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CHROMATOGRAPHIC RESOLUTION OF LYSOZYME VARIANTS

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SUMMARY

There are seven avian lysozyme variants of nearly identical three-dimensional structure which have amino acid substitutions broadly distributed on their surface. By using these protein variants, it was possible to study the relationship between protein structure and chromatographic retention. It was determined that according to the mode of separation various regions of the proteins surface determine chromatographic retention. At one extreme, immunosorbents targeted a very small region on the protein surface. Hydrophobic interaction chromatography was an intermediate case in which one surface domain of the lysozymes controlled chromatographic behavior. At the opposite extreme, cation-exchange columns probed most of the protein surface. It was concluded that identification of random variations in protein structure will be most successfully detected by a separation mode that broadly targets the surface of a protein.

INTRODUCTION

There are at least seven avian lysozyme variants that have been shown by X-ray structure analysis to have nearly identical three-dimensional structure[1]. When these isoenzymes were examined by hydrophobic interaction chromatography (HIC), a region of the molecular surface opposite the catalytic cleft, extending from residue 41 to 102 and from residue 75 to the α-helical region starting at residue 89 dominated chromatographic retention[2]. This region of contact between a chromatographic sorbent and a solute has been referred to as the chromatographic contact region[3]. Amino acid substitutions in lysozyme variants within this region altered chromatographic behavior, whereas substitutions in other external faces of the protein had no influence on chromatographic retention[2]. All but two of the seven lysozyme variants examined were resolved by HIC.

The objectives of this paper were to determine: (i) whether other modes of liquid chromatography were equally capable of resolving variant proteins; and (ii) whether each mode has a unique chromatographic contact region. Separation modes examined were reversed-phase chromatography (RPC), cation-exchange chromatography (CEC), and immuno-affinity chromatography (IAC).

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EXPERIMENTAL

Reagents

CM-Cellulose was purchased from Sigma (St. Louis, MO, U.S.A.). CM-Sephadex was obtained from Pharmacia (Uppsala, Sweden). Buffers were made with [2-(N-morpholino)ethanesulfonic acid] (MES) (Calbiochem-Boehringer, LaJolla, CA, U.S.A.), tris(hydroxymethyl)aminomethane (Tris) (Boehringer Mannheim Biochemicals, Indianapolis, IN, U.S.A.), sodium borate (MCB Manufacturing Chemists, Cincinnati, OH, U.S.A.), sodium chloride, ammonium acetate (Fisher, Fair Lawn, NJ, U.S.A.). Trifluoroacetic acid (TFA) was purchased from Pierce (Rockford, IL, U.S.A.). All solutions were filtered through a Rainin Nylon-66 (0.45 μm) filter (Woburn, MA, U.S.A.) and degassed. The pH was adjusted with appropriate base or acid. Organic solvents used for RPC were of HPLC grade (American Burdick & Jackson, Muskegon, MI, U.S.A.).

Lysozyme purification

Peking duck eggs were purchased from Gallo Duck Farm (Patchogue, NY, U.S.A.). Lyophilized turkey lysozyme was obtained from Dr. Eli Sercarz of the Microbiology Department of the University of California at Los Angeles, U.S.A. Ring-necked pheasant (RNP) and Japanese quail (JPQ) egg whites were obtained from Dr. Michael Laskowski, Jr., of the Chemistry Department of Purdue University. Peking duck, RNP and JPQ lysozymes were purified from egg white by a procedure based on that of Prager and Wilson[4] and described in a previous paper[2].

Chromatography

Determination of the Z numbers by CEC was performed by eluting the proteins isocratically from a Mono-S column (Pharmacia, Piscataway, NJ, U.S.A.) at increasing salt concentration of sodium chloride in 10 mM MES (pH 6.0), or 10 mM potassium phosphate (pH 7.0), or 10 mM Tris (pH 8.0). A flow-rate of 0.5 ml/min was produced with a Hewlett-Packard 1084B liquid chromatograph, equipped with a 79875A scanning UV detector, a 79850 LC terminal and a 79841A variable-volume injector (Hewlett-Packard, Waldbron, F.R.G.).

