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Presence of a preferred anion-exchange binding site on cytochrome b_5: structural and thermodynamic considerations

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Abstract

A preferred chromatographic contact region for adsorption of recombinant soluble tryptic fragment of rat cytochrome b_5 on the hydrophilic anion-exchanger Mono Q has been identified using conservative carboxylate-to-amide mutations of charged residues. Equilibrium adsorption isotherms were measured under conditions of full reversibility by high ionic strength, as confirmed by explicit mass balances performed for each experiment. Although cytochrome b_5 displays several clusters of negative charge, mutations in one cluster consistently reduce binding affinity and the stoichiometric displacement parameter Z by much greater factors than do mutations in other areas of the molecule. Adsorption heterogeneity derived by fitting isotherms to the Hill equation is reduced by factors which reduce the overall affinity of adsorption. Van 't Hoff analyses gave uniformly positive enthalpies of adsorption, and mutational changes in adsorption enthalpy were relatively independent of the site of mutation. These results suggest that enthalpy does not play a dominant role in either affinity or selectivity of anion-exchange adsorption in this system.

1. Introduction

Ion-exchange chromatography is widely used for the high-resolution purification of proteins [1,2]. For optimal design of processes and adsorbents for protein ion exchange, it is desirable to have a clear understanding of the structural and thermodynamic factors governing its performance. Ion-exchange adsorption is conventionally regarded as requiring that the net charge of the protein be opposite that of the exchanger. However, both neutral proteins and proteins with net charge of the same sign as the exchanger have been shown to adsorb in certain cases [3–6]. This is because the three-dimensional geometry of the protein sterically precludes all the charged residues contacting the ion-exchange surface simultaneously [7]. If the distribution of charged amino acids on the protein surface is sufficiently asymmetric, then electrostatic screening can make a cluster of charges on the protein surface a preferred chromatographic contact region which dominates adsorption behavior.

The phenomenon of "patch-controlled" adsorption was first predicted by Boardman and Partridge [8]. The first experimental demonstra-

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tion was provided by Brautigan et al. [9] using cation-exchange separation of chemically modified variants of cytochrome *c*. More recent work has clearly documented the adsorption of proteins with net charge equal to zero, or of the same sign as that of the ion exchanger [3–5]. Direct evidence of the dominant role of specific amino acid clusters in adsorption has been provided by chromatographic studies of charge mutant forms of single proteins [10–13]. The importance of local charge clusters has been further investigated by computational analysis [14,15]. We have also recently applied computational electrostatics modeling to understanding cytochrome b_5 adsorption [16].

We have characterized the anion-exchange adsorption of recombinant soluble rat cytochrome b_5 in order to test the contributions of specific amino acid residues to the thermodynamic driving forces for adsorption. We employed batch adsorption isotherm measurements under equilibrium conditions to avoid potential complications associated with flow systems. The equilibrium adsorption data support interpretation of the results in terms of thermodynamic driving forces. We have chosen conservative (carboxylate to amide) site-directed mutant forms to systematically examine the surface of cytochrome b_5 for preferred chromatographic contact region(s). Isotherm data were analyzed in several ways. Values for the equilibrium affinity parameter (K) were derived from Hill analysis [17] and were interpreted in terms of the stoichiometric displacement model of Kopaciewicz et al. [5] to obtain the apparent number of binding sites on the protein surface (Z). Hill analysis was also used to estimate the degree of heterogeneity of adsorption. Apparent enthalpies of adsorption were calculated using Van 't Hoff analysis.

2. Materials and methods

Recombinant soluble wild type rat cytochrome b_5 and its surface charge mutants were prepared in *Escherichia coli*. Synthetic genes for most of these proteins were the generous gift of Dr. Stephen Sligar (see Ref. [18]). The E96Q mutation was introduced into the wild type gene by replacing an *Nru*I-*Eco*RI fragment of the gene with synthetic oligonucleotides as described elsewhere [19]. Proteins were purified by ion-exchange and size-exclusion chromatography, and characterized using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), spectrophotometry, and HPLC as described previously [18,20,21].

The monodisperse polymeric quaternary amine-based strong anion exchanger Mono Q (Pharmacia) was used as the adsorbent. It was equilibrated according to the manufacturer's protocol [22] and then recovered from fast protein liquid chromatography (FPLC) columns. The fresh, equilibrated Mono Q was used for initial adsorption experiments and then regenerated by washing with 50 volumes of high ionic strength buffer (10 mM Tris, pH 8.0 + 0.1 mM EDTA + 1.0 M NaCl) followed by washing (10 steps of 50 volumes each) with 10 mM Tris, pH 8.0 + 0.1 mM EDTA. The effectiveness of regeneration was confirmed by observation of the absorbance of the wash solution at 280 nm. Adsorption behavior was not observably affected by several cycles of regeneration after adsorption at low loading; the results reported here were obtained using adsorbent which had been regenerated in this manner.

