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High-performance liquid chromatography of amino acids, peptides and proteins

C.[a] Characterisation of coulombic interactive regions on hen lysozyme by high-performance liquid anion-exchange chromatography and computer graphic analysis[b]

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ABSTRACT

The molecular characteristics of the dominant anion-exchange binding site of hen egg white lysozyme (HEWL) has been investigated using a combination of high-performance liquid chromatographic techniques and computer graphic analysis of the X-ray crystallographic structure. These studies have indicated that the site of highest electrostatic potential, in terms of the density of negatively charged amino acid side chains, is located around the catalytic cleft area. The four residues tentatively identified to be involved in the electrostatic binding domain were aspartic acid 48, 52, 101 and glutamic acid 35. The number of these charged groups correlated with the maximum value of the chromatographically determined retention parameter (Z_c value). Variations in the range of experimental Z_c values obtained under different elution conditions have been interpreted in terms of conformational flexibility of the structural domains of HEWL which result in the opening or closure of the catalytic cleft during the retention process.

[a] For Part XCIX, see ref. 47.

[b] It is with great pleasure that the authors submit this manuscript to the Honour Volume for Professor J. Calvin Giddings as a token of their good wishes on his 60th birthday. As an outstanding scientist and inspired wilderness traveller, he has provided a great stimulus to the field of separation sciences on an international front. Acknowledging the intellectual innovation which he has represented for over three decades, this manuscript is dedicated to the scientific objectives which he has so incisively championed.

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INTRODUCTION

Protein retention in high-performance ion-exchange chromatography (HPIEC) arises from electrostatic interactions between the solute and the stationary phase surface. In analytical chromatography the retention behaviour of protein solutes is commonly characterised by the dependence of the capacity factor, k', on the concentration, c, of the displacer salt. Over a limited range of the salt concentration the dependence of log k' on log $(1/c)$ can be approximated by a linear relationship in the form

$$\log k' = \log K + Z_c \log (1/c) \quad (1)$$

where K is the ion-exchange distribution coefficient obtained by extrapolation of plots of log k' *versus* log $(1/c)$. While the precise physicochemical significance of the Z_c term remains to be determined, this parameter has frequently been taken to represent the number of charged interactive binding sites established between unique ionic patches on the protein surface, called *ionotopes*[1], and the stationary phase support.

In anion-exchange systems the magnitude of Z_c has been found to be influenced by a number of mobile phase components, such as buffer pH[2,3], the salt concentration[4,5], the type of displacer salt[4–6], the buffer concentration and the elution mode[3,5]. Variation in the magnitude of Z_c is associated with subtle changes in the interactive surface structure or ionotope of the protein. The non-uniform distribution of charged groups arising from the unique primary structure of the protein results in patches of high electrostatic potential, which participate in binding to the support surface. These areas of localised charge can occur as a result of either sequentially linked amino acids or via topographic arrangements involving through space alignment of amino acid side chains. Under a particular set of chromatographic conditions the distribution, charge density and surface accessibility of these groups generate a specific charge vector which controls the orientation, the contact area A_{cont}, and the approach distance a, of the ionotope in the interaction established between the protein and the stationary phase[1,8]. Furthermore, the molecular composition of these interactive segments, which present themselves at the chromatographic surface, ultimately determines the thermodynamic properties of the solute binding at the solid–liquid interface. The ability to locate these interactive sites on the surface of a protein solute under a defined set of experimental parameters would therefore provide significant insight and predictive power into the chromatographic behaviour of a particular protein.

In the present study the X-ray crystallographic structures determined for lysozyme variants[9,10] have been analysed to locate potential anion-exchange binding sites on the surface of hen egg white lysozyme (HEWL). The three-dimensional structure of this protein was visualised by computer simulated graphics of the X-ray structural atomic coordinates. Patches or clusters of glutamic acid and aspartic acid residues, which are negatively charged under the chromatographic elution conditions, were located and assessed on the basis of their surface accessibility, charge density and steric interference. This information was then utilised in the development of an anion-exchange binding model for HEWL.

MATERIALS AND METHODS

All chromatographic experiments were performed with a Pharmacia (Uppsala, Sweden) fast protein liquid chromatography (FPLC) system as previously described[7].

Computer assisted molecular modelling

A Silicon Graphics (Mountain View, CA, U.S.A.) Iris 3120 computer system combined with a University of California, San Diego, U.S.A. (UCSD) Molecular Modelling System (version 1.3) was used to visualise the three-dimensional structure of various proteins. X-ray crystallographic coordinates for HEWL were obtained from the Brookhaven protein database. Photographs of these computer simulated molecular models were taken directly from the monitor using a Nikon F3 SLR 35 mm camera with a 55 mm lens. The camera aperture was set at f 2.8 and shutter speeds of 1/4 or 1/8 s were used.