The other experiments in CEC and RPC were carried out on a Varian 5500 gradient pumping system (Varian, Walnut Creek, CA, U.S.A.). Detection was monitored at 280 nm with a LC 85B variable-wavelength UV detector (Perkin-Elmer, Norwalk, CT, U.S.A.). The injector was a Rheodyne sampling loop 7125 (Rheodyne, Berkeley, CA, U.S.A.). The polystyrene bulk packing material was obtained from Polymer Labs. (Shropshire, U.K.). The SynChropak CM-300 bulk packing and the SynChropak S-300 column were purchased from SynChrom (Linden, IN, U.S.A.).

The immuno-affinity column was made by covalently immobilizing a monoclonal antibody on a hydrophilic poly(styrene–divinylbenzene) matrix, which had been activated with carbonyl diimidizole. The antibody was generously donated by Dr. Sandra Smith-Gill at the National Institute of Health. IAC separations were carried out on a tandem column HPLC system in which the immuno-affinity column was coupled to an RPC column through a switching valve. Antigens adsorbed by the immuno-affinity column were subsequently desorbed onto the RPC column and separated with a TFA–acetonitrile gradient. Glycine buffer of 100 mM (Nutritional

Biochemicals Corporation, Cleveland, OH, U.S.A.) (pH 2.2) was used as the antigen displacing agents. Desorbed antigen(s) were concentrated at the top of an RP-8 Syn-Chropak column and then eluted by a TFA–acetonitrile gradient. This system allows direct chromatographic analysis of materials eluted from IAC[5].

RESULTS AND DISCUSSION

Seven avian lysozymes of defined amino acid sequence (Fig. 1) were used in this study. The three-dimensional structure of hen egg white (HEW) lysozyme is given in Fig. 2 as a reference for amino acid substitutions in the variants. As noted above, the X-ray structure of HEW lysozyme is very similar to that of the variants in Fig. 1[1].

Reversed-phase chromatography

RPC and HIC are similar in retention mechanism in that hydrophobic interactions are the basis for solute sorption. The primary difference between these two modes of chromatography is that in RPC the stationary phases are more hydrophobic and elution is achieved with organic solvents instead of aqueous buffers. HIC allowed the separation of all the variants but two (duck A and B)[2]. Surprisingly, RPC gave little resolution of the lysozyme variants on either alkyl silane or divinylbenzene sorbents, eluted with a variety of mobile phases. TFA–acetonitrile, TFA–2-propanol, and 60% formic acid–acetonitrile were all used unsuccessfully as mobile phases in attempts to resolve all of the lysozyme variants by RPC. Varying the concentration of the acidic component of the mobile phase did not give any improvement. Although there were increases in solute retention and slight changes in selectivity as the concentration of TFA was increased, the changes were not sufficiently large to achieve more than a 40-s difference in retention between any two species (Fig. 3).

	1	5	10	15	20	25	30	35	40	45	50	55	60	65
Chicken	K	V	F	G	R	C	E	L	A	A	A	M	K	R
Japanese quail		Y												
Ring-necked pheasant	G	Y				M								
Turkey		Y				L								
Duck A		Y	S			L								
Duck B		Y	S			L								
Duck C		Y	S			L								

	66	70	75	80	85	90	95	100	105	110	115	120	125
Chicken	D	G	R	T	P	G	S	R	N	L	C	N	I
Japanese quail													
Ring-necked pheasant													
Turkey													
Duck A													
Duck B													
Duck C													

	66	70	75	80	85	90	95	100	105	110	115	120	125
Chicken	P	C	S	A	L	L	S	S	D	I	T	A	S
Japanese quail													
Ring-necked pheasant													
Turkey													
Duck A													
Duck B													
Duck C													

