ELSEVIER

Journal of Chromatography A, 704 (1995) 339–349

JOURNAL OF CHROMATOGRAPHY A

Preferred high-performance liquid chromatographic anion-exchange chromatographic contact region for recombinant rat cytochrome b_5

David J. Roush[1], Davinder S. Gill[2], Richard C. Willson*

Department of Chemical Engineering, University of Houston, 4800 Calhoun Avenue, Houston, TX 77204-4792, USA

Received 5 December 1994; accepted 8 February 1995

Abstract

The HPLC anion-exchange isocratic retention behaviors of site-directed charge mutants of the recombinant soluble core of rat cytochrome b_5 on Mono Q HR 5/5 were investigated as a function of sodium chloride concentration, at a fixed temperature and eluent flow-rate. The retention behavior of the charge mutants was observed to depend on both the net charge of the protein and on the distribution of charged residues on the protein surface. Site-directed mutants of the same net charge differed significantly in retention behavior; differences in retention were observed to increase with decreasing ionic strength. The retention results were interpreted in terms of the stoichiometric displacement model (SDM) to obtain the apparent number of binding sites in the contact region, Z. Elimination of a single negatively charged residue, in an apparent preferred chromatographic contact region, resulted in disproportionately large changes in the retention behavior as compared with elimination of a single negatively charged residue on other areas of the protein surface. The experimentally determined preferred chromatographic contact region compares favorably with the results of batch equilibrium adsorption studies on the same system and with previously reported results of computational molecular electrostatic modeling. The results of this study indicate that the observed protein ion-exchange retention represents the summation of many fractional electrostatic interactions of varying interaction energies. Therefore, protein ion-exchange retention is a function of both the number of contacts and the individual electrostatic interaction energies of the contacts between the protein and the ion-exchange surface.

1. Introduction

Electrostatic interactions between oppositely charged groups on the protein and adsorbent surfaces are the primary interactions in the ion-exchange chromatography of proteins. However, the precise contributions of the charged groups located on the surfaces of the protein and the ion-exchanger to retention have not been well characterized. Several studies have indicated that the distribution of charges on the protein surface can influence the observed retention behavior to the extent that a protein may be retained on an ion-exchange surface of the same sign as the net charge of the protein [1,2]. The ion-exchange

* Corresponding author.

[1] Present address: Merck and Co., Bioprocess R&D, P.O. Box 2000, Rahway, NJ 07065, USA.

[2] Present address: Department of Surgery, Massachusetts General Hospital and Harvard Medical School, Boston, MA 02114, USA.

0021-9673/95/$09.50
SSDI 0021-9673(95)00180-8

adsorption of a protein has also been observed to be dominated by patches or clusters of charge on the protein's surface [1–6]. These observations are in contrast to the traditional assumption that ion-exchange retention is governed by the net charge of the protein so that a protein will be retained on an anion-exchange surface at any pH above the protein's isoelectric point. The surface topography of the protein has also been noted to sterically restrict electrostatic interactions between a protein and an ion-exchange surface [6].

The non-mechanistic stoichiometric displacement model (SDM) originally proposed by Boardman and Partridge [7] and first applied to HPLC by Kopaciewicz et al. [1] has been widely employed to determine the regression parameter Z, the apparent number of contacts between the protein and the ion-exchange surface participating in the adsorption/desorption process [1,3,5,6,8–10]. Z-values have been found to be fractional and have been observed to change as a function of loading [4,11,12]. The change in the observed value of Z with different loading levels has been attributed to changes in the orientations of absorbed protein molecules as the concentration of the absorbed protein increases. The existence of a heterogeneous distribution of orientations for adsorbed protein molecules is supported by observations from other systems mediated by electrostatic interactions, especially protein–protein interactions. For example, the cytochrome b_5/cytochrome c complex has been suggested to adopt multiple binding orientations which appear to vary as a function of ionic strength [13–15]. These studies experimentally confirmed molecular modeling studies by Wendoloski et al. [16], who determined that the flexibility of the two proteins allows for the sampling of multiple binding conformations. This investigation of the existence of multiple binding orientations for an electrostatically mediated protein–protein complex can also aid in understanding of protein ion-exchange interactions, as fractional chromatographic Z-values can also be interpreted to represent the summation of many fractional interactions between the protein and ion-exchange surface.