RESULTS AND DISCUSSION

HEWL is a small enzyme made up of a single polypeptide chain of 129 amino acids, the primary structure of which is shown in Fig. 1. X-ray crystallography studies[9] have revealed that the HEWL molecule is approximately ellipsoidal with dimensions 45 × 30 × 30 Å. A deep cleft located on one side divides the molecule into two domains. The smaller domain is sheet-like with the thickness of only one chain and is comprised mainly of hydrophilic residues which are exposed to the outside solvent or the cleft of the molecule. Hydrophobic residues are found mainly in the larger domain where they form a central core. HEWL is involved in the destruction of gram-negative bacteria by cleaving the polysaccharide component of their cell walls. X-ray crystallographic[12] and solution[13,14] studies on enzyme-substrate and enzyme-inhibitor complexes have identified the cleft as the active site for the degradation of particular gram-negative bacterial cell wall polysaccharides, where negatively charged residues located in the cleft play a major role in the degradation process. In particular, Asp 52 and Glu 35 are thought to contribute directly to the cleavage of the glucosidic bond via an acid–base catalysis mechanism. Furthermore, Asp 101, Trp 62 and Trp 63 are believed to support the polysaccharide substrate in the cleft through hydrogen-bonding interactions.

HEWL is a basic protein with an isoelectric point (pI_{HEWL}) of 11.0. In anion-exchange chromatographic systems where the buffer pH is less than pI_{HEWL},