	66	70	75	80	85	90	95	100	105	110	115	120	125
Chicken	V	N	C	A	K	K	I	V	S	D	G	N	G
Japanese quail								V H				N	
Ring-necked pheasant			K		H						K H	N V	
Turkey			K					A G				H	
Duck A		K		K	A	G	V	R	E A R	R		R	S K
Duck B		K	R	K	A	G	V	R	E A R	R		R	S K
Duck C		K	R	K	A	G R	V	R	E A R	R		R	S K

	66	70	75	80	85	90	95	100	105	110	115	120	125
Chicken	A	W	R	N	R	C	K	G	T	D	V	Q	A
Japanese quail													
Ring-necked pheasant													
Turkey													
Duck A													
Duck B													
Duck C													

	66	70	75	80	85	90	95	100	105	110	115	120	125
Chicken	W	I	R	G	C	R	L						
Japanese quail													
Ring-necked pheasant													
Turkey													
Duck A													
Duck B													
Duck C													

Fig. 1. Primary structure of the seven avian lysozymes[2].

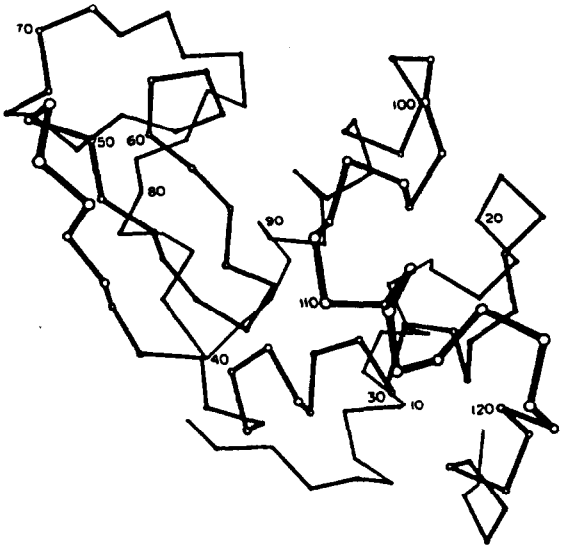

Fig. 2. The three-dimensional structure of HEW lysozyme[2].

These findings are quite remarkable for the following reasons. First, the turkey and HEW lysozymes vary at 7 of 129 amino acid residues. It is difficult to imagine how such a large difference could go undetected in lysozyme when it has been reported that single amino acid substitutions have been detected in large polypeptides by RPC[3,6]. Second, the lysozyme variants are so well resolved by HIC that it is

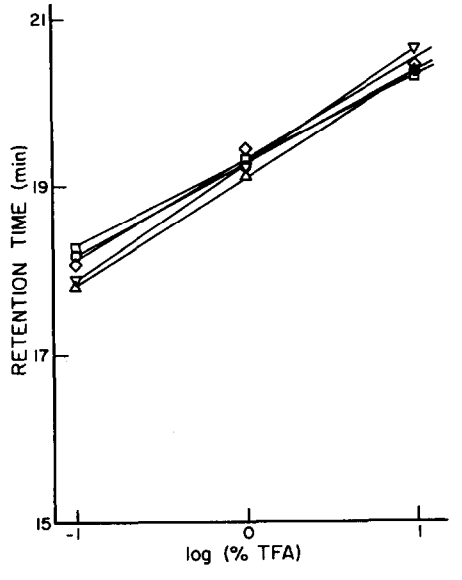

Fig. 3. Retention time *vs.* concentration of acid in the mobile phase on a polystyrene column. Elution was achieved with a 40-min linear gradient, ranging from 0.1% aq. TFA to 100% acetonitrile. ○ = Duck A; △ = duck C; □ = HEW; ◇ = RNP; ▽ = turkey.

equally difficult to understand how all resolutions could collapse on a RPC column. The most plausible explanation is that some region of the structure which is conserved in all the lysozyme variants dominates retention in RPC. If amino acid substitutions in the variants are outside of this chromatographic contact region, they would play no role in retention, and all of the variants would appear to be identical. The validity of this explanation awaits more detailed studies on the interaction of lysozyme with hydrophobic surfaces under denaturing conditions.