Equilibrium batch adsorption experiments were performed in triplicate, as previously described [20]. Briefly, protein samples were equilibrated with the adsorbent in microcentrifuge tubes rotated at 3 rpm for 1 h. This period had been found in control experiments to be more than sufficient to ensure equilibration as judged by isotherm invariance. The tubes were completely filled with liquid to avoid protein denaturation at the gas–liquid interface. Samples were prepared in 10 mM Tris buffer adjusted to pH 8.0 in an environmental room at the experimental temperature and supplemented with NaCl to the desired concentration. Measurements were carried out at pH 8.0 to minimize the variability of ionization of weakly acidic and basic groups (histidine 19 and the termini) and to ensure that the surface charge was dominated by the highly

ionizable acidic groups of aspartic and glutamic acids in addition to heme propionates, and basic groups of lysine and arginine. At pH 8.0, far from their pK_a values, mutations made in these residues lead directly to a unit change in the net charge of the protein. Tris buffer was chosen because neither of its buffer species were expected to interact with the positively charged adsorbent. The amount of protein adsorbed was always less than 400 nmol protein per 10^8 adsorbent particles, less than 20% of the demonstrated capacity of Mono Q for cytochrome b_5.

The amount of protein remaining in the supernatant after equilibration was calculated from the absorbance at 412 nm using the known molar absorbance in the strong cytochrome b_5 Soret band at this wavelength (130 mM^{-1} cm^{-1}; the oxidized form of the protein was used throughout). The amount of protein adsorbed was separately determined by elution of adsorbed protein with 10 m*M* Tris, pH 8.0 + 0.5 *M* NaCl over 1 h. Salt at this concentration was found to achieve quantitative elution within 30 min, and also did not alter the molar absorbance of the protein. An explicit mass balance on the free and adsorbed protein was calculated for each sample to control for the potentially confounding effects of denaturation, proteolysis, incomplete elution, etc. Mass balances for all experiments reported here closed to within 5%. Some of the cytochrome b_5 mutants were found to be less tightly adsorbed to Mono Q than was the wild type protein under similar conditions. These mutants were studied over a range of salt concentrations (75 to 150 m*M* NaCl) extending to somewhat lower values than used for the wild type protein (100–175 m*M*).

Values for the equilibrium affinity parameter (K) were derived from Hill analysis [17]. Each K value reported here was derived from an isotherm defined by at least six points. The standard deviations reported were calculated from the results of triplicate experiments. Values of the stoichiometric displacement parameter Z were obtained through linear least squares fits of log K versus log (reciprocal ionic strength) for four salt concentrations. Van 't Hoff enthalpies of adsorption were calculated from fits of log K versus reciprocal absolute temperature for at least five temperatures in the range of 4 to 37°C.

The mutations studied in this work were chosen based on the high-resolution X-ray structure determined for cytochrome b_5 [23]. The mutations used were conservative, with typically a single negatively charged aspartic or glutamic acid residue at the surface mutated to the corresponding neutral amide (asparagine or glutamine). An important consideration was that mutant forms of cytochrome b_5 be used that were not structurally different from the wild type protein. Characterization of rat cytochrome b_5 and its mutants by two-dimensional NMR indicates that none of the mutations studied induces any significant change in the tertiary structure of the protein [24], with the exception of the double mutant E47,48Q. Mutating residue 47 to glutamine abolishes a salt link to Arg51, which may perturb the local structure of the protein [25]. It should also be noted that mutants E82Q and E96Q have not been subjected to characterization by NMR. As discussed below, the acidic residue at each of these positions is thought to be involved in one or more salt bridges whose disruption may alter the local tertiary structure.