It is important to note that the observed protein ion-exchange retention behavior reflects the contributions of both the protein and the adsorbent. Wu and Walters [17] investigated the effects of varying the charge density of a cation-exchanger on the isocratic retention of lysozyme and cytochrome c. The value of Z and even the elution order was observed to vary as a function of the adsorbent charge density, illustrating the importance of the adsorbent's contributions to retention behavior [17]. These researchers [17] also noted that the heterogeneity of the ion-exchange surface can affect the observed protein ion-exchange retention behavior, in agreement with the results of Gill et al. [10].

This study examines retention of wild type and site-directed charge mutants of recombinant soluble tryptic core of rat cytochrome b_5 on the strong anion-exchanger, Mono Q. This protein was chosen as an experimental model because of the wealth of related structural [18–21] and biochemical data available and the existence of an efficient system for its expression in *E. coli* [22]. The protein is well-suited for use in anion-exchange adsorption studies because of its great stability in solution, strong chromophore, moderate molecular mass (M_r = 13 603) and negative net charge at pH values near neutrality; pI 4.6 by isoelectric focusing (IEF); 23 negative groups (including the protoporphyrin) and 15 positive groups (allowing for partial titration of histidines) resulting in net charge −9.4 at pH 8.0.

The goals of this investigation were to: (a) determine the contributions of the individual charged amino acid residues located on the protein's surface to the observed anion-exchange retention behavior, (b) to verify the existence of a "preferred chromatographic contact region" on the protein surface for interaction with the anion-exchange surface and (c) to determine the contributions of the other charged residue clusters by providing a more complete map of the protein surface. The HPLC anion-exchange retention results are compared to equilibrium batch adsorption data for the same protein/ion-exchanger system by Gill et al. [12].

2. Experimental

2.1. Reagents and proteins

The reagents, proteins, and protein purification and characterization methods used in these investigations were the same as those previously described [9,10,12].

2.2. Equipment

The experimental system and the control experiments verifying the precision and estimating the accuracy of the experimental apparatus have been previously described in detail [9] and are therefore described only briefly. The entire HPLC system, with the exception of the computer (NEC 386/25 CPU; NEC Technologies, Foxborough, MA, USA), and the computer interface (System Interface Module; Waters, Milford, MA, USA) was located in a temperature-controlled environment room (Norlake, Hudson, WI, USA) regulated to within ±0.5°C. Data were analyzed using Maxima 3.0 (Dynamic Solutions) software. All chromatographic experiments were performed with a Waters HPLC system consisting of two Model 510 positive displacement pumps, a WISP 710B automated sample injection system and either a Model 441 UV–Vis single wavelength detector operating at 405 nm or a Model 481 variable-wavelength detector. The 405 nm wavelength was used for rat cytochrome b_5 retention measurements because it is close to the oxidized protoporphyrin Soret band maximum at 412 nm.

Mono Q (Pharmacia) prepacked strong anion-exchange columns (HR 5/5, 50 × 5 mm I.D.) were used in all studies. Each determination of the SDM parameter Z was carried out entirely on a single column to avoid any effects of inter-column variations. Columns were replaced when the plate count fell below the Pharmacia specification of 25 000 theoretical plates/m.

2.3. Chromatography

Protein samples were prepared in 10 m*M* Tris, pH 8.0 plus the appropriate NaCl concentration corresponding to the intended elution conditions. Protein samples of 50 μl were injected and eluted isocratically with eluents containing 10 m*M* Tris, pH 8.0 and NaCl in the range of 150 to 700 m*M*, matching the injection buffer. Protein sample concentrations were 0.26 ± 0.01 mg/ml for the experiments designated 0.25 mg/ml. Retention was studied at a fixed nominal temperature of 25°C (25.0 ± 0.25°C) with a nominal eluent flow-rate of 0.5 ml/min (0.48 ± 0.01 ml/min). For each eluent flow-rate, the protein was assigned a non-retained volume corresponding to the elution volume at 700 m*M* NaCl, corrected for non-column system volume, based on control experiments which showed that retention does not vary significantly in the range of 600 to 700 m*M* NaCl.

Protein recovery calculations were performed as described previously [9]. The recoveries of cytochrome b_5 were 100 ± 6%. A gradient cleaning run, employing 10 m*M* Tris, pH 8.0 and 2 *M* NaCl, was performed after isocratic runs of less than 300 m*M* NaCl. The ratio of peak area to baseline noise integrated over a period equal to the peak width at baseline was typically ca. 75 under conditions of strong retention.

As discussed previously [9,23], the k' value can be a strong function of temperature. Therefore, the protein samples and eluents were pre-equilibrated at the experimental temperature. Previous studies on the effect of variations in the mean system temperature in the range of 25.0 ± 0.4°C indicated that at 150 m*M* a variation in temperature of 0.4°C would produce a change in k' or Z of ca. 5% and 0.4%, respectively.

3. Data analysis

The capacity factor, k' was calculated based on the protein retention volume, V_R and the protein non-retained volume, V_o, corrected for non-column system volume. Retained and non-retained volumes were obtained from the retention time and the eluent volumetric flow-rate determined gravimetrically using the measured

eluent density for each run. Retention data were also analyzed through application of the stoichiometric displacement model [1] to yield the apparent number of protein-adsorbent contacts, Z. For the one-to-one electrolyte employed in this study, NaCl, the value of Z equals half the slope of the plot of log k' versus the log of the reciprocal ionic strength. Values of the capacity factor, k' calculated for representative runs from the peak maximum and from the mean retention time for the wild type protein were in agreement within experimental error [9]. The k' data presented in this work were determined from the peak maxima.

The mutations examined in this work consisted of conservative carboxylic acid to amide substitutions located on surface residues. The mutations examined are designated using the single letter code for each amino acid where the site-directed mutation(s) occurred; e.g. E47,48Q represents the mutation from residue E (glutamic acid) to Q (glutamine) at both residues 47 and 48. Representative conservative site-directed charge mutants used in this study have been previously examined by NMR spectroscopy [18] to determine if any substantial tertiary conformational change resulted from the charge mutation. The NMR results indicated that only the double charge substitution E47,48Q resulted in a significant difference in tertiary structure when compared with the wild type; more conservative mutations did not result in an experimentally observable tertiary structural change. The other site-directed mutants studied by Rodgers et al. [18] also involved the loss of one negative charge and are denoted as D60N and E48Q, where D represents aspartic acid and N represents asparagine. Based on the tertiary structural results for D60N and E48Q (which represent the same type of conservative mutation as the other mutant proteins examined) and in the absence of any direct structural data, the other site directed mutants employed in this study will be assumed to have essentially the same tertiary structure as the wild type protein. The sole exception is the mutant D70K which represents a substitution of a positively charged lysine residue for a negatively charged aspartic acid. This amino acid mutation involves residues of significantly different side-chain geometry, and the charge reversal and associated local structural changes associated with this mutation might substantially alter the tertiary structure of the protein in the local environment of the substitution. Hence, the conclusions based on the investigation of the D70K mutant will take the possibility of mutation-induced local conformational changes into account.

4. Results

The contributions of various charged amino acid residues to the overall protein retention were inferred by comparing the retention values of the site-directed mutant proteins to that of the wild type protein, as a function of NaCl concentration. Retention data for the mutant and wild type proteins over a range of NaCl concentrations are presented in Fig. 1.

As illustrated in Fig. 1, the resolution among the mutant proteins decreases as the ionic strength increases. The loss of resolution (R_s) is so pronounced that for NaCl concentrations of 300 and 400 mM, the mutant proteins are not well resolved from the wild type protein; average R_s ($\pm$ S.D. over mutant proteins) decreases from 0.48 ± 0.30 (300 mM) to 0.20 ± 0.21 (400 mM). Resolution is clearly enhanced as the NaCl concentration is reduced from 400 to 150 mM. A potential explanation for this change is that as the ionic strength increases, the electrostatic screening of the protein surface potentials increases, masking the differences among the mutants on all but the local length scale (less than 10 Å). Therefore, patches or clusters of charge on the protein surface could dominate retention behavior on a local scale.

The differences in the relative contributions of the various charged amino acid groups to retention can be analyzed from the site-directed mutant protein retention data presented in Fig. 1. All of the site-directed unit charge change mutant proteins studied have the same net charge at pH 8.0 of -8.4, yet their retention behaviors vary significantly. Examples are the

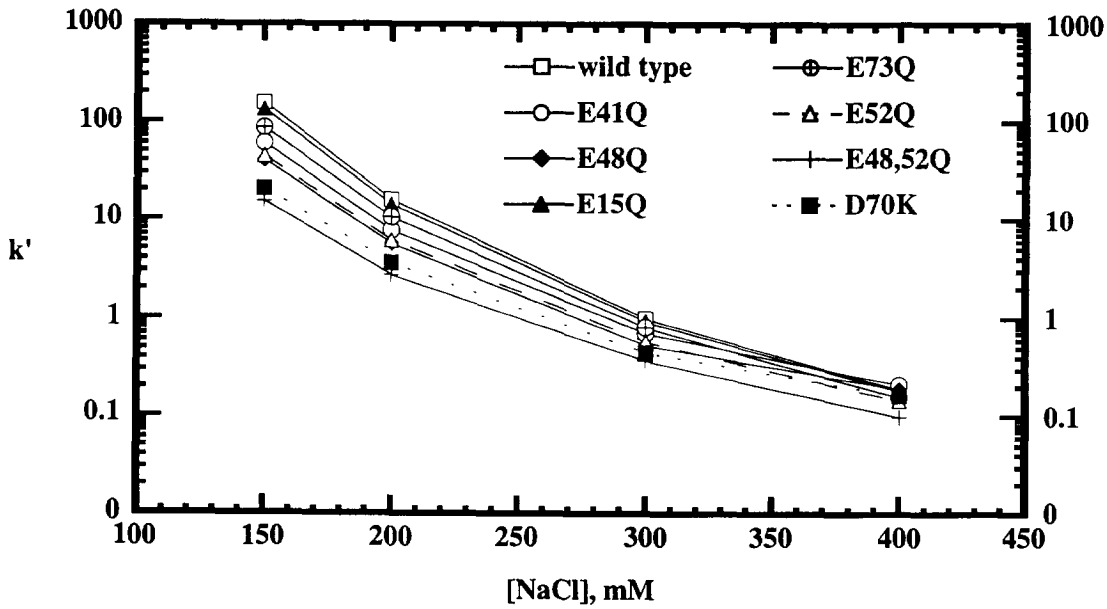

Fig. 1. A semilog plot of the capacity factor, k', versus the NaCl concentration, comparing the retention of wild type and site-directed mutant forms of rat cytochrome b_5 on Mono Q. All retention data were acquired at a nominal eluent flow-rate of 0.5 ml/min and at a mean system temperature of 25°C. The data presented are the results of duplicate retention measurements. Error bars have been omitted for clarity. The average percentage errors from duplicate retention measurements are 5.5 (150 m*M*), 3.6 (200 m*M*), 4.8 (300 m*M*) and 14.0 (400 m*M*).

mutant proteins E15Q, E41Q, and E48Q which have the respective isocratic capacity factors (in 10 m*M* Tris, pH 8.0 and 150 m*M* NaCl) of 132.8 ± 5.5, 59.6 ± 0.6, and 40.0 ± 1.7, compared to 155.6 ± 21.2 for the wild type protein. Deletion of a single charge, therefore, can have only a minor effect on retention (15% loss of retention for E15Q) or may significantly reduce retention (62 and 74% for E41Q and E48Q) as compared to the wild type protein. The reduction in the net charge of the protein by two units to −7.4, either through the double mutant E48,52Q or the double charge change mutant D70K, resulted in even more dramatic losses in relative retention of 87 and 94%, respectively. However, results obtained with the double charge mutants must be viewed carefully since these mutant proteins could have a slightly different tertiary structure than the wild type protein. These retention results, combined with the results from stoichiometric displacement model analysis of the wild type protein retention data (Z of 3.4 at 25°C), clearly indicate that only a small fraction of the 23 negative residues of rat cytochrome b_5 available for interaction with the anion-exchange surface of Mono Q are actually fully involved in the anion-exchange adsorption process. The anion-exchange retention results for the site-directed charged mutants indicate that the relative contribution of a particular charged residue is a strong function of its location on the protein surface.

The SDM analyses allow one to determine the change in the value of Z (ΔZ) resulting from charge mutagenesis. For comparison, the values for Z and of ΔZ of the site-directed mutants and the wild type cytochrome b_5 obtained from both HPLC (with the corresponding mean system temperatures) and equilibrium batch adsorption experiments [10,12] are tabulated in Table 1. The Z values obtained by the two experimental methods are in good agreement with respect to the relative contributions of the various residues. For instance, both methods indicate that the residue E15 does not appear to substantially contribute to the anion-exchange retention of

Table 1
The values for Z obtained from isocratic, isothermal HPLC retention measurements at 0.5 ml/min (in 10 m*M* Tris, pH 8.0 and the associated NaCl concentration) for the wild type and site-directed mutant forms of rat cytochrome b_5 at a mean system temperature of 25.0 ± 0.25°C for each set of Z determinations (HPLC)

Protein	Z (HPLC)	ΔZ (HPLC)	Z (Batch)	ΔZ (Batch)
Wild type	3.42 ± 0.01	N/A	2.92 ± 0.32	N/A
E15Q	3.37 ± 0.04	−0.05	2.95 ± 0.25	0.03
E41Q	2.89 ± 0.07	−0.53	2.18 ± 0.27	−0.74
E48Q	2.76 ± 0.07	−0.66	2.33 ± 0.34	−0.59
E52Q	2.91 ± 0.04	−0.51	N.D.	N.D.
E48,52Q	2.56 ± 0.08	−0.86	N.D.	N.D.
E73Q	3.20 ± 0.03	−0.22	N.D.	N.D.
D70K	2.49 ± 0.08	−0.93	N.D.	N.D.

Z-values determined from equilibrium batch adsorption experiments [10,12] at a nominal temperature of 25°C are included for reference. Errors are standard deviations for Z-values determined from replicate experiments.

cytochrome b_5, since Z values obtained for E15Q are not statistically significantly different from those obtained for the wild type protein. Both methods indicate approximately the same contribution (within experimental error) for the residues E41 and E48, based on the ΔZ values obtained for the mutant proteins E41Q and E48Q.

The importance of the location of a charged amino acid residue to its contribution to the observed retention behavior of cytochrome b_5 can be better understood by examining the tertiary structure of the protein [21]. The locations of the site-directed mutations and the associated ΔZ values resulting from the charge mutations are illustrated on the Cα (carbon backbone) trace in Fig. 2, for both HPLC and batch equilibrium adsorption experiments [12]. Fig. 2 reveals the presence of a patch on the protein surface which appears to preferentially mediate protein anion-exchange adsorption. Deletion of a negative charge (glutamate to glutamine site-directed mutant) from the residues E41, E48 or E52 results in the largest observed change in the apparent number of contacts, Z (Table 1) and this appears to be a dominant patch mediating retention. These residues, along with E47 and E42, form a patch (refer to Fig. 2) on the left side of the protoporphyrin heme prosthetic group. As a comparison, elimination of a negative charge on the opposite side of the prosthetic group, as in D64N ($\Delta Z = -0.17$, batch adsorption), E60Q ($\Delta Z = -0.06$, batch adsorption) and E73Q ($\Delta Z = -0.22$) results in only a small change in the value of Z. Deletion of two charges (resulting in a protein of net charge −7.4) to form mutant D70K, however, did result in a substantial change in the apparent number of contacts with respect to the wild type

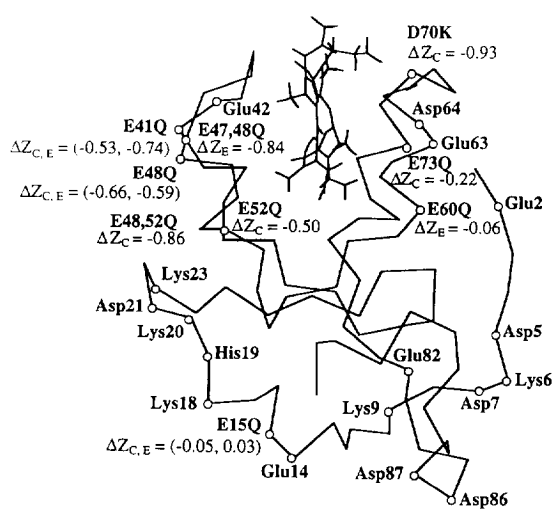

Fig. 2. A Cα trace of rat cytochrome b_5 [21] depicting the site-directed mutant proteins examined in this investigation (single letter amino acid designations) and the associated ΔZ values (with respect to the wild type protein). Results from the isocratic, isothermal HPLC retention measurements [9] and equilibrium batch adsorption experiments [10,12] are denoted by C and E, respectively.

protein, $\Delta Z = -0.93 \pm 0.08$. The retention results for this mutant, combined with those for E73Q, indicate that the patch of negative residues (D64, D70 and E73) located on the opposite side of the heme from the dominant patch is somewhat involved in anion-exchange retention of the protein. These results are also in agreement with earlier results for the mutants D70K and E73Q (see Fig. 1), which indicated that elimination of a single negative charge resulted in a substantial loss in retention (87 and 45%, respectively, relative to the wild type protein). These comparisons indicate that HPLC is a robust method for analyzing changes in retention behavior resulting from charge mutations and that HPLC can be used to approximate the results of equilibrium batch adsorption studies.

The varying contributions of different charged amino acid residues to the anion-exchange retention behavior of the protein are further illustrated by the distribution of retention times of the site-directed mutant proteins of the same net charge (Fig. 1). These results indicate that certain residues contribute more strongly to the ion-exchange retention behavior than do others. The combination of these two analyses is presented in Fig. 3; a plot of the relative retention of the mutant protein (with respect to the wild type protein), k'_r, versus the change in apparent number of contacts (ΔZ) resulting from mutagenesis. The results from both the HPLC and equilibrium batch adsorption experiments [12] are presented to provide a better map of the protein surface, although some differences did exist between the protocols for HPLC and batch adsorption experiments. Both data were acquired at a nominal temperature of 25°C (see Table 1) in 10 m*M* Tris, pH 8.0 (batch experiments also included 0.1 m*M* EDTA in the buffer). The HPLC data were acquired over a range of NaCl concentrations of 150 to 400 m*M* whereas the batch adsorption data were acquired

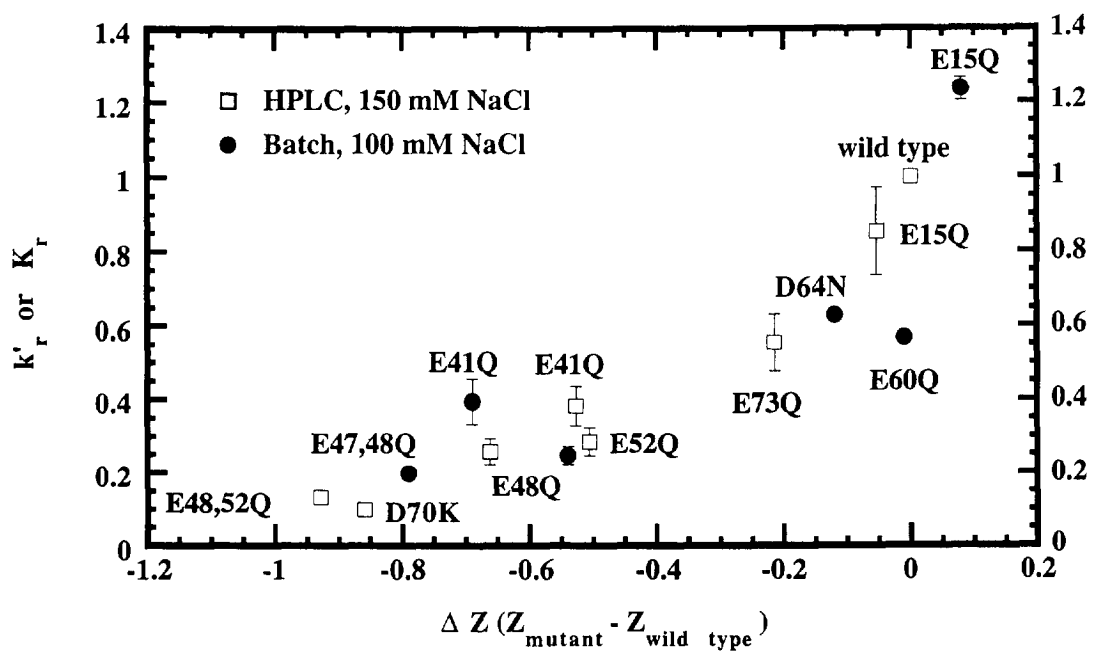

Fig. 3. Plot of the reduced or relative capacity factor, k'_r, ($k'_r = k'_{mutant}/k'_{wild\ type}$) versus the change in the apparent number of contacts, ΔZ, (determined using the stoichiometric displacement model) resulting from the mutation. The value for the analogous relative Hill binding constant (K_r) from equilibrium batch adsorption experiments [10,12] have been included to provide a better map of the protein surface and for comparison purposes. HPLC and batch adsorption experiments were both performed at a nominal temperature of 25°C. Error bars represent standard deviations based on the results of replicate experiments.

over a range of NaCl concentrations of 75 to 175 mM, the range in which equilibrium adsorption is most accurately quantifiable.

Despite the differences in data acquisition protocols between the HPLC and batch adsorption methods, several important comparisons can be made among the data. Combining these two analyses (relative retention and change in apparent number of contacts) indicates that even when the apparent number of interactions (Z-value) is similar, relative retentions are different. For instance, the apparent number of contacts between the protein and the anion-exchange surface, Z, and the relative retention with respect to the wild type protein (in parentheses) for E73Q and E15Q are 3.20 ± 0.03 (0.55) and 3.37 ± 0.04 (0.85), respectively. Thus, large changes in retention can result from only a small change in Z. This comparison can be generalized across the data resulting in the following observations.

First, the elimination of a single charged residue (through site-directed mutagenesis) on the protein surface produces less than a unit decrease in Z, for all the proteins examined in both HPLC and batch adsorption experiments (Table 1, Fig. 3). The fractional change in Z resulting from a unit change in charge suggests that the overall number of contacts between the protein and the ion-exchanger is the summation of many fractional interactions or is the statistical average over diverse orientations of individual charge–charge interactions. The nature of the electrostatic interactions between the protein and the ion-exchanger has been further investigated through computational electrostatic modeling calculations [24]. The electrostatic modeling results indicate that two patches of residues, including residues E47, E48, D70, R72, and E73 (surrounding the heme prosthetic group) (patch 1) and residues E60, D64, H67 (patch 2) are primarily involved in the anion-exchange adsorption behavior of cytochrome b_5, in agreement with the experimental results.

Deletion of two negative charges by site-directed mutagenesis (e.g., in E48,52Q) produces a value of ΔZ less than the sum of the ΔZ's of the independent E48Q and E52Q mutations (Table 1). This observation is clarified by comparing the relative changes in retention for the single charge mutants E48Q and E52Q to the relative change in retention of the double mutant E48,52Q. Values of reduced k' ($k'_{\text{mut}}/k'_{\text{wild type}}$) are 0.25 and 0.28 for the single mutants E48Q and E52Q and 0.1 for the double mutant E48,52Q. Therefore, these two mