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A cytochrome C homologous library with varying charge distribution was employed to study the effects of protein surface modification on protein retention in cation exchange chromatography. Varying quantities of succinic anhydride were added to horse cytochrome c to control the degree of succinylation of protein surface lysine residues. Cation exchange chromatography was then carried out using a succinylated protein mixture containing primarily single lysine modifications and the collected fractions were evaluated using direct infusion and tryptic digest–mass spectrometry to determine the degree of succinylation and the sites of modification on the protein surface. Electrostatic potential (EP) maps of the native protein and the variants were generated to provide insight into the elution order of the variants and the results indicated that there were two major binding regions on the protein surface. Variants showing the largest change in retention time as compared to the native protein had modified residues within the binding regions. On the other hand, variants which showed a smaller change in protein retention time had succinylated residues at locations which were not part of the two distinct binding regions. This study demonstrates that the use of controlled protein surface modification offers a convenient means of studying the relationship between protein surface properties and chromatographic binding affinity.

Keywords electrospray ionization mass spectrometry; homologous protein library; ion exchange chromatography; protein adsorption; protein surface chemical modification

INTRODUCTION

Ion exchange chromatography is a separation process that is commonly used in the downstream purification of biomolecules in the biopharmaceutical industry. However, in order to further optimize and improve ion exchange resins for separation processes, it is essential to gain a better understanding of protein interactions in these systems. Protein surface charge density and distribution play significant roles in determining protein retention in ion exchange chromatography (1,2).

Homologous libraries of protein molecules can be used to provide significant insight into the relative importance of protein surface charge residues in determining protein retention on the ion exchanger. Due to the similarity in the physical structure of the homologous proteins, changes in protein retention on the ion exchanger can be correlated to the modifications in the protein surface properties.

Site-directed mutants and protein variants from different species have previously been employed to generate homologous libraries of protein molecules. Through the use of single site mutants, Chicz and Regnier have shown that significant changes in protein retention can arise if the mutation occurred at the sites of protein-resin interaction (3). Using rat cytochrome b5 variants in combination with electrostatic potential surfaces and electrostatic interaction free energies, Roush and coworkers (4,5) have simulated the binding of rat cytochrome b5 mutants to an anion exchange surface. Yao and Lenhoff (6,7) examined the electrostatic interaction free energies for cytochrome c variants from different species binding onto a cation exchanger and modeled the protein retention behavior using energy-minimized 3D models. We have used a homologous library of site directed mutants of cold shock protein B (CspB) to examine the effects of charge modification on the retention behavior in cation exchange chromatography and to elucidate preferred binding regions on the protein surface (8).

Despite the advantages offered by homologous mutant libraries, the generation of an extensive mutant library is a labor intensive and costly procedure. The use of protein variants from different species is less labor intensive but is limited by the number of variants that are available.

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Chemical modification of the protein surface presents an easier, faster and less costly alternative. Dismer and Hubbuch (9) used a dye labeling process to examine the binding orientation of lysozyme on various cation exchange surfaces. Brautignan et al. (10) modified the surface of horse cytochrome c with 4-chloro-3, 5-nitrobenzoate to study the binding of the protein on a CM–cellulose cation exchanger. While that work demonstrated the ability to identify the elution order of chemical variants of proteins, their analysis of the data was limited due to a lack of the availability of appropriate modeling techniques. We have recently employed lysozyme charge ladders to examine the retention of this complex protein library on a cation exchange surface and have determined the preferred binding regions on the protein surface using detailed electrostatic potential maps (11). However, that analysis was complicated by the presence of a very large number of chemical variants.

In this paper, we employ succinic anhydride under controlled conditions to generate homologous horse cytochrome c variants of specified degrees of modification. Succinic anhydride alters the net charge on the protein surface by replacing the positive charge on the lysine side-chain with a negatively charged moiety. The modified protein mixture is then used to examine the effects of protein surface charge reversal on protein binding affinity and preferred binding regions in cation exchange chromatography (CEX). Direct infusion electrospray ionization mass spectrometry (ESI-MS) is performed on CEX eluent fractions to determine the presence of variants. Enzymatic digest and MS are subsequently performed to determine the sites of succinylation on the protein variant surface. Finally, electrostatic potential maps of these variants are generated to provide further insight into the elution order of the variants.

EXPERIMENTAL

Materials and Reagents

Horse cyotchrome c, tris-base, glycine, hydrochloric acid, mono- and di-basic sodium acetate, sodium chloride, trypsin, trifluoroacetic acid, ammonium bicarbonate, and ammonium acetate were purchased from Sigma Chemical Co. (St. Louis, MO). succinic anhydride, sodium hydroxide, and acetonitrile were purchased from Fisher Scientific (Fair Lawn, NJ). Ethanol was purchased from Aldrich (Milwaukee, WI). Dithiothreitol was purchased from Promega Corp. (Madison, WI). ZipTips were purchased from Millipore Corp. (Billerica, MA). Strong cation-exchange (SCX) columns (sulfopropyl on crosslinked polymethacrylate polyol ester) Protein-Pak SP8HR (8 μm, 100 mm × 4.6 mm ID) were a gift from Waters (Milford, MA).