Cation-exchange chromatography

Ion-exchange chromatography on both weak and strong cation-exchange columns was found to be very effective in the resolution of lysozyme variants. Even if all the lysozymes cannot be resolved in the same chromatogram, it is remarkable that the three Peking duck A, B, and C lysozymes, which vary by single charges, were resolved (Fig. 4). Resolution of all species was achieved at approximately pH 7 (Fig. 5). Selectivity was relatively constant as a function of pH for all species, except for the HEW and RNP lysozymes. Nothing in the amino acid composition or structure explains the behavior of the HEW and RNP lysozymes.

An attempt was made to identify the chromatographic contact region in CEC by using the ionization of histidine in the range of pH 6–8 and the stoichiometric displacement model (SDM) of retention for ion-exchange chromatography[7,8]. In the stoichiometric displacement model, Z represents the average number of cations that are required to displace lysozyme from the CEC column. It is seen in Table I that ΔZ/histidine is approximately 0.24 in all variants except RNP, where ΔZ/histidine is 0.37. These results strongly imply that histidines located on multiple faces of lysozyme contribute to chromatographic retention. This would mean that the chromatographic contact region in CEC is different from that in HIC. For example, the histidines at positions 15, 41, 77, 103, 114, and 121 are clearly making a contribution

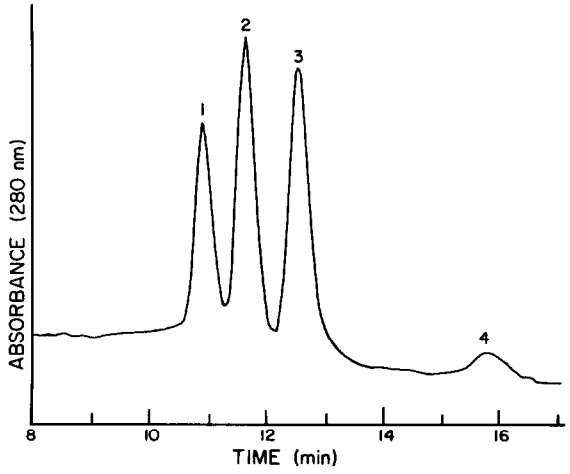

Fig. 4. The separation of duck and RNP lysozymes on a SynChropak S-300 strong-cation-exchange column. Elution was achieved with a 30-min linear gradient, ranging from 10 mM borate buffer (pH 9.2) to 1 M sodium chloride in 10 mM borate buffer (pH 9.2). 1 = RNP; 2 = duck A; 3 = duck B; 4 = duck C.

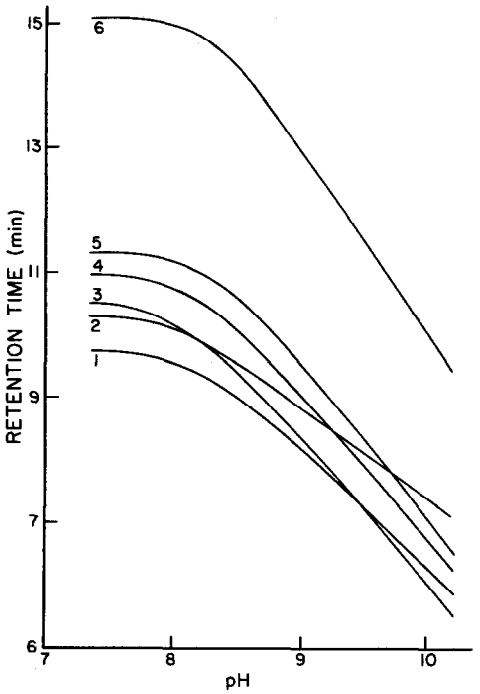

Fig. 5. Retention time of lysozyme *vs.* pH on a SynChropak CM-300 column. Elution was achieved with a 20-min linear gradient from 100% buffer A to 100% B. When the pH was less than 8, buffer A was 10 m*M* phosphate buffer. When the pH was greater than 8, buffer A was 10 m*M* borate buffer. Buffers A and B were identical with the exception that buffer B contained 1 *M* sodium chloride. 1 = HEW; 2 = JPQ; 3 = RNP; 4 = duck A; 5 = duck B; 6 = duck C.

to retention in CEC, in contrast to HIC, where only residues 41, 77, and 103 participated[2].

It is still unexplained how ionic groups on opposite sides of a molecule can both participate in chromatographic retention. There are several possible explana-

TABLE I

HISTIDINE CONTRIBUTION TO *Z*

Species	*No. of His*	*Position*	*pH*	*Z*	*ΔZ*	*ΔZ/His*
Duck A	0	–	8	2.34	0.00	–
	0	–	6	2.34	0.00	–
HEW	1	15	8	2.28	0.00	–
	1	15	6	2.50	0.22	0.22
JPQ	2	15 and 103	8	2.33	0.00	–
	2	15 and 103	6	2.82	0.49	0.25
Turkey	2	41 and 121	8	2.64	0.00	–
	2	41 and 121	6	3.12	0.48	0.24
RNP	2	77 and 114	8	2.11	0.00	–
	2	77 and 114	6	2.85	0.74	0.37

tions. One possibility is that the molecule has multiple contact sites that are energetically equivalent. Rotational translation of the solute across the sorbent surface would cause all of these equivalent contact regions to interact with the sorbent on a transitory basis. This would make the contribution of a single cationic amino acid within one of these contact regions directly proportional to the relative amount of time it spends in contact with the surface and account for fractional Z values. A second explanation is that amino acids do not have to come in direct contact with the sorbent surface to contribute to sorption and retention. If the dielectric constant within the interior of a protein is sufficiently low, the electrostatic potential of an amino acid can be felt on the opposite side of a molecule. Russel and Fersht[9] have shown that the pK_a of active-site histidines can be influenced by charged groups >15 Å away.

The larger ΔZ/histidine value for RNP implies that the contribution to chromatographic retention of one or both of the histidines in this species is larger than in the other variants. The same phenomenon is seen in the duck B and C variants where the ΔZ/arginine values are different. These results indicate that, although charged groups from many different locations in a molecule are sensed by a sorbent, their contributions may not be equivalent.

Immuno-affinity chromatography

The immunosorbent used in these studies was prepared with a monoclonal antibody directed against HEW lysozyme. Antibody binding was at an epitope, consisting of amino acid residues 102, 103, and the amino acids surrounding the C-terminus[10]. RNP, HEW, and duck A lysozymes are all identical in this region. As expected, the monoclonal immunosorbent did not differentiate between these variants when the antigens were desorbed with weak acids.

CONCLUSIONS

Based on this study and the previous work of Fausnaugh and Regnier[2] it may be concluded that both HIC and CEC are capable of differentiating between most of the natural lysozyme variants. However, the chromatographic contact region for these two separation modes is different. Chromatographic retention is determined in HIC by amino acids on a single face of the protein opposite the catalytic cleft. Substitutions outside of this region have little influence on retention. In contrast, cationic groups on multiple faces of the lysozyme variants contribute to retention in CEC.

The findings indicate that the chromatographic contact region of a polyeptide is defined both by amino acid distribution within the molecule and the nature of the sorbent surface. When all of the amino acids participating in the retention process are localized in one small area of the molecule, the chromatographic contact region will be small. In contrast, a broad distribution of participating amino acid residues will result in a large chromatographic contact region.

Finally, it is possible to conclude that when searching for small random modifications of errors in a polypeptide, a separation mode with a broadly distributed chromatographic contact region is the most likely to be successful. Chromatographic modes such as IAC will have a much higher probability of failure because interaction with the protein surface is focused on fewer than ten amino acids.

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