenic site and is reminiscent of results obtained with some myoglobin derivatives modified at end residues of an antigenic reactive site (Atassi *et al.*, 1975). Both Lys-33 and Lys-116 were concluded to be parts of an antigenic site in lysozyme (Lee *et al.*, 1975). Earlier studies had shown that nitration of Tyr-20 and Tyr-23 in lysozyme effected immunochemical changes (Atassi & Habeeb, 1969) which were not caused by conformational changes in the derivative (Atassi *et al.*, 1971). Modification of these two tyrosine residues caused a decrease in the antigenic reactivity by 21 and 23% with antisera G9 and G10 respectively. Conversely, antisera to nitrated lysozyme reacted 75–82% with lysozyme relative to the homologous reaction. Reduction of the nitrotyrosine residues to aminotyrosine restored fully (99–100%) the antigenic reactivity with antisera to lysozyme. It was therefore concluded that one or both of Tyr-20 and Tyr-23 is located in, or is very close to, an antigenic site in lysozyme (Atassi & Habeeb, 1969; Atassi *et al.*, 1971). Finally, a disulphide peptide corresponding to the sequence $Gly_{(22)}$-$Lys_{(33)}$-($Cys_{(30)}$-$Cys_{(115)}$)-$Cys_{(115)}$-$Lys_{(116)}$ possessed a substantial inhibitory activity toward the immune reaction of lysozyme and was specifically bound by immunoadsorbents carrying lysozyme antibodies (Atassi *et al.*, 1973). 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 disulphide bond 30–115. It was therefore suggested (Lee & Atassi, 1976) that Lys-33 is at one end and the tyrosine residues are at the other end of the antigenic site.

The assignment of the site and description of its location were achieved (Lee & Atassi, 1976) by application of a novel and unorthodox 'surface-simulation' synthetic approach which was first devised in our laboratory for the delineation of another antigenic site (Site 2) in native lysozyme (Atassi *et al.*, 1976*d*). This approach linked the relevant spatially adjacent residues constructing the site into a single peptide. Since the three-dimensional structure of lysozyme is known (Blake *et al.*, 1967; Imoto *et al.*, 1972), its examination revealed that the residues Tyr-20, Tyr-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 (Lee & Atassi, 1976). 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 Fig. 1. Two peptides were thus synthesized (Lee & Atassi, 1976) with glycine spacers where necessary (peptides II and III in Fig. 1) to achieve the correct separations between their side chains. These studies established for the first time that the site was indeed formed by conformationally adjacent residues on the surface which are not necessarily in direct peptide linkage with one another (Lee & Atassi, 1976). The studies also showed 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. The present studies were designed to narrow down with accuracy the exact boundaries of the antigenic site and to investigate if the site has a preferred 'direction'. The latter question is rather intriguing, since on the surface of a globular protein, there is no clear 'beginning' or 'end' for an antigenic site of this kind which differs from those sites made up of residues directly linked in the sequence, as in myoglobin (Atassi, 1975). The existence or otherwise of a preferred direction for a spatially constructed site should, in either case, represent an important finding.

Accurate definition of the site

With each of the antisera studied, peptide I had a substantially lower inhibitory activity than either of peptides II or III. In fact with some antisera (L21, Table 2), peptide I had only a negligible inhibitory activity. Clearly, therefore, the antigenic site had a preferred direction on the surface of the globular protein molecule. In spite of the fact that on the surface of an ideal sphere all directions are presumably equivalent and that the protein molecule has an unrestricted rotational freedom, the antigenic site may be accepted by the antibody-combining site, only if it is presented in one way. This is of course not entirely unexpected in specific protein–protein interactions, where certain complementary side chains must attain favourable proximity. However, it is somewhat surprising to find that it does in fact matter even with the surface-simulation synthetic sites, since only the amino acid side chains should participate in the antigen–antibody binding (Lee *et al.*, 1976). Peptides I and II, for example, have the same representation and arrangement of side chains, but only differed in the direction of the peptide bonds linking the residues which are not in any case present

in the intact molecule. 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 with the previously reported (Lee & Atassi, 1976) peptides II and III are extended here for completion to include two rabbit antisera. Again, replacement of Tyr-23 by a glycine spacer in peptide III in fact improved its immunochemical reactivity relative to that of peptide II, confirming that Tyr-23 is not an essential part of the antigenic site. Deletion of the residue equivalent to Tyr-20 (peptide IV) had no adverse effect on the immunochemical reactivity with any of the antisera (Table 2). Deletion of Lys-33 while recoupling Tyr-20 (peptide V), so that the sizes of peptides IV and V are equal, caused a substantial loss in immunochemical reactivity of the peptide. Clearly Tyr-20 is not part of the antigenic site, whereas Lys-33 makes an important contribution to the reactivity of the site. With this conclusion, it became questionable whether Arg-21 is in fact part of the site. Arg-21 was implicated because it is an intervening residue in the imaginary line between Lys-116, Tyr-23 and Tyr-20. But since Tyr-23 and Tyr-20 have been proved here to make no contribution to the reactivity of the site, there remained no reason why Arg-21 should be implicated. Investigation of peptide VI was therefore undertaken and its immunochemical behaviour demonstrates 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 (see Table 3), as can be derived from the effect of modifying both Lys-33 and Lys-116 in lysozyme (Lee *et al.*, 1975).

To ascertain that the maximum inhibitory activity of peptide VI was a true representation of its immunochemical reactivity, its direct binding of anti-lysozyme antibody was examined by using an immunoadsorbent of the peptide. The amount of antibody from antiserum G10 bound directly in this manner accounted for 29.5% of total antibody to lysozyme, which is in excellent agreement with the value of 30.3% found for the inhibitory activity of the peptide. Clearly therefore, the inhibition values provide a faithful measure of the immunochemical reactivity of the peptide. This correspondence has also been reported for the synthetic antigenic sites of myoglobin (Atassi & Koketsu, 1975) and for two inhibitory fragments of bovine serum albumin (Atassi *et al.*, 1976*b*; Habeeb & Atassi, 1976).

Comments about Tyr-20

As outlined in the above discussion we had originally concluded (Atassi & Habeeb, 1969; Atassi *et al.*, 1971) that one or both of Tyr-20 and Tyr-23 is located in, or very close to, an antigenic site in lysozyme. In fact in the first 'surface-simulation' approach to this site (Lee & Atassi, 1976), they were incorporated into the synthetic scheme. Even then, when Tyr-23 was found to make no contribution to the reaction of the site, Tyr-20 was believed to be at one extreme end of the site. However, since the present findings unequivocally show that Tyr-20 is not part of the site, a rationalization for the findings becomes essential.

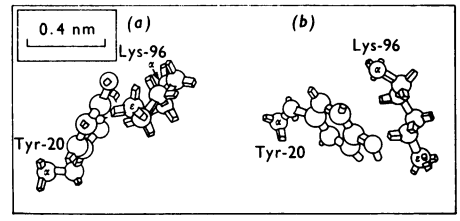

Fig. 5. *Schematic diagram showing the closeness of the phenolic side chain of Tyr-20 to the side chain of Lys-96*
The diagram is taken from a constructed model of lysozyme: (*a*) and (*b*) represent two views, at right-angles to one another, of the same two residues.

In the three-dimensional structure of native lysozyme, the phenolic ring of Tyr-20 is extremely close (0.3–0.4 nm) to the hydrocarbon chain of Lys-96 (Fig. 5), which is a critical residue in site 2 (Lee & Atassi, 1975; Atassi *et al.*, 1976*a*,*d*). The immunochemical effect of nitrating Tyr-20 may therefore be due to a secondary effect exerted on the ability of a neighbouring residue (Lys-96), itself in an antigenic site, to participate in binding. On nitration of the tyrosine residues at the *ortho* position, the inductive effect of the nitro group on the aromatic nucleus will increase the acidity of the phenolic hydroxyl group, thus promoting its ionization, and the resultant anion will be stabilized by the electron-withdrawing mesomeric effect (Atassi, 1968). The increased acidity is shown by a decrease in the pK_a value from 10.1 for tyrosine to 7.2 for 3-nitrotyrosine (Sokolovsky *et al.*, 1967). The pK_a value for 3-aminotyrosine is 10.0 (Sokolovsky *et al.*, 1967). Obviously the presence of a newly created negatively charged group within interaction distance of Lys-96 should be expected to disturb drastically its ionic environment and consequently its immunochemical interaction properties. This effect is completely removed, as to be expected, when the nitrotyrosine residues are reduced to aminotyrosine.