Selection of mutants was based on examination of the structure of cytochrome b_5 using the molecular graphics utilities in QUANTA (version 3.2) and CHARMM 20.3 (Polygen) on a Silicon Graphics 4D/20 workstation. Although no complete structural determination for rat cytochrome b_5 is yet available, nearly complete high-resolution coordinates for the crystal structure of the 93% homologous bovine protein are available through the Brookhaven Protein Data Bank [23]. We have recently reported [26] a homology model for rat cytochrome b_5 based on the bovine cytochrome b_5 structure. The missing termini were added to the core in configurations derived from the coordinates of homologous proteins identified by an exhaustive search of the Brookhaven Protein Data Bank. The six amino acid differences between the bovine and rat proteins occur at the surface, and are largely conservative. These mutations were built into the bovine protein, and the resulting structure explicitly solvated (a 10 Å annulus consisting of

942 TIPS3P waters) and minimized over 500 steps of the adopted-basis Newton–Raphson algorithm. The homology-based model structure provided a basis for selection of mutants for this study as well as interpretation of the results, as described below.

The solvent-accessible surface of cytochrome b_5 displays several clusters of negative charges. The assignment of residues to clusters depends on the cutoff radius used. Based on a Cα to Cα distance of ca. 5.0 Å, the clusters of two or more charges on wild type cytochrome b_5 are: (1) Glu14 and Glu15; (2) Glu41, Glu42 and Glu47; (3) Glu47, Glu48 and Glu52; (4) Glu60, Glu63 and Asp64; and (5) Asp70 and Glu73 (see Fig. 1). The protoporphyrin propionates may contribute to adsorbent interactions involving residues Glu47, Asp70 and others nearby. Additional

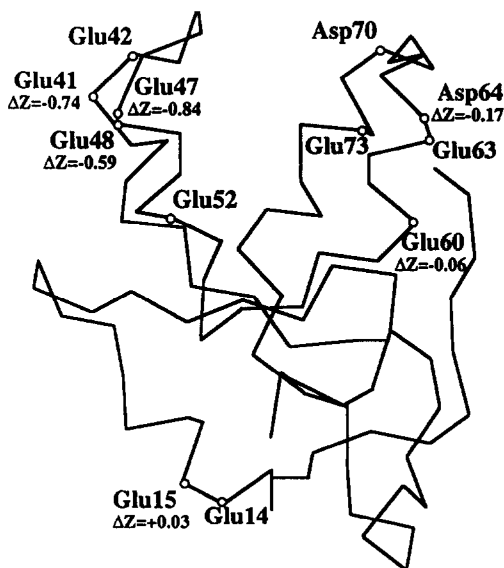

Fig. 1. Cα trace of the three-dimensional structure of rat cytochrome b_5 (see Ref. [26]) depicting the distribution of negative charge on the protein surface. Only surface charges in clusters of two or more are shown. The molecule is oriented with the N-terminus on the right and the heme-binding pocket shown as the open area at the top of the diagram. Circles represent positions of negatively charged amino acids where substitutions were studied. Wild type residues are labeled using the three-letter code for amino acids. ΔZ is the difference in the value of the stoichiometric displacement parameter Z relative to that for the wild type protein.

charge is contributed by carboxylic acid residues which are not clustered with any patch and/or which are salt linked with positive residues, as discussed below. Although it was difficult to predict a priori whether a given part of the protein surface would prove to be a preferred chromatographic contact region, the major clusters were targeted by the mutations initially used. We avoided mutations in residues salt-bridged with neighboring, positively charged amino acids (i.e., Glu47–Arg51, Glu63–Arg72) as these would be only partially available to interact coulombically with the ion-exchange surface. Except for E47,48Q, all mutations studied were single carboxylate-to-amide substitutions (Table 1).

3. Results and discussion

Values of the Hill affinity constant (K) at 25°C in the presence of 100 mM NaCl, the apparent number of contacts estimated from the salt-dependence of adsorption using the stoichiometric displacement model (Z), and Van 't Hoff enthalpies of adsorption (ΔH_{ads}) are shown in Table 1. The values given for K, Z and ΔH_{ads} for the wild type protein have been reported previously [20].

The largest clusters of negative charge on rat cytochrome b_5 are two groupings of three negative charges, one on each side of the heme-binding pocket (Fig. 1). Residues 41, 47 and 48 comprise a cluster located at one end of the heme plane while residues 60, 63 and 64 lie on the other side (Fig. 1). If any cluster functions as a chromatographic contact region in anion-exchange adsorption, mutations that neutralize one or more of the negative charges in the cluster would be expected to reduce the observed Z number and adsorption affinity. From Table 1, it can be seen that compared to the wild type protein ($Z = 2.92$), the single-charge mutants E41Q and E48Q show reductions of Z by 0.74 and 0.59, respectively, and also produce the largest reductions in adsorption affinity at 100 mM NaCl. Each of these mutations affects residues lying in the major cluster composed of

Table 1
Values of the Hill binding constant K, stoichiometric displacement parameter Z, and Van 't Hoff enthalpy of adsorption ΔH_{ads} for adsorption of wild type cytochrome b_5 and its mutants on Mono Q in 0.010 M Tris buffer at pH 8.0

Protein	K	Z	ΔZ	ΔH_{ads} (kcal/mol)	$\Delta\Delta H_{ads}$ (kcal/mol)
Wild type	0.081 ± 0.002	2.92 ± 0.32	0.00 (0.0)	2.87 ± 0.26	0.00
E41Q[a]	0.032 ± 0.005	2.18 ± 0.27	−0.74 (−1.0)	2.51 ± 0.47	−0.36
E48Q	0.020 ± 0.000	2.33 ± 0.34	−0.59 (−1.0)	2.44 ± 0.52	−0.43
E47,48Q	0.016 ± 0.001	2.08 ± 0.42	−0.84 (−2.0)	2.51 ± 0.60	−0.36
E15Q	0.100 ± 0.002	2.95 ± 0.25	0.03 (−1.0)	2.32 ± 0.37	−0.55
E60Q	0.046 ± 0.001	2.86 ± 0.05	−0.06 (−1.0)	2.49 ± 0.25	−0.38
D64N	0.051 ± 0.000	2.75 ± 0.33	−0.17 (−1.0)	2.40 ± 0.28	−0.47

Reported values of Hill binding constants (K ± S.D.) were measured at 25°C in buffer containing 0.100 M NaCl. Values of Z were obtained by linear regression of log K versus log (1/[NaCl]); values obtained at 25°C are shown. ΔH_{ads} values were calculated from a Van 't Hoff linear regression of log K versus $1/T$ for at least five temperatures in the range 4–37°C. Z and ΔH_{ads} values for wild type cytochrome b_5 are from Ref. [20]. ΔZ denotes the difference of the Z value for the mutant from Z obtained for the wild type protein. Numbers in parentheses beside the ΔZ values indicate the expected change in net charge for the given mutation, as compared to the wild type protein (see Ref. [16]). $\Delta\Delta H_{ads}$ are the differences from the wild type ΔH_{ads}. The reported standard deviations were calculated from the results of triplicate experiments.

[a] In naming the variants the single-letter amino acid code for the wild type carboxylate residue is followed by the position of the amino acid, followed by the single-letter amino acid code for the new amide residue. For example, E41Q indicates that glutamic acid (E) in position 41 was mutated to glutamine (Q). D = Aspartic acid; N = asparagine.

residues 41, 47 and 48. In contrast, neutralization of Glu15 at the N-terminus of the protein produces no statistically significant change in the value of Z, and appears to slightly *increase* the affinity of adsorption at 100 mM NaCl. Neutralization of Asp64 or Glu60 in the secondary charge cluster produces an intermediate change in the values of Z and K. These results strongly imply that the major charge cluster plays a dominant role in adsorption of the protein.

The observed values of Z reflect fractional contributions from multiple charged residues, rather than a direct correspondence between Z and the number of charged residues in a particular cluster. No single mutation in the major contact region produces a change in Z of more than −0.74. The double mutant E47,48Q shows a reduction in Z value of −0.84, well below the $\Delta Z = -2.0$ expected from unit contributions by dominant contact residues. The further reduction in Z produced by incorporating the E47Q mutation into the E48Q protein ($\Delta\Delta Z = -0.25$) is larger than the change produced by the first mutation in other charge clusters. This behavior presumably reflects the continued importance of this cluster in adsorption even after deletion of one charge by mutation. The small ΔZ produced by the second mutation also illustrates the lack of a one-to-one correspondence between Z and the number of residues interacting with the adsorbent surface.

The differences in Z number between proteins bearing mutations in the apparent major contact region (E41Q and E48Q) and proteins bearing mutations outside this region (E15Q, E60Q, D64N) are larger than the sum of the standard deviations of the individual values (Table 1). The complex nature of the measurement procedure, however, introduces more scatter than one would prefer. Further limitations arise from the fact that the largest ΔZ expected for single substitutions is only −1.0. Double or triple substitutions would increase the expected difference, but would risk perturbing the tertiary fold of the protein. These measurements do show reduced scatter compared to the first (and only other) study to report Z numbers measured using equilibrium adsorption experiments [27]. The principal conclusions drawn from the ΔZ values are supported by the disproportionate

responsiveness of binding affinity (K) to mutations in this region. In addition, the present results are in good agreement with (significantly lower scatter) Z values for this system derived from our independent HPLC studies of a partially overlapping set of mutants [28]. Thus, structural data derived from site-specific mutagenesis suggest that the region surrounding glutamic acid residues 41, 47, and 48 is the preferred chromatographic contact region for the anion-exchange adsorption of cytochrome b_5.