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        *       *       *       *       *       *
KVFGRCELAAAMKRHGLDNYRGYSLGNWVCAAKFESNFNTQATNRNTDGSTDYGILQINS
        *       *       *       *       *       *
RWWCNDGRTPGSRNLCNIPCSALLSSDITASVNCAKKIVSDGNGMNAWVAWRNRCKGTDV
        *
QAWIRGCRL
```

Fig. 1. Primary structure of hen egg white lysozyme.

HEWL will have an overall net positive charge and on the basis of the net charge hypothesis is not expected to be retained. However, as shown in our earlier studies[6,7] on the HPIEC of proteins, HEWL is retained on high-performance anion-exchange supports at high pH, through interaction of localised areas of negative charge. These localized areas of negative-charge groups will be manifested as coulombic patches —or ionotopes— accessible to the chemical moeities of complementary charge present on the sorbent surface. These quaternary ammonium ligands, in the case of strong anion-exchange systems, thus function as molecular probes for the region(s) of highest electron density. The interaction will be characterised, in part, by the parameter Z_c, which will vary according to the extent of ionisation and the extent of ion-interaction of the dominant ionotope. As such, the value of Z_c is anticipated to correlate with the numbers of charge groups on the ionotope and its accessibility to the chromatographic ligand. Table I shows Z_c values, representing the number of charged interactions between HEWL and the positively charged Mono-Q column, derived with elution systems of pH 9.60 and a variety of neutral salts. Values of Z_c were found to vary between *ca.* 0.5 and 4.5 over these elution conditions. While it is known that the magnitude of the Z_c value may also be influenced by hydrophobic interaction effects[2,3,6], experimental conditions can be chosen (*e.g.* as used in the present study) to maximise the electrostatic effects and thereby minimising the contribution of hydrophobic effects to Z_c. Under such conditions, a high correlation in the linearity of the log k' *versus* log $(1/c)$ plots is predicted from thermodynamic, near equilibrium and stoichiometric models for electrostatic retention behaviour of proteins in HPIEC. The correlation coefficient for the derivation of Z_c values in Table I was $R^2 \geqslant 0.90$ which is in accord with the concept that electrostatic effects dominate the retention behaviour.

TABLE I

Z_c VALUES FOR LYSOZYME AT pH 9.60

Salt	*Elution mode*		
	Varied t_G[a]	*Varied flow*[b]	*Isocratic*[c]
LiCl	2.50 ± 0.26	4.46 ± 0.25	0.80 ± 0.20
LiBr	4.21 ± 0.66	3.96 ± 0.77	–[d]
		1.96 ± 0.09[f]	
NaF	2.99 ± 0.31	2.91 ± 0.12	0.74 ± 0.31
NaCl	1.48 ± 0.17	1.88 ± 0.23	–[e]
NaBr	2.41 ± 0.44	3.30 ± 0.33	1.59 ± 0.21
		0.56 ± 0.00[f]	
KF	3.58 ± 0.14	3.23 ± 0.50	–[d]
KCl	1.16 ± 0.15	1.73 ± 0.11	–[e]
KBr	3.28 ± 0.24	4.52 ± 0.75	–[d]
	0.98 ± 0.14[f]	2.53 ± 0.28[f]	

[a] Varied gradient time, constant flow elution conditions.
[b] Varied flow-rate, constant gradient time elution conditions.
[c] Constant flow-rate isocratic elution conditions.
[d] Not eluted with this salt.
[e] Mixed modal retention.
[f] A non-linear dependency of log k' on log $(1/c)$ resulted in more than one Z_c value.

The origins of the differences between Z_c values obtained by isocratic and gradient elution have been discussed in detail elsewhere[4,7].

Several studies[14–16] have shown good correlation between the number of charged groups on highly charged biomolecules, such as oligonucleotides, and the resultant chromatographically derived Z_c values. However, when solute molecules are sufficiently large that they can adopt some degree of tertiary structure, lower correlation between Z_c and the total number of charges on the molecule has been reported[17]. In terms of average charge interactions, one positive charge at the surface of the quaternary ammonium sorbent will be required for full electrostatic interaction and the establishment of charge electroneutrality with a unitary negative charge associated with an ionised amino acid side chain at the surface of the protein. Experimentally observed non-integer values could conceivably arise from (1) system effects such as the precision of instrumental measurement, (2) solute effects such as the distribution, orientation and solvation of charged or polarised groups in the micro-environment of the contact regions, (3) changes in the hydrogen bonding structure associated with salt bridges and (4) restricted accessibility and clustering of the positively charged ligands on the sorbent. In the current study, the distribution of negatively charged Glu and Asp residues at the surface of the net positively charged HEWL molecule, allows a unique opportunity to examine the relationship of the chromatographically derived Z_c values with the number of potential interacting residues on the protein surface. This correlation also gives valuable insight into the anion-exchange retention mechanisms for proteins.