Equipment

Analytical linear gradient experiments were performed using a chromatographic system from Waters (Milford, MA), which consisted of a 600E Multi-solvent Delivery System, a PDA 996 photodiode array detector, and a 712

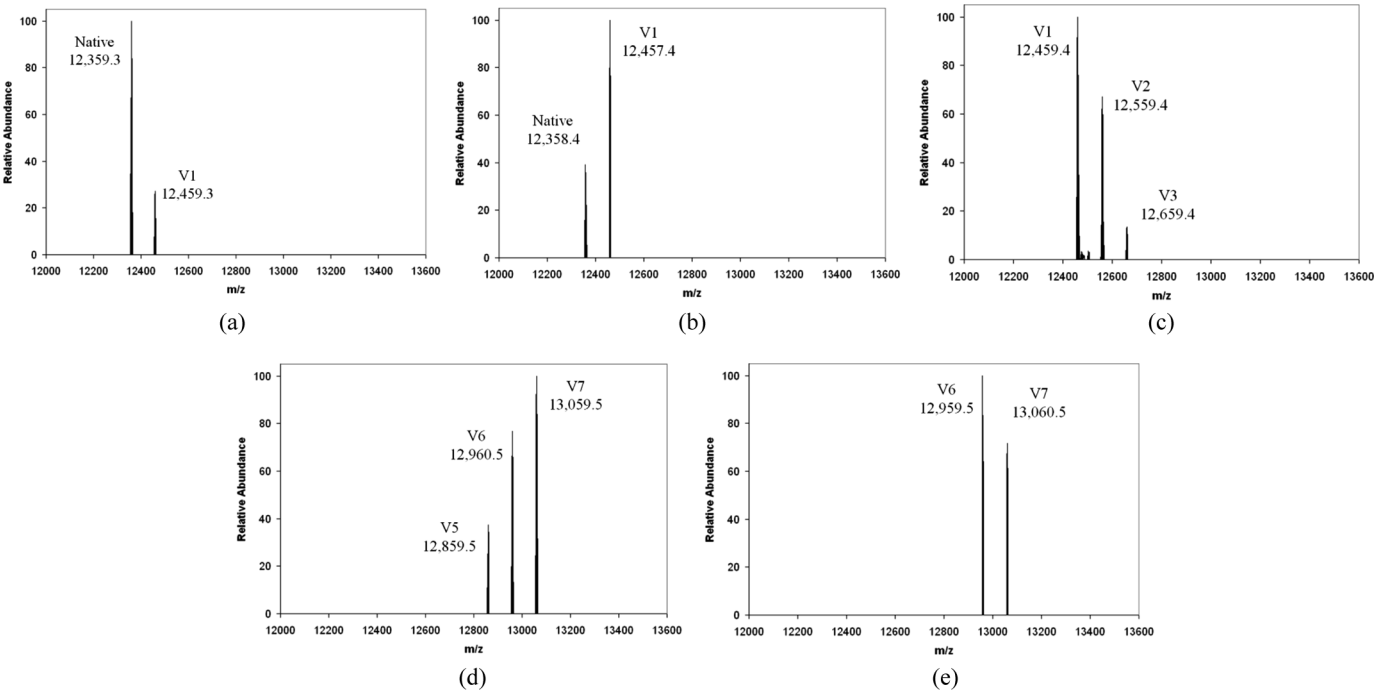


FIG. 1. Mass spectra of 1 mM horse cytochrome C reacted with (a) 10 mM succinic anhydride; (b) 25 mM succinic anhydride; (c) 50 mM succinic anhydride; (d) 75 mM succinic anhydride; and (e) 100 mM succinic anhydride.

WISP auto sampler with a cooling module. A 2 ml Waters Protein-PAK 8HR SP chromatographic column (1000 Å pore size, 8 μm particle size, 10 × 100 mm) was used in variant separation. Fractions of the column eluent were collected using an Advantec SF-2120 Super fraction collector. A Thermo LTQ Orbitrap XL linear trap-orbitrap mass spectrometer was used to carry out direct infusion mass spectrometry analysis on selected column eluent fractions.

PROCEDURES

Succinylated Protein Mixture Formation

1 ml of 1 mM horse cytochrome c in 3 mM ammonium acetate (pH 7) was prepared in a 1.5 ml eppendorf tube. Appropriate concentrations of succinic anhydride were added to attain final concentrations of 10 mM, 25 mM, 50 mM, 75 mM, or 100 mM and the mixtures were vortexed for 1 hour. Subsequently, all mixtures were dialyzed overnight in 10 mM sodium phosphate buffer, pH 7, using a dialysis membrane with a molecular weight cut-off of 3000 Da.

Cation Exchange Chromatography

A Waters Protein-PAK 8HR SP column was used for the analysis and separation of the various succinylated protein mixtures. Separate injections of 200 μL of the protein mixture that had been reacted with 25 mM succinic anhydride and 20 μL of unreacted horse cytochrome c (1 mM) were loaded onto the column and analyzed for their retention times. Linear gradient elution runs from 100% buffer A (20 mM sodium acetate, pH 5.0) to 100% buffer B (20 mM sodium acetate containing 500 mM of sodium chloride, pH 5.0) in 20 column volumes at a flowrate of 0.5 ml/min were carried out to obtain retention time data on the variants. The column effluent was monitored at 280 nm and eluent fractions were collected at a rate of 1 fraction per minute.

Direct Infusion Elecrospray Ionization Mass Spectrometry Analysis

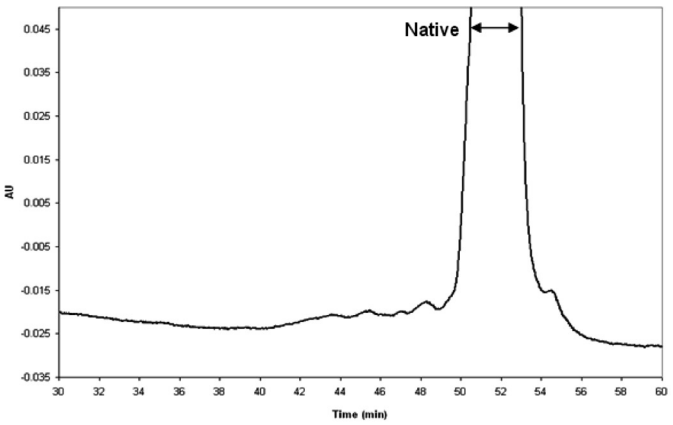
All samples were desalted using Millipore reversed-phase ZipTip pipette tips before mass spectrometry analysis. The samples were eluted from the ZipTips using a 90% ACN, 0.1% TFA, and 9.9% (v/v) water solution. Electrospray ionization mass spectra were obtained on a Thermo LTQ Orbitrap XL linear trap-orbitrap mass spectrometer. Samples were introduced into the ion source using an autosampler without prior enzymatic digestion delivered at a flow rate of 50 μL/min. The source voltage was set to 3.9 kV and the capillary voltage set to 275°C. Data was collected in the positive ion modes in a mass range from m/z of 500 to 2000 Da. The mass spectrometer was calibrated and optimized for the detection of the succinylated horse cytochrome c feed mixture. Instrument control, data acquisition, and data processing were performed using Xcalibur 2.0 software (Thermo).

Enzymatic Digestion of Collected Fractions

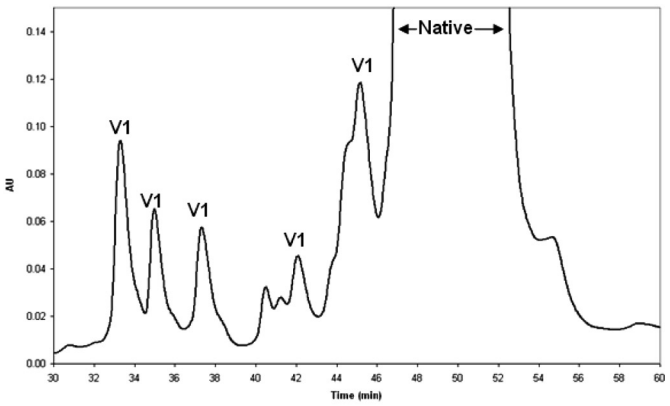
Dithiothreitol (DTT) was added to each fraction to a concentration of 4 mM. This solution was heated to 60°C for 1 hour. The pH was adjusted to 8.0 and trypsin was added at a substrate:enzyme ratio of 30:1 and incubated at 37°C for 24 hours with periodic mixing. The digest was allowed 1 hour to cool to room temperature and glacial acetic acid was added to stop the digest by bringing the pH to 3.0. This peptide mixture was then stored frozen at – 80°C until mass spectrometry analyses were performed. Aliquots were allowed to thaw completely and were mixed prior to further processing.

Analysis of the Electrostatic Potential Surfaces of Native and Protein Variants

To study the distribution of electrostatic potential near the protein a series of Poisson-Boltzmann calculations were preformed on the native protein as well as succinylated variants. Each variant was developed by manually mutating the



(a)



(b)

FIG. 2. Chromatogram of (a) 1 mM native horse cytochrome C; (b) succinylated horse cytochrome c mixture (25 mM succinic anhydride was added to 1 mM horse cytochrome C) on a Waters protein-PAK SP8HR cation exchange column.

pdb file then minimizing using the AMBER 94 force field. These structures were then parameterized with the PARSE force field due to its ability to properly account for implicit solvent effects. The Adaptive Poisson-Boltzmann Solver (APBS) (12–15) package was used to generate electrostatic distributions which were then depicted and analyzed using the VMD molecular graphics viewer (16).

RESULTS AND DISCUSSION

Succinylated Protein Mixture Formation

Homologous protein libraries provide an effective means of gaining insight into the effects of protein surface properties on its retention in chromatographic systems. Chemical modification of a protein surface allows for the rapid generation of a homologous library of protein variants. Succinylation of a lysine residue reverses the charge of the residue sidechain. The small size of the chemical modifier allows for succinylation of one site on the protein surface without affecting the reactivity of other neighboring lysine residues. In addition, it has been reported in the literature that the reaction of the protein with succinic anhydride causes no change in the protein structure, thereby providing a convenient and rapid means of generating a homologous library of surface charge modified protein variants (17). Further, addition of varying quantities of succinic anhydride to the protein enables the degree of protein succinylation to be controlled.

Succinylated cytochrome c mixtures were prepared as described in the experimental section. Figures 1a–e are the mass spectra obtained for 1 mM horse cytochrome c solutions that have been reacted with 10 mM, 25 mM, 50 mM, 75 mM, and 100 mM succinic anhydride, respectively. As can be seen in Fig. 1a, a concentration of 10 mM resulted in the formation of a relatively small amount of variant 1 (V1), proteins where one lysine residue has been succinylated. A concentration of 25 mM resulted in the formation of more V1s, with no higher variants being formed

TABLE 1
(a) Abbreviated list of peptide fragments used in the identification of the sites of succinylation on the surface of horse cytochrome c and (b) List of variants identified using tryptic digest mass spectrometry

(a) Label	Modification site	Peptide mass (charge)	Residues	Sequence
1^{H}	Lys^{25}	525.6 (+1)	23–27	GGKHK
2^{N}	Lys^{27}	1433.6 (+1), 716.8 (+2), 477.9 (+3)	26–38	HKTGPNLHGLFGR
3^{N}	Lys^{72}	1623.8 (+1), 811.9 (+2), 541.3 (+3)	61–73	EETLMEYLENPKK
4^{N}	Lys^{79}	1438.8 (+1), 719.4 (+2)	74–86	YIPGTKMIFAGIK
5^{N}	Lys^{86}	907.2 (+1), 453.6 (+2)	80–87	MIFAGIKK
6^{N}	Lys^{39}	1598.7 (+1), 799.4 (+2)	39–53	KTGQAPGFTYTDANK
7^{N}	Lys^{60}	2081.4 (+1), 1040.7 (+2)	56–72	GITWKEETLMEYLENPK
1^{s}	Lys^{25}	625.7 (+1)	23–27	GGK_SHK
2^{s}	Lys^{27}	1533.8 (+1), 766.9 (+2), 511.3 (+3)	26–38	$HK_sTGPNLHGLFGR$
3^{s}	Lys^{72}	1723.9 (+1), 862.0 (+2), 574.6 (+3)	61–73	$EETLMEYLENPK_sK$
4^{s}	Lys^{79}	1538.9 (+1), 769.5 (2)	74–86	$YIPGTK_sMIFAGIK$
5^{s}	Lys^{86}	1007.4 (+1), 503.7 (+2)	80–87	$MIFAGIK_SK$
6^{s}	Lys^{39}	1698.8 (+1), 849.4 (+2)	39–53	$K_sTGQAPGFTYTDANK$
7^{s}	Lys^{60}	2181.5 (+1), 1090.8 (+2)	56–72	$GITWK_sEETLMEYLENPK$

(b) Fraction time (min)	Assignment	Sites of Lys succinylation	Peaks observed: Not in native (native peaks absent upon succinylation)
33	V1	Lys^{86}	(4^{N}), (5^{N}), 5^{s}
35	V1	Lys^{25}	(1^{N}), (2^{N}), 1^{s}
37	V1	Lys^{72}	(3^{N}), 3^{s}
42	V1	Lys^{79}	(4^{N}), (5^{N}), 4^{s}
45	V1	Lys^{27}	(1^{N}), (2^{N}), 2^{s}
50	Native	–	$1^{N} - 7^{N}$
66	Native	–	$1^{N} - 7^{N}$
68	Native	–	$1^{N} - 7^{N}$
75	Native	–	$1^{N} - 7^{N}$
79	Native	–	$1^{N} - 7^{N}$

(Fig. 1b). A higher concentration of 50 mM succinic anhydride resulted in the formation of V1s and V2s (Fig. 1c). When 75 mM and 100 mM of succinic anhydride were used, variants with still higher degrees of succinylation were obtained. For example, a final concentration of 75 mM succinic anhydride resulted in the formation of V5s, V6s, and V7s (Fig. 1d), while the sample containing 100 mM of succinic anhydride yielded V6s and V7s (Fig. 1e).

Prakash and Mazumdar (17) have reported that seven (Lys 25, Lys 27, Lys 39, Lys 60, Lys 72, Lys 79, Lys 86) of the nineteen lysine residues on the surface of horse cytochrome c were highly susceptible to succinylation. Of these seven residues, five (Lys 25, Lys 27, Lys 72, Lys 79, Lys 86) had a higher reactivity than the remaining two. Thus, it is likely that the V1s and V2s obtained using 10 mM, 25 mM, and 50 mM succinic anhydride were succinylated at one or more of the five highly reactive lysine residues. The V5s, V6s, and V7s that were observed in the samples with 75 mM and 100 mM succinic anhydride were probably succinylated at one or both of the less reactive lysines (Lys 39, Lys 60) in addition to the five highly reactive lysine residues.

Clearly, the use of varying quantities of succinic anhydride enables controlled succinylation of horse cytochrome c. In order to study the effects of modifying a protein's surface charge on its retention behavior in ion exchange chromatography, the sample that had been treated with 25 mM succinic anhydride was employed. Cation exchange analysis was carried out with this sample since it had a higher relative peak intensity of V1s as compared to native protein (Fig. 1b). Further, the use of primarily V1s facilitated the enzymatic digest-MS analysis described below.

Cation Exchange Chromatography and Mass Spectrometry Analysis

Prior to conducting the chromatographic separation, enzymatic digest-MS was performed on a sample of the feed mixture (i.e., the protein sample treated with 25 mM succinic anhydride). The MS analysis showed five succinylated lysine residues (Lys 25, Lys 27, Lys 72, Lys 79, and Lys 86). Since only V1s and the native protein were present in the initial feed mixture (Fig. 1b) this indicates that five V1s were present in the feed mixture. Figures 2a,b presents chromatograms of the native horse cytochrome c and the succinylated feed mixture that were separated on the cation exchange system. By comparing the chromatograms it can be seen that several additional peaks were observed for the succinylated feed. The column eluent fractions were then subjected to direct infusion ESI MS to confirm the presence of V1s within these peaks. As can be seen in Fig. 2b, all V1s eluted before the native protein which was expected since succinylation of a lysine residue replaces the positive charged species with a negative charge. The elution times of the V1s ranged from 33 to 45 minutes. Brautigan and coworkers (10) found that cytochrome c variants that had a single lysine modified with 4-chloro-3,5-dinitrobenzoate also eluted before the native cytochrome c in cation exchange chromatography on a CM cellulose column (a more direct comparison of our results with this previous paper is presented below). In order to determine the site of modification on the variants, enzymatic digest-mass spectrometry was carried out.

Enzymatic Digest Mass Spectrometry Analysis

Column eluent fractions that were identified to contain V1s from the direct infusion ESI MS analysis were then evaluated using tryptic digest – MS. Table 1a presents an abbreviated list of peptides used in the determination of the succinylation at a particular lysine site on the protein surface. This table includes the name of each peptide (label), the mass, the amino acid residues from the protein, and the actual sequence of the peptide. Labels with the superscript N correspond to the native protein and those with superscript S correspond to succinylated peptides. Shown in Fig. 3 are the mass spectra of a digested native horse

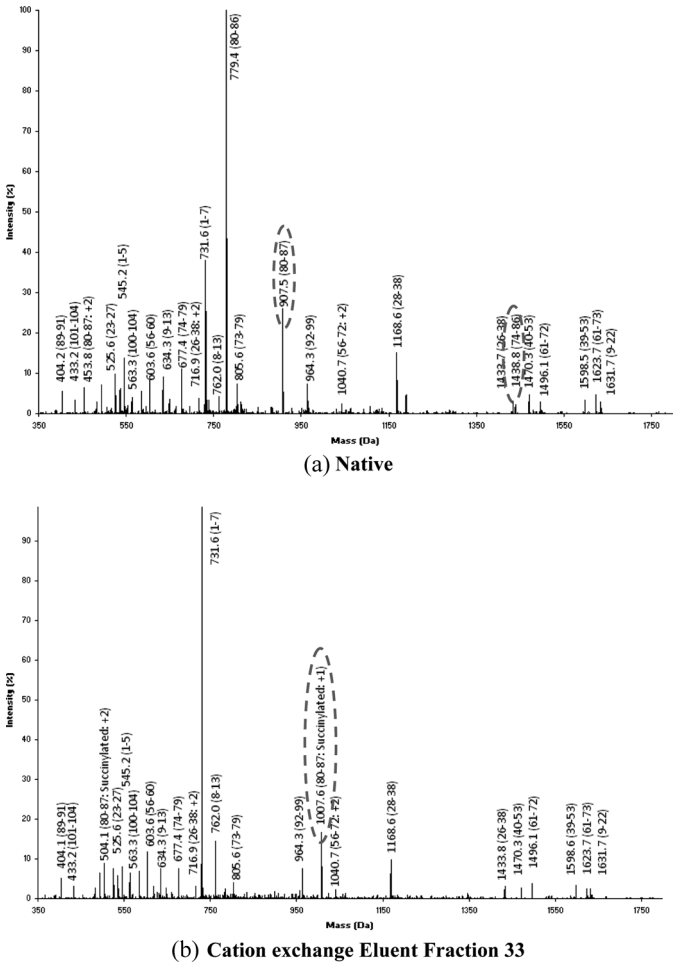


FIG. 3. Tryptic digest mass spectra of (a) native horse cytochrome C and (b) cation exchange eluent fraction 33.

cytochrome c sample and CEX eluent fraction 33. In the native sample, peptide fragments with masses of 907.5 Da and 1438.8 Da (which correspond to peptide fragments with a non-succinylated lysine at position 86) were present. Succinylation of a lysine residue increases the molecular weight of the peptide fragment by 100.1 Da. In eluent fraction 33, the peptide mass fragments of 907.5 Da and 1438.8 Da were absent, while a new peptide fragment with a mass of 1007.6 Da (which corresponds to the molecular weight of the fragment with a succinylated lysine at position 86) appeared. Table 1b shows the assignment of the variants that were determined from the presence and loss of various peaks from the mass spectra lists. $V1_{(86)}$ is used to denote a variant 1 with Lys 86 succinylated. From this analysis the elution order of the variants from weakest to strongest retention was determined to be $V1_{(86)}$, $V1_{(25)}$, $V1_{(72)}$, $V1_{(79)}$, and $V1_{(22)}$.

Examination of the Electrostatic Potential Maps of Native and Succinylated Horse Cytochrome C

It has been previously reported that the electrostatic potential (EP) is a major determinant in protein retention on ion exchange surfaces (4–8). Hence, it is instructive to examine the electrostatic potential of the native protein and its succinylated variants to determine the effect of succinylation on the electrostatic potential of the variants. From Table 1b, succinylation of Lys 86 caused a larger drop in variant retention as compared to Lys 72, indicating that Lys 86 may be closer to, if not part of a preferred binding region as compared to Lys 72. Figure 4a shows an EP surface representation of native horse cytochrome c. As seen in the figure, Lys 5, 7, 8, 13, 86, and 87 are positive charge residues which make up a major charge region on the protein surface. The binding region is indicated by the dotted area A in Fig. 4a. Lys 72 is a protruding charge residue that is located behind Lys 86 in binding region A; however, it is not directly involved in this major binding region. As seen in Fig. 4b, succinylation of Lys 86 weakens the positive EP in the binding region due to the reversal of the positive charge moiety. This reduction of positive charge in this binding face of the protein results in the earlier elution of $V1_{(86)}$ than the native protein on the cation exchange column. Figure 4c is the EP representation of

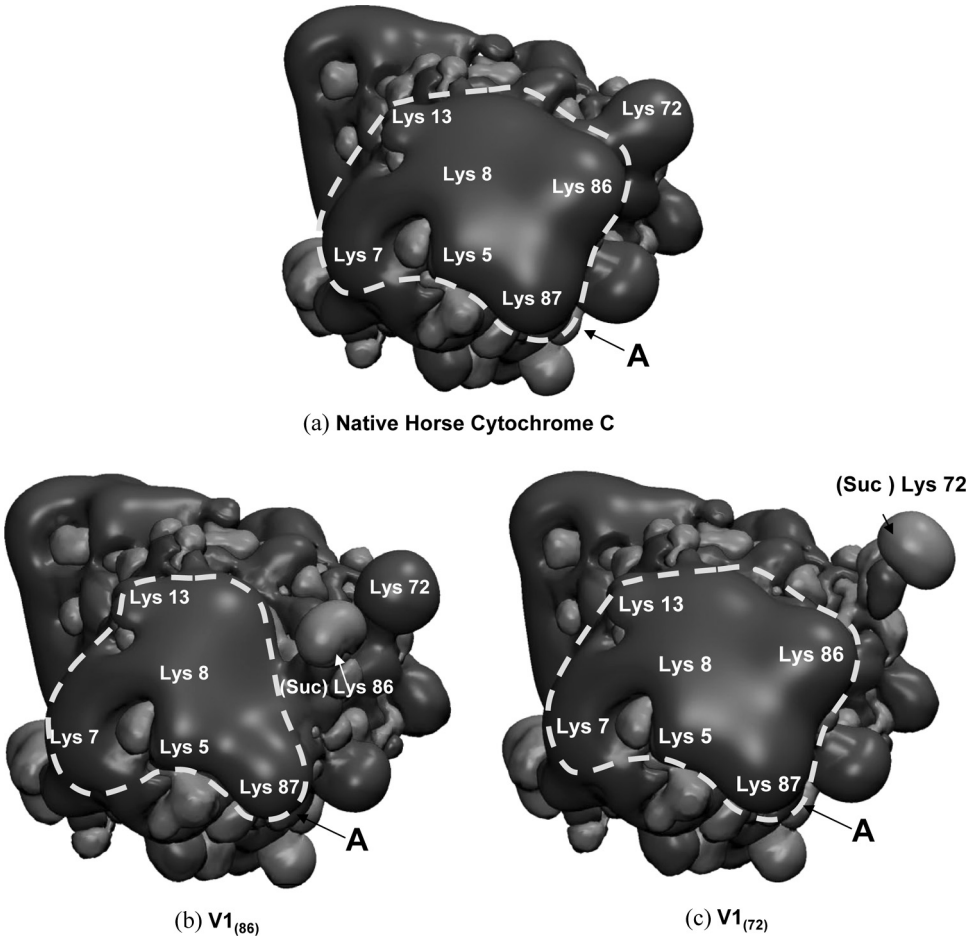


FIG. 4. Electrostatic potential surface representations of (a) native horse cytochrome C; (b) $V1_{(86)}$; and (c) $V1_{(72)}$.

$V1_{(72)}$. Upon succinylation, the repulsive effect of the negative charge moiety at Lys 72 does not directly hinder interactions with the resin surface since the binding region A is still intact. However, the positive EP around Lys 86 is slightly weakened by the close proximity of the negative charge moiety at Lys 72. Thus $V1_{(72)}$ still eluted before the native protein, although the drop in retention time was not as significant as compared to $V1_{(86)}$.

From the data in Table 1b it can be seen that $V1_{(25)}$ eluted off the cation exchanger before $V1_{(79)}$ and $V1_{(27)}$, indicating that Lys 25 played a larger role in affecting protein retention than Lys 27 or Lys 79. Since Lys 25 was not part of the binding region shown in Fig. 4a, it may be part of a separate preferred binding region located at another region on the protein surface. This proposed second region is presented in Fig. 5a for native horse cytochrome c and consists of Lys 22, 25, 39, 60, and Arg 38 and is indicated by the dotted area B. Shown in Fig. 5b is the EP representation of $V1_{(25)}$. The effect of replacing a positive charge with a negative charge moiety caused significant weakening of the positive EP within this binding region. In addition, the repulsive effect brought about by the presence of the negative charge moiety will also play a major role in the binding affinity since the succinylated Lys will now protrude further from the protein surface, enhancing the repulsive effect of this negative charge. Both of these effects are likely playing a role in causing $V1_{(25)}$ to elute before the native protein.

Lys 27 and 79 are located behind binding region B in Fig. 5a. Thus, any modifications to these amino acids would be expected to play less of a role on the retention time since the binding region B would remain intact. However, as was discussed above for $V1_{(72)}$, the introduction of a negative charge moiety in the vicinity of a binding region can still weaken the positive EP within the binding region and result in earlier elution than the native protein. This can be seen in Figs. 5c and d which present the EP maps for $V1_{(79)}$ and $V1_{(27)}$, respectively. In both cases, these variants have a minimal effect on the EP of binding region B. However, while $V1_{(79)}$ affects the EP of lys25 and 39 (Fig. 5c), $V1_{(27)}$, will only affect the EP of Lys 22 (Fig. 5d). (note: when modifications are made from positive to negatively

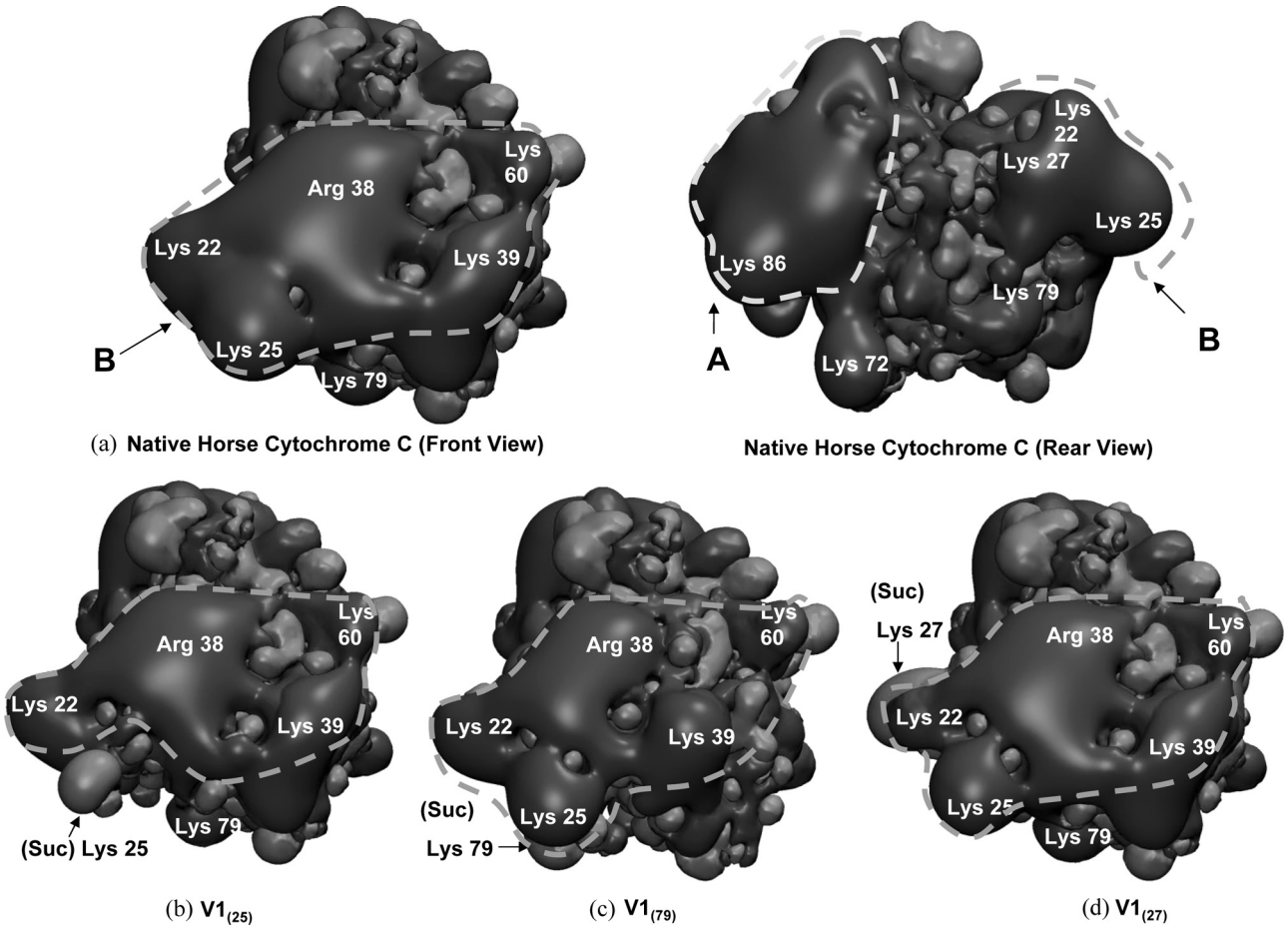


FIG. 5. Electrostatic potential surface representations of (a) native horse cytochrome C (front view); native horse cytochrome C (rear view); (b) $V1_{(25)}$; (c) $V1_{(79)}$; and (d) $V1_{(27)}$.

charged side chains, the subsequent minimization of the protein structure can effect the EP beyond the specific location of the modification). This may explain why $V1_{(27)}$ was more strongly retained on the cation exchanger than $V1_{(79)}$, while still eluting before the native protein.

Finally as shown in Fig. 5a (rear view), the relative importance of the lysines at positions 25 and 86 which are part of distinct regions as compared to those at 79, 27, and 72 (which are ''behind'' these binding regions) indicates that the two binding regions A and B are likely distinct and that binding of the protein to the surface occurs with one binding region at a time and does not span the intermediate region. This is in contrast to the previous work presented by Brautigan and co-workers which examined the retention behavior of a different set of horse cytochrome c variants on a different cation exchange column (CM cellulose) (10). In that paper, they hypothesized that horse cytochrome c had a single binding region that spanned a large proportion of the protein surface.

The differences in the identified binding region for horse cytochrome c in these two studies could be due to the different stationary phases employed (CM cellulose versus SP Sepharose) and/or the different techniques used for interpretation of the results. While there may be subtle differences in the strength of the electric field generated by these two resin surfaces, it is unlikely that this would have a dramatic impact on the preferred orientation of the protein if the interactions are primarily electrostatic. Further, since electrostatic effects in protein binding are long-range, it is unlikely that the resin backbone would have a significant impact on protein orientation. Brautigan et al. identified the binding region in their study based on the change in retention times observed with the variants along with a qualitative examination of the protein crystal structure. In contrast, the current study employed EP maps to provide a three-dimensional view of the changes in EP for each variant, thus allowing for more accurate determination of the important binding regions on the protein.

CONCLUSIONS

The advantages of using homologous protein libraries to study protein binding affinity on different chromatographic surfaces have previously been established in our research group (8,11,18). Chemical modification of a protein surface provides a rapid means of obtaining a homologous library of protein variants. In this study, the reaction of succinic anhydride with horse cytochrome c under controlled conditions resulted in the generation of five protein variants, each with one site of succinylation. The sites of succinylation on the protein surface were determined using tryptic digest MS. While separation of the variant mixture on a cation exchanger resulted in all variants eluting before the native protein due to the reduction in net surface charge, variations in the elution times of the variants were observed. The site of modification on each variant was determined through tryptic digest MS analysis of column eluent fractions and the elution order of the V1s with respect to the site of modification was Lys 86 (earliest eluting variant), Lys 25, Lys 72, Lys 79, and Lys 27 (latest eluting variant).

Examination of the EP maps of native horse cytochrome c showed that there were two major positive charge clusters on the protein surface which could potentially act as preferred binding regions for interaction with the negatively charged cation exchanger. The residues Lys 86 and Lys 25 are located within preferred binding regions A (Fig. 4) and B (Fig. 5), respectively. Examination of the EP maps of these two variants showed notable weakening in the positive EP of the preferred binding regions upon succinylation which can explain the significant reductions in variant retention times observed.

In contrast, Lys 27, Lys 72, and Lys 79 are located ''behind'' these proposed binding regions. Thus, when the protein is adsorbed onto the resin in the proposed two preferred binding orientations, these residues will be shielded from the resin surface by the residues within the binding region. This will minimize the electrostatic interactions between the resin surface and these three residues, resulting in less of an impact on the retention of these variants. However, succinylation at positions 27, 72, and 79 will still weaken the positive EP around neighboring charge residues in the preferred binding regions, resulting in a moderate reduction in retention.

This study demonstrates that the use of controlled protein surface modification offers a convenient means of studying the relationship between protein surface properties and chromatographic binding affinity. Future work will involve examination of these and other protein libraries for their behavior in multimodal chromatographic systems.

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