In order to cover the surface of the protein as completely as possible, we also considered two mutations believed potentially to alter the structure of the protein. The first of these was E82Q in the carboxy end of the protein, away from any of the charge clusters mentioned above. Unlike other mutations studied, residue 82 is not completely solvent accessible, but is partially buried (14 $Å^2$ solvent accessible surface area, measured with a 1.4 Å probe radius, as compared to an average of 92 $Å^2$ accessible area for the residues discussed above). Limited studies of the adsorption behavior of the E82Q protein revealed a moderate reduction in binding affinity and a large change in the apparent stoichiometry of binding ($K = 0.068$; $\Delta Z = -1.03$). The structure of cytochrome b_5 suggests that Glu82 participates in a double salt bridge with Lys32 (3.7 Å average distance between carboxylate oxygens of Glu82 and amino nitrogens of Lys32) as well as with Arg88 (3.6 Å). We speculate that these salt links act to stabilize this region of the protein, and their abolition may disrupt the secondary structure in the C-terminus of the protein, producing the observed changes in adsorption behavior. It is also possible that Glu82 could be involved in an alternative chromatographic contact region, but this possibility is disfavored by the absence of clustered negative charges and the proximity of the positively charged residues Lys32 and Arg88. We observed similar behavior with a mutant in which Glu96, which also lies mainly in the interior of the protein (4 $Å^2$ solvent accessible surface area), is mutated to glutamine. This protein gave $K = 0.031$, $\Delta Z = -1.33$. A change in the protein's conformation as a result of this mutation is indicated by a reduced value of R_z, the ratio of molar absorbances at 412 nm and 280 nm. This value, a commonly used index of structural integrity for cytochromes, is only 5.0 for E96Q as compared with over 5.6 for pure wild type protein.

The Van 't Hoff enthalpies of adsorption (ΔH_{ads}) of the wild type and mutant proteins are uniformly positive (Table 1). For spontaneous, high-capacity adsorption (ΔG_{ads} negative), positive enthalpies of adsorption imply an entropic driving force. This increase in entropy could arise from liberation of ions or waters of solvation, from either the ion-exchange surface or the protein. Values of $\Delta\Delta H_{ads}$ of mutation range from -0.36 to -0.55 kcal/mol (1 cal = 4.184 J) (13–19% of the wild type ΔH_{ads}), with no statistically significant dependence on the location of the mutation. These results show that the enthalpy of adsorption does not control either the affinity or the selectivity of adsorption in this system, under the conditions studied.

The heterogeneity index (n_H) calculated from Hill regression of equilibrium adsorption isotherm data was used to assess the uniformity of protein adsorption. Table 2 presents values of n_H calculated from 25°C adsorption isotherms measured at various ionic strengths. As noted above, the range of ionic strengths at which adsorption affinity is reliably measurable varies among the mutant proteins. Therefore, n_H data are available for each mutant only at a subset of the NaCl concentrations studied. The value of n_H is substantially below unity for all cases examined, implying that apparent adsorption affinity decreases with protein loading. A similar conclusion can be reached from a Scatchard analysis of isotherm data, and has been drawn in our previous work on the adsorption of wild type cytochrome b_5, and by others [27,29].

The effects of mutation on adsorption heterogeneity provide insight into the sources of heterogeneity. In the interest of brevity, we discuss in detail only the data collected at 100 mM NaCl; similar conclusions follow from the results obtained under other conditions. At 100 mM NaCl, the n_H value for the wild type protein is 0.32 ± 0.02. Mutations E15Q, E60Q and D64N, which affect residues outside the dominant chro-

Table 2
Hill heterogeneity indices of adsorption of cytochrome b_5 variants on Mono Q as a function of added NaCl concentration in 10 m*M* Tris buffer, pH 8.0 at 25°C