That Tyr-20 is not part of an antigenic site makes for a more acceptable antigenic structure. If Tyr-20 were indeed part of antigenic site 3, this would mean that sites 2 and 3 are untenably close. It would then be sterically impossible for two antibody molecules

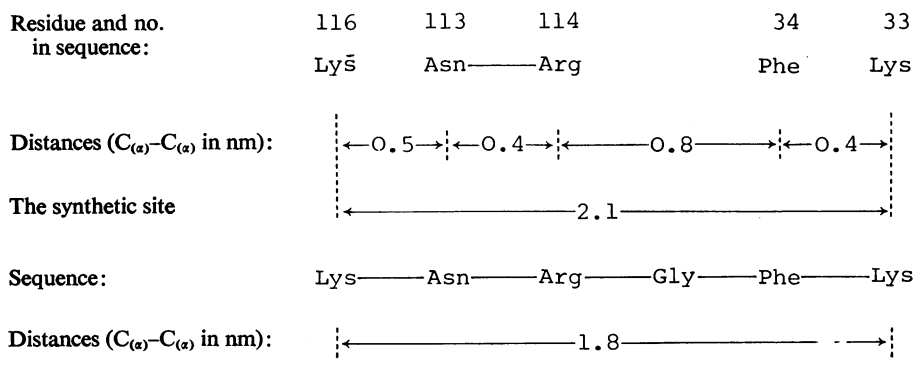

Fig. 6. *Diagram showing the spatially contiguous surface residues established in the present work to comprise an antigenic reactive site*

The distances (in nm) separating the consecutive residues of the reactive site are given as $C_{(\alpha)}$–$C_{(\alpha)}$ distances, together with the overall linear dimension of the site. The primary structure of the synthetic peptide VI is also given in the lower part. The total length of the synthetic peptide shown above assumes an ideal $C_{(\alpha)}$–$C_{(\alpha)}$ distance of 0.362nm.

to occupy those two sites simultaneously on a given lysozyme molecule.

The immunochemical effect of nitrating Tyr-20 reveals another facet, hitherto unsuspected in protein immunochemistry. Thus immunochemical changes, observed as a result of selective chemical modification of a residue in a derivative that suffers no conformational change, do not necessarily imply the participation of the modified residue in an antigenic site. This face-value interpretation is no longer valid unless independent data lend it additional weight. Furthermore it is not hard to see similar situations being generated by single amino acid evolutionary substitutions outside an antigenic site but sufficiently close to influence its ionic and binding characteristics. The immunochemical relationships of related proteins from various species are not necessarily linearly related to sequence similarities and we had previously consistently attributed this to considerable or even local and subtle conformational differences (for reviews, see Atassi, 1977; Atassi & Habeeb, 1977). Now a new factor, the ionic or inductive effect of a substitution on another very close residue that is a critical part of an antigenic site, has to be taken into consideration in the interpretation of the immunochemistry of protein mutants.

Conclusions

To sum up, antigenic site 3 is made up (Fig. 6) 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 of the native protein (Plate 1), has an overall dimension of 2.1nm [taking $C_{(\alpha)}$–$C_{(\alpha)}$ distances]. In interaction with antibody, these residues of the site function as if in direct peptide-bond linkage (Lee & Atassi, 1976). Site 3 is analogous in spatial construction to another antigenic site (site 2) previously reported (Atassi *et al.*, 1976*d*). 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 (Imoto *et al.*, 1972). Therefore antigenic site 3 overlaps with the enzymic binding site (Lee & Atassi, 1976). This is the last of the three antigenic sites on native lysozyme to be delineated, and antibodies against this synthetic site have now been prepared (M. Z. Atassi & C.-L. Lee, unpublished work). Synthesis and immunochemistry of the other two sites have been reported (Atassi *et al.*, 1976*c*,*d*).