Fig. 2 shows the computer simulated structure of HEWL and the location of all glutamic acid (yellow) and aspartic acid (orange) residues which may be involved in the retention process. Noticeably, the acidic side chain groups are sparsely located over the surface of the molecule except for Asp 101, Glu 35, Asp 52 and Asp 48. All of these latter group of amino acid residues lie in close proximity to each other and are positioned in, or adjacent to, the active site cleft area. If it is assumed that stoichiometric interactions occur between HEWL and the chemical ligands on the stationary phase surface, *i.e.* one quarternary ammonium group is required to bind to each acidic amino acid side chain, then the number of charged groups in this cleft area accessible to the ligands would be between one and four depending on the salt or pH condition. These values correlate well with the range of Z_c values shown in Table I. From spatial considerations, the remaining amino acid side chains are sufficiently distant or on opposite faces of the protein to preclude simultaneous interaction as would be required for a Z_c value of 4. Fig. 3 shows the distribution of positively charged arginine (blue) and lysine (pink) groups in HEWL. X-ray crystallographic[9] and enzyme-activity[18] studies have determined that the six lysyl amino groups are on the surface of the molecule and not in the active-site cleft. Fig. 4 also shows that access to the cleft is not obstructed to any great extent by arginine residues wich project away from the plane of the four acidic amino acids. Furthermore, Blake *et al.*[9] noted that intermolecular contacts observed in the protein crystal may also represent the attachment sites during the dimerisation and polymerisation of HEWL reported to occur in solution at high pH[19]. Again these intermolecular contacts would not obstruct access to the cleft.

In Figs. 5 and 6 the topography of the cleft is further examined. It can be seen in Fig. 4 that a number of additional amino acid residues may enhance or interfere with

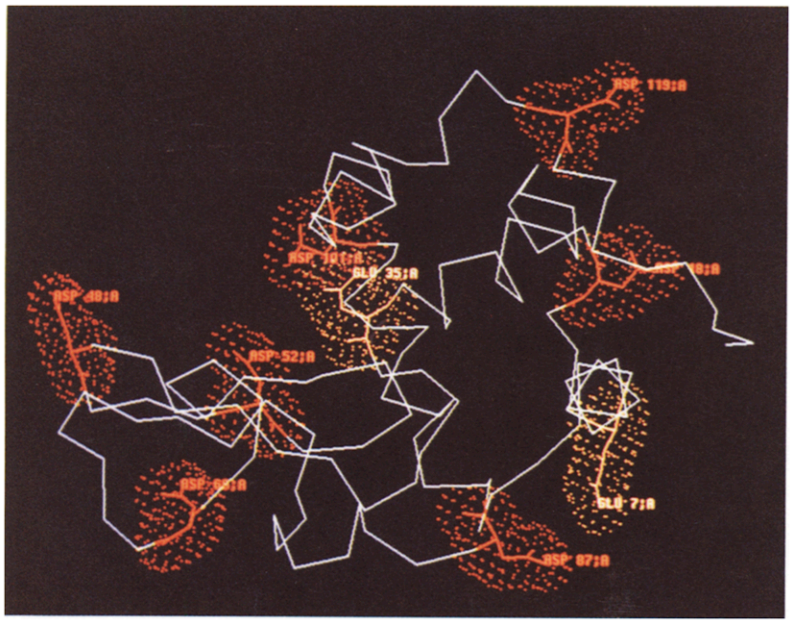

Fig. 2. Computer graphic simulation of the X-ray crystal structure of HEWL indicating the location of all glutamic acid (yellow) and aspartic acid (orange) residues. Residues Asp 101, Glu 35, Asp 52 and Asp 48 were identified as the dominant anion-exchange binding domain. Picture produced by a computer program written by Lesk and Hardman[46].

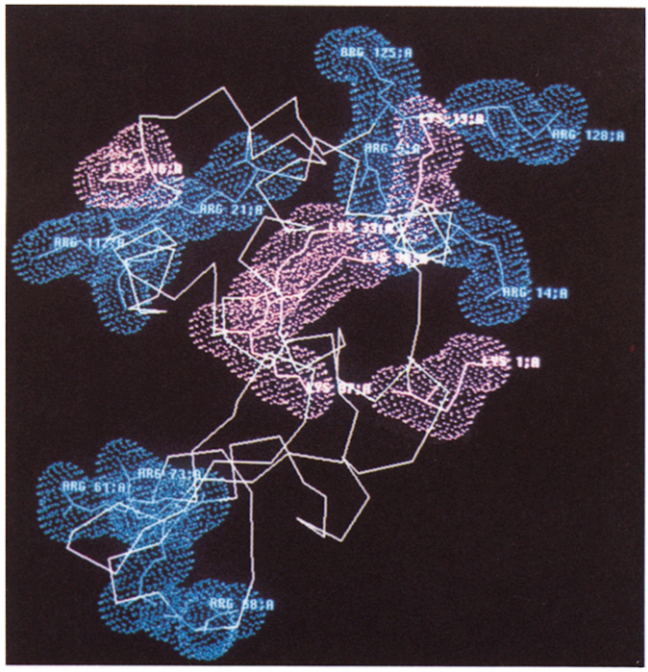

Fig. 3. Computer graphic simulation of the HEWL crystal structure showing the distribution of positively charged lysine (pink) and arginine (blue) residues. Picture produced by a computer program written by Lesk and Hardman[46].

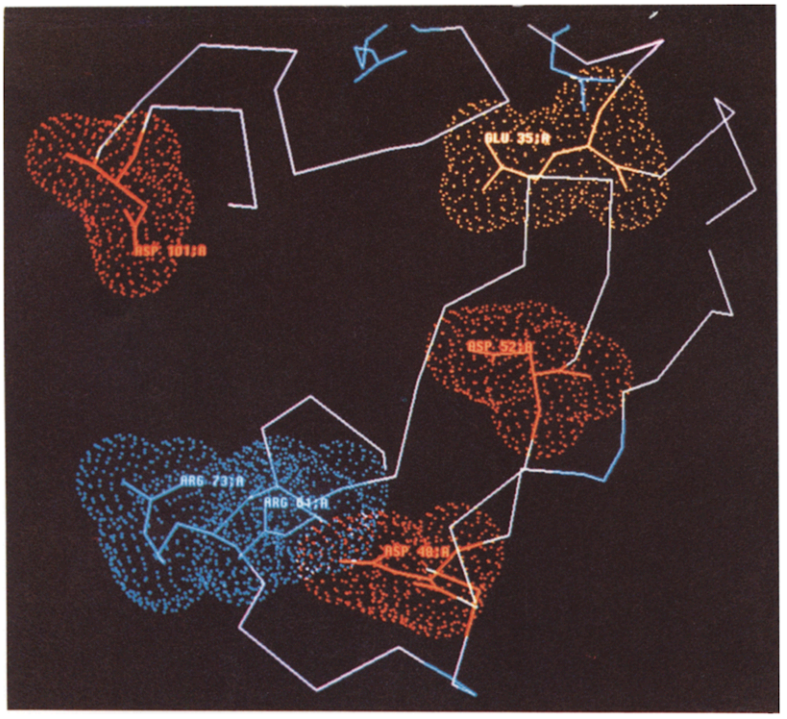

Fig. 4. Computer graphic representation of the spatial proximity of Arg 61 and Arg 73 (blue) around the negatively charged groups in the active site. Picture produced by a computer program written by Lesk and Hardman[46].

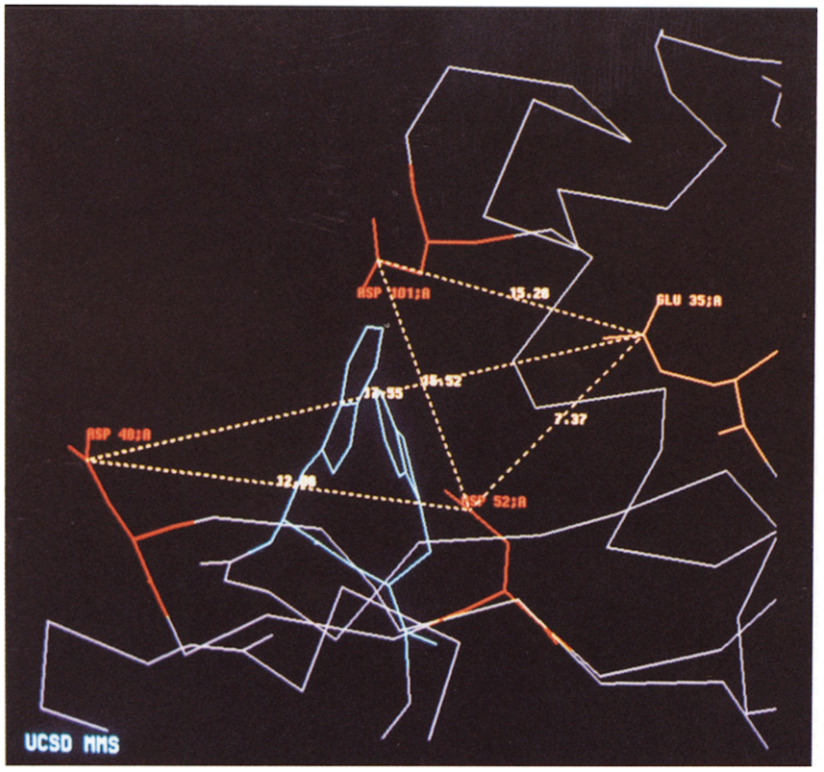

Fig. 5. Computer simulation demonstrating the spatial topography of Trp 62, 63 and 108 (light blue) around the active cleft. Contour lines have been drawn to measure the distances between Asp 101, Glu 35 and Asp 52. The area of the dotted triangle was used to calculate the charge density of 0.55 charge per 10 Å^2. Similarly, the charge density of the binding domain encompassing Glu 35, Asp 52 and Asp 48 was calculated to be 0.85 charge per 10 Å^2. Picture produced by computer program written by Lesk and Hardman[46].

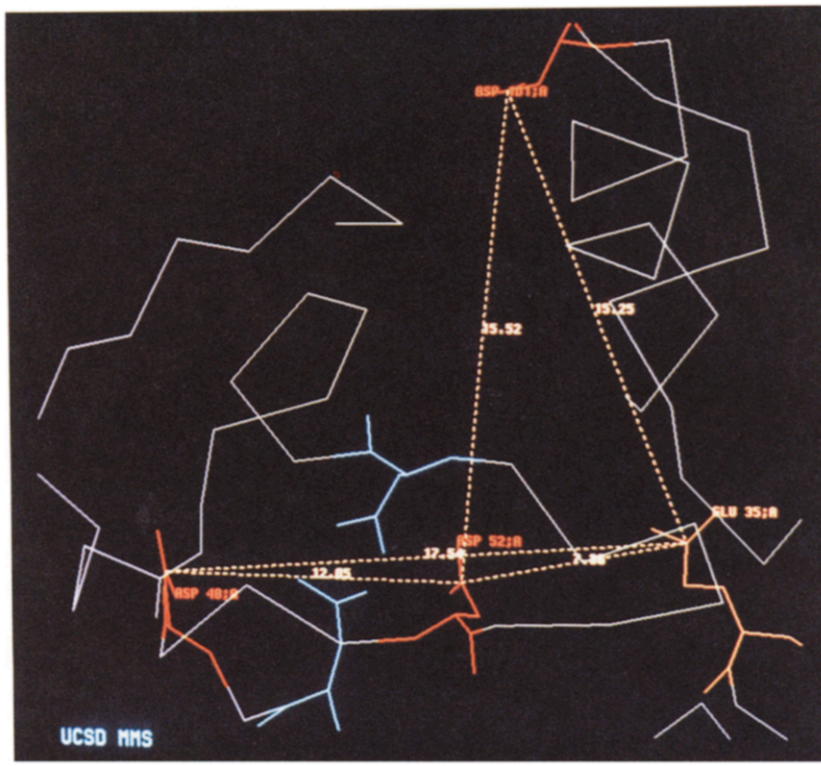

Fig. 6. Computer graphic representation of the spatial proximity of Asn 46 and Asn 59 (blue) around the active cleft of HEWL.