Protein	Hill heterogeneity index					
	0.05 *M* NaCl	0.075 *M* NaCl	0.1 *M* NaCl	0.125 *M* NaCl	0.15 *M* NaCl	0.175 *M* NaCl
Wild type	ND	ND	0.32 ± 0.02	0.46 ± 0.03	0.70 ± 0.03	0.82 ± 0.11
E41Q	ND	0.33 ± 0.01	0.51 ± 0.05	0.64 ± 0.05	0.84 ± 0.05	ND
E48Q	0.28 ± 0.08	0.35 ± 0.06	0.46 ± 0.09	0.79 ± 0.09	0.88 ± ND	ND
E47,48Q	0.25 ± 0.00	0.40 ± 0.00	0.59 ± 0.02	0.77 ± 0.05	ND	ND
D64N	ND	ND	0.35 ± 0.01	0.49 ± 0.03	0.71 ± 0.05	0.81 ± 0.04
E60Q	ND	ND	0.42 ± 0.01	0.60 ± 0.01	0.79 ± 0.01	0.92 ± 0.03
E15Q	ND	ND	0.36 ± 0.00	0.52 ± 0.03	0.71 ± 0.01	ND

Calculated from linear regression of adsorption isotherm data using the Hill equation (see Ref. [16]). The salt concentrations used with each variant were selected according to its adsorption behavior, as described in the text. ND = Not determined.

matographic contact region, produce relatively small changes in heterogeneity (average n_H = 0.37, range 0.35–0.42). The n_H values for mutants E41Q and E48Q in the primary contact region are significantly higher at 0.51 and 0.46, respectively; n_H is 0.59 ± 0.02 for the double mutant E47,48Q. In general, therefore, n_H values are most increased (heterogeneity is most reduced) for those mutants where the amino acid substitution affects *K* (and *Z*) most significantly.

Heterogeneity of protein ion-exchange adsorption can arise from heterogeneous protein orientation on uniform adsorbent sites, and/or an intrinsically heterogeneous population of adsorbent sites. We discount the possible role of lateral protein–protein interactions in the present work as all data were collected at low surface coverage. It should be noted, however, that proteins could be brought close enough to interact, even at low loadings, by heterogeneous site distribution. Heterogeneous protein adsorption on uniform adsorbent sites would involve competition among binding orientations of differing affinities. Mutations reducing charge within the preferred contact region would create a more equal competition among possible orientations. This could increase heterogeneity by allowing significant occupation of a larger set of orientations. Alternatively, in the absence of any large redistribution of protein molecules among adsorbed states, it could reduce heterogeneity by making the populated orientations more energetically similar. Only the latter possibility is consistent with our observations, as heterogeneity is reduced (n_H increased) by all mutations tested, and most effectively reduced by mutations in the contact region.

Adsorption heterogeneity could also reflect an intrinsically heterogeneous population of sites on the adsorbent. Such heterogeneity is to be expected from the irregular topography of the hydrophilic adsorbent surface, and from the random chemical derivatization by which charges are introduced. Heterogeneity from this source would be reduced by factors which restrict the energetic diversity of sites occupied under experimental conditions, such as mutation, or changes in temperature and in ionic strength. (These factors could produce reorganization of the surface as well as reducing the overall affinity of adsorption, although the linear nature of the Van 't Hoff and stoichiometric displacement plots argues against this interpretation). The reduction in heterogeneity produced by mutations in the contact region could be ascribed to a reduction in the protein's intrinsic affinity for the surface, leading to disqualification of some sites previously acceptable by the (wild type) protein. This model is also consistent with the reduced heterogeneity observed at higher NaCl concentrations and at lower temperatures. Under each of these conditions the overall affinity is reduced,

and a smaller population of sites is expected to be energetically acceptable.

4. Conclusions

In this work we have systematically used site-directed mutant forms of cytochrome b_5 to study the structural and thermodynamic elements of protein ion-exchange adsorption. This has also allowed us to put the widely accepted stoichiometric displacement model to a rigorous test under equilibrium conditions for the first time. Our results show that the stoichiometric displacement model can effectively correlate adsorption data for this small globular protein under the low surface coverage conditions tested.

We have identified a major cluster of negative charges on the protein surface as a probable adsorbent contact region, mutations within which produce disproportionately large reductions in affinity and in the stoichiometric displacement parameter Z. Van 't Hoff analysis shows that the enthalpy of adsorption is positive (unfavorable) and is also relatively unresponsive to mutations which greatly alter adsorption affinity. For ion-exchange adsorption in this system, therefore, enthalpy does not play a dominant role in the affinity or the selectivity of adsorption. Analysis of the changes in the apparent heterogeneity of adsorption produced by mutations and by variations in ionic strength suggests the possible roles of orientation and site heterogeneity in protein adsorption. Further work, however, will be required for a complete understanding of these phenomena.

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