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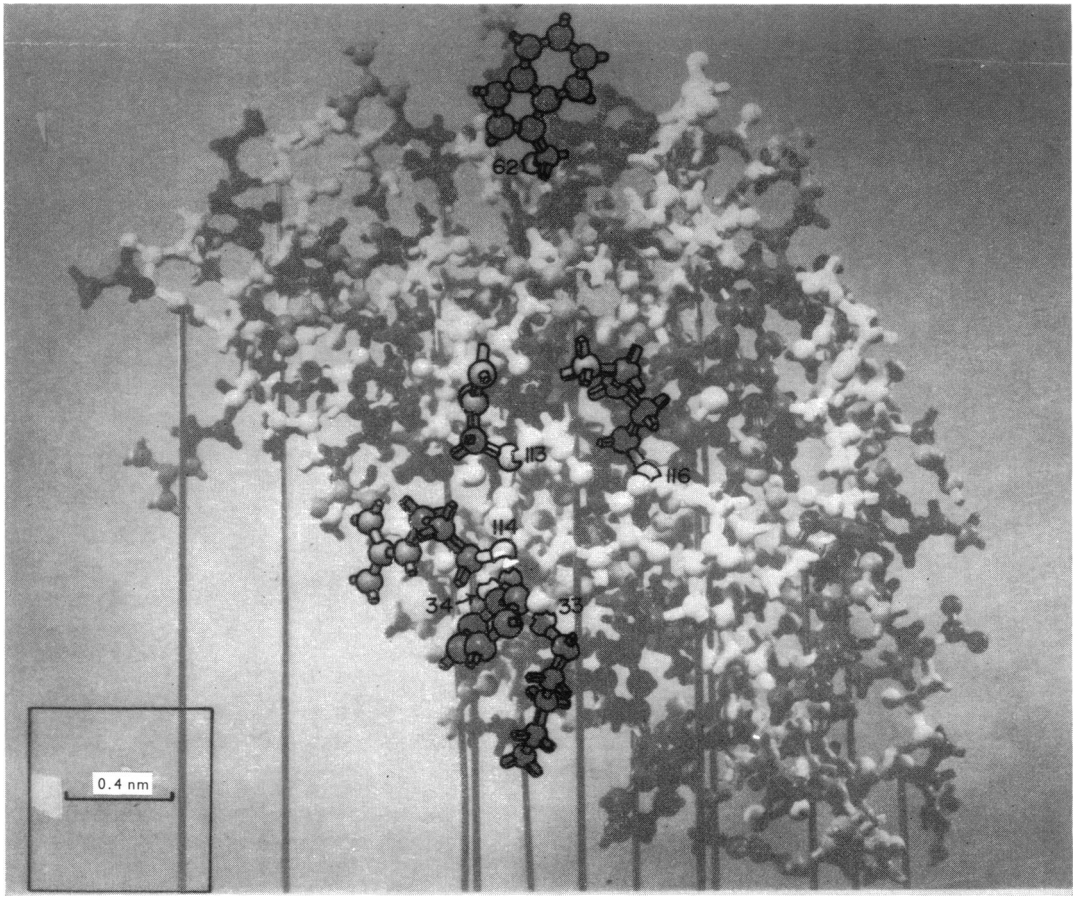

EXPLANATION OF PLATE 1

Photograph of a lysozyme model showing the relative positions of the residues constituting antigenic site 3

The side chains of the relevant residues are outlined, whereas the background representing the remainder of the molecule is somewhat muted to attain maximum clarity. Trp-62, which represents the first residue of antigenic site 2 (Atassi *et al.*, 1976*d*) is also outlined to provide a perspective of the relative locations of the two sites. From this view, only Trp-62 of site 2 can be seen and the remainder of site 2 continues on the back side.

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