the electrostatic interactions at the protein–stationary phase interface. Three of the six tryptophan residues of HEWL (that is, Trp 62, 63 and 108) are located in the active site[11]. Trp 62 has been identified as the most surface accessible of these three residues via X-ray crystallographic methods and its reactivity in solution with N-bromosuccinomide[20]. Fig. 5 shows the spatial arrangement of Trp 62 and Trp 63 (light blue) which are the closest to the four negatively charged amino acid residues in the cleft. Both tryptophan residues occupy a substantial volume and would be expected to sterically interfere with the ion-exchange binding process. However, binding studies involving HEWL and polysaccharide inhibitors have found that the alicyclic rings in sugars and the tryptophan rings in the enzyme rotate to accomodate each other when the cleft is occupied[21]. Rotation of these tryptophans induced by specific salt interactions could also occur during HPIEC to facilitate the binding of the negatively charged residues in the cleft to the support surface. Studies with lysozyme[21,22] and other proteins[23], in salt solutions using intrinsic fluorescence quenching techniques have demonstrated this behavioural characteristic of tryptophan residues. Furthermore, as aromatic residues can potentially act as electron donors, the tryptophan residues may also enhance the binding process through ion-induced dipole interactions.

The amino acid residue Asp 48 has been shown not to be involved with

enzyme–substrate binding due to its location outside the bottom of the cleft. If Z_c does reflect the total number of electrostatic interactions occurring between the solute and stationary phase, then the chromatographic data in Table I indicate that up to four negatively charged groups are involved in the electrostatic binding. Since Asp 48 is located in close proximity to the cleft and is spatially aligned with the other side-chain carboxyl groups this amino acid residue has been assigned to be the fourth negatively charged group participating in the HPIEC retention process. However, several neighbouring (but not charge interacting) residues may hinder its participation in the binding process. From Fig. 6, which illustrates views taken from above and opposite the cleft, it can be concluded that Asp 48, Asp 52 and Glu 35 have an essentially linear topographic arrangement. However, the side chains of Asn 59 and Asn 46 (light blue) are both located close to the space occupied by Asp 48 and Asp 52. If a rigid structure is assumed, according to the known atomic coordinates of lysozyme[9], then the side chains of these two amino acid residues could sterically interfere with a linear electrostatic binding site incorporating Asp 48, Asp 52 and Glu 35. As evident from Fig. 4 the interactive capability of Asp 48 may be further diminished by the close proximity of the positively charged Arg 61 and Arg 73 residues. If Asp 48 does participate in the binding process (*i.e.*, when $Z_c \approx 4$) these positively charged arginine residues may be instrumental in determining the orientation of the cleft at the stationary phase surface.

During ion-exchange chromatography, localised areas of high electrostatic potential on the protein surface will generate an overall charge vector, which will control the approach and the orientation of the protein solute to the stationary phase surface. The identification of these areas of high electrostatic potential will assist in determining the chromatographic retention mechanisms of protein solutes. In Figs. 5 and 6 dotted three-dimensional contours have been drawn between the negatively charged residues in and around the cleft. The areas of these three-dimensional contours were then calculated and used to estimate the charge density in these two regions. For the area incorporating Asp 101, Glu 35 and Asp 52 a charge density of 0.55 negative charge per 10 Å^2 was determined. The area encompassed by Glu 35, Asp 52 and Asp 48 was estimated to have a higher charge density of 0.85 negative charge per 10 Å^2 and hence should play a more dominant role in steering lysozyme toward the stationary phase surface from the bulk solvent. The concept that high electrostatic charge density, arising from the sequential or spatial clustering of charged groups, can direct the site of electrostatic interaction has been used to design specific protein purification protocols. For example, a recombinant protein, β-urogastrone has been produced with a C-terminal polyarginine (polyArg) tail which enhanced its purification on a cation exchanger[24]. This polyArg tail functions to define a unique region within the total three-dimensional structure which directs and orientates the recombinant β-urogastrone to the cationic surface of the sorbent. In terms of the total number of amino acids in the folded protein, the polyArg region was small, *i.e.* only 6 amino acids out of a total 59 residues. Nevertheless, binding still occurs mainly through the alignment of the arginine residues with the cationic ligands, whilst the other side-chain groups on the polypeptide surface create secondary interactive preferences. In the case of HEWL, once the molecule is in the vicinity of the double layers of the solvated ligand and during the final stage of adsorption, other amino acid groups on the protein surrounding the active site cleft, such as Arg 61 and Arg 73, can

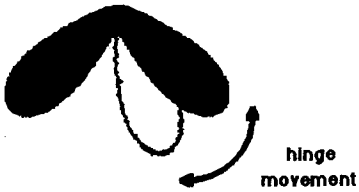

Fig. 7. Diagramatic representation of the hinge-bending mechanism for HEWL.

be anticipated to influence in an analogous manner the orientation and magnitude of the interactive ionotope on the protein surface.

The adsorption of proteins onto the stationary phase surface involves dynamic processes which may also induce conformational changes in the protein solute. These processes could involve the motion of only a few atoms or could result in movements of entire structural subunits or domains. If the negatively charged residues in the cleft participate in the ion-exchange binding process, then the fluctuation in Z_c values, between *ca.* 1 and 4, with various elution conditions suggests that there is a change in accessibility to these residues. More specifically, the degree of structural fluctuations arising from conformational changes in the interactive ionotope, and induced by the elution conditions, can be assessed through changes observed in protein Z_c values. It is well known that portions of polypeptide chains frequently fold into compact, local semi-independent units or domains. There are numerous examples of proteins, including HEWL, that have elongate double-lobed structures comprising two domains separated by an active cleft[25–27]. X-ray crystallographic studies have shown, for some double-lobed enzymes, that these domains can move relative to each other through a hinge-bending mode of vibration as shown in Fig. 7. This hinge-bending mode also plays an important role in their catalytic function[28,31]. For example, when glucose binds to yeast hexokinase the small lobe of the enzyme molecule rotates by 12° relative to the large lobe, moving the polypeptide backbone as much as 8 Å (ref. 30). These conformational rearrangements result in the closure of the cleft and a reduction in the radius of gyration for hexokinase[31]. There is substantial experimental evidence that the two structural domains in HEWL can also move relative to each other via a hinge-bending mode of vibration[32–34]. In the absence of solvent molecules and with an unoccupied cleft, the hinge-bending mode for HEWL has been calculated[33] to have a vibrational frequency of $1.3 \cdot 10^{11}$ Hz. Furthermore, energy calculations indicate that hinge-bending rotations of several degrees are permissible in the HEWL molecule. In solution the lobe vibration is reported to be dampened by the hydrodynamic friction of the lobe motion and the two domains move relative to each other in a diffusive manner.

As regions of highest charge density and electrostatic potential represent the possible site of electrostatic interaction, the range of Z_c values suggests that the interaction of HEWL with the charged anion-exchange sorbent may also be mediated by a similar hinge-bending mechanism associated with the catalytic cleft as shown diagrammatically in Fig. 7. The number of negatively charged residues in the cleft area that interact with the stationary phase surface will then be determined by the extent of domain motion. The Z_c value will, therefore, reflect the degree of opening or closure of the cleft on HEWL. A similar retention mechanism has also been proposed by Chicz

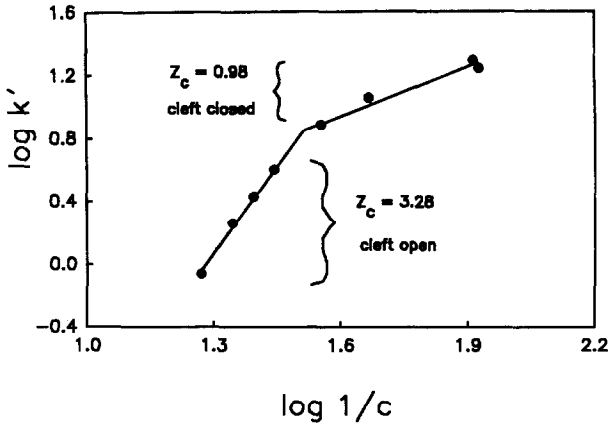

Fig. 8. Plots of log k' *versus* log $(1/c)$ for lysozyme separated on a Mono-Q anion exchanger by gradient elution at pH 9.6 with KBr as displacer salt, with gradient times between 8.6 and 171 min and a flow-rate of 1 ml/min. The Z_c values corresponding to the open cleft and closed cleft were 3.28 ± 0.24 and 0.98 ± 0.14 respectively.

and Regnier[35] for subtilisins chromatographed on a cation exchanger where the structural state of an ion bridge on the surfac of subtilisin was observed to be dependent on the ionic strength of the displacer salt.

Various studies[36–38] have shown that neutral salts influence the stability, solubility and biological activity of macromolecules in solution. Neutral salts can also interfere with the intermolecular forces involved in the association/dissociation of proteins and their subunits[39,40]. The resulting changes in the structure of the macromolecule arise as a consequence of complex processes which regulate the layer of hydration and electrostatic potential about the surface of the molecule. In addition to responding to these subtle changes in structure, HEWL is also known to polymerise at high pH. Hence, the choice of neutral salt will regulate the approach to, and orientation of, HEWL at the stationary phase surface through manipulation of the size and shape of the monomer and also of the degree of polymerisation.

As is evident from Table I, Z_c values of HEWL were found to vary as a result of a number of systematic changes to the mobile phase composition. As discussed elsewhere[4], the type of displacer salt and the elution mode can have a profound influence on the magnitude of Z_c. This behaviour is particularly evident with HEWL. For example, as demonstrated in Fig. 8, the magnitude of Z_c for HEWL was observed to be dependent on the concentration of bromide salts under varied flow or varied gradient time elution conditions. Similarly, a non-linear dependence of log k' on log $(1/c)$ has been reported for subtilisins eluted with sodium chloride[35]. In these instances, retention plots of log k' *versus* log $(1/c)$ can be divided into two linear regions (*i.e.*, with different slopes) with a distinct inflexion point. The dependence of the Z_c value of HEWL on the bromide salt concentration is consistent with the chaotropic or water-structure-breaking properties of bromide ions. Unlike heavily hydrated anions such as fluoride, bromide ions are relatively less hydrated. In aqueous-based chromatographic systems bromide ions can reduce the surface area of the solute exposed to water molecules by preferentially binding to the stationary phase or protein

surfaces. Therefore, at higher bromide concentrations, it is possible that the cleft of HEWL is forced open by a large number of bromide ions condensing onto the surface of the cleft which results in the larger Z_c values observed in Table I. At a critical concentration, differing for each bromide salt, the number of bromide ions in the cleft is sufficiently reduced to allow hinge movement and the relative closure of the cleft. This behaviour will result in a decrease for Z_c observed for the lower bromide concentrations. The observation that ion-exchange-adsorbed HEWL and other enzymes show decreased catalytic activity which is restored on elution with salts and that substrate-specific elution can be achieved for enzyme adsorbed to ion-exchange or biomimetic-affinity sorbents[41,42] provides further support to the concept that, in the

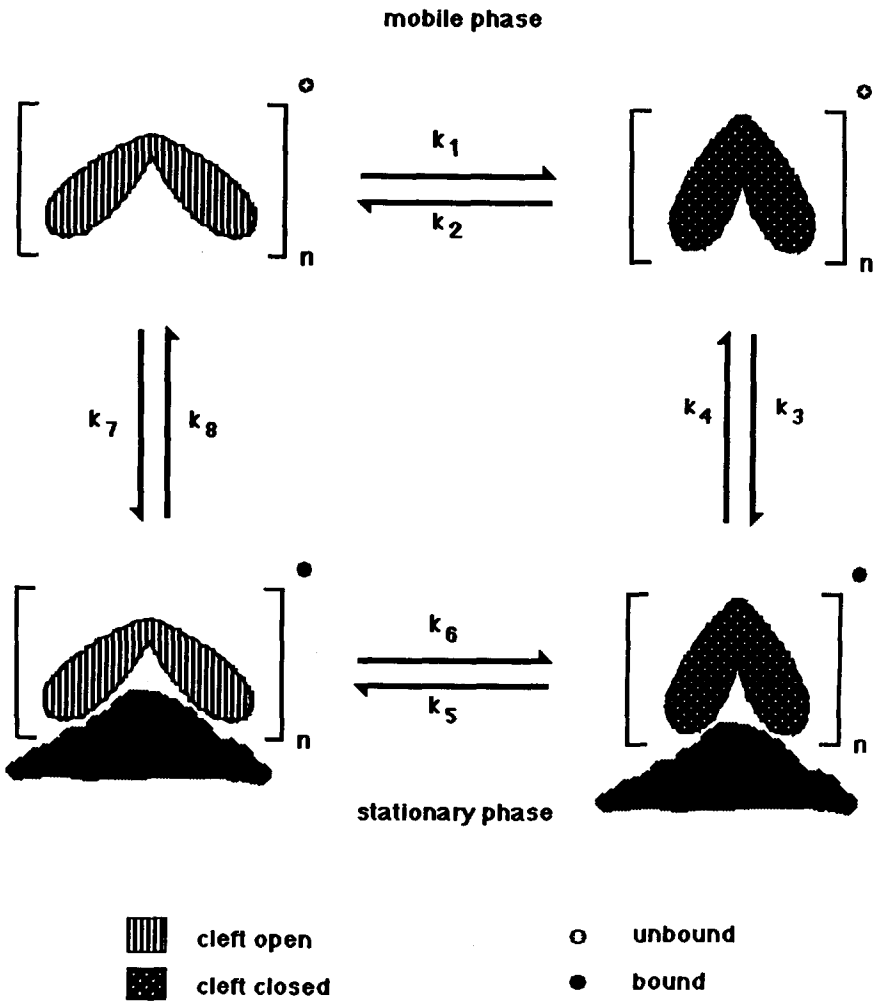

Fig. 9. Schematic representation of the structural equilibria associated with the binding of lysozyme to the anion-exchange surface, involving the opening and closure of the active cleft as a result of a hinge-bending mechanism.

case of HEWL, the cleft region is involved with coulombic recognition with the cationic ligand.

Inspection of the data in Table I also reveals that the choice of elution mode, *i.e.* either varied gradient time, varied flow-rate or isocratic elution, can influence the binding process and the hinge-bending movement of HEWL. For example, as shown in Table I, the elution of HEWL with LiCl results in significantly different Z_c values for these two different elution modes. Hence, the length of exposure to the stationary phase surface which is controlled by the rate of change in the displacer-salt concentration also determines the extent of opening or closure of the lysozyme cleft.

In this study, changes in Z_c value have been quantitatively related to the movement of the catalytic cleft of HEWL. Fig. 9 schematically depicts the structural equilibria associated with the binding of HEWL to the anion-exchange surface. Assuming HEWL is a monomer, *i.e.* when $n = 1$, the binding process which occurs at the cleft–stationary phase interface can be characterised in terms of the degree of opening or closing of the cleft and will be governed in part by the nature of the displacer-salt. The variation in entropy resulting from these salt-induced structural changes will contribute toward the free energy required for hinge-movement in HEWL. The hinge-bending process will be governed by the rate constants k_1 to k_8. When the cleft is closed, *i.e.* when $Z_c = 1$ only the surface residue will be accessible whilst the negatively charged groups located inside the cleft are sterically prevented from direct electrostatic interaction with the support surface (*i.e.* the structure on the right-hand side of Fig. 9). In contrast, larger Z_c values, *i.e.* $Z_c = 3$–4 correspond to the opened cleft which results in maximal electrostatic interactions (the left-hand side of Fig. 9). The thermodynamics and kinetics of adsorption will be further complicated under conditions where the polymerisation of HEWL can be induced, *i.e.* when $n > 1$. The dependence of the hinge-movement equilibria on the bromide concentration, which is reflected by non-continuous retention plots such as the data shown in Fig. 8, can also be accomodated by this model where the interconversion process will be controlled by the rate constants k_3, k_4, k_5 and k_6.

CONCLUSIONS

The present study introduces an approach to the correlation of anion-exchange chromatographic data with protein three-dimensional structures in order to allow the identification and characterisation of the molecular identity of the electrostatic interactive binding site on proteins. In the case of HEWLs, the mechanism by which HEWL approaches and orientates itself at the stationary phase surface can be determined through the analysis of the spatial disposition of neighbouring positively charged and hydrophobic residues. The existence of conformationally flexible structures and analysis of the influence of elution conditions on the underlying equilibrium processes also provide significant insight on the experimental factors which can be used to control the interaction between proteins and chromatographic surfaces. The interactive behaviour of lysozyme variants on both a cation-exchange resin and a hydrophobic interaction stationary phase has been recently investigated[43,44] and partly correlated with amino acid sequence differences. The potential therefore now exists to chromatographically map the surface topography of proteins through the correlation of retention properties in different interactive modes with the three-dimensional structure of a protein.

The identification, analysis and prediction of the surface-accessible interactive regions of biomacromolecules is also central to a wide range of biological systems which are controlled by biorecognition, interfacial phenomena or surface–surface interactions. For example, the residues Asp 48 and 52 of HEWL, which are located in the anion-exchange binding site, have also been implicated as forming part of the antigenic determinants recognised by specific T-cell hybridomas[45]. While the intrinsic chemical and stereospecific features which immunologically distinguish the most reactive and the least reactive sites of a protein are not fully understood, the most frequently recognised antigenic and immunogenic sites form three-dimensional superassemblies characterised by high local mobility, convex surface shape and negative electrostatic potential. Furthermore, the prevalence of complementary hydrophobic and aromatic residues in the binding sites of antibodies[40] also suggests an important binding role for buried hydrophobic side chains. Clearly, high-performance liquid chromatographic techniques have the potential to characterise the relative spatial disposition of amino acid residues within the three-dimensional structure of *e.g.*, a protein. Definition of the molecular basis of the interactions between the biomacromolecule and a biological or synthetic surface has not only significant implications for the optimisation of separation performance *per se* but also would impact on the design and development of synthetic peptidic vaccines and new peptidic analogues for use in therapy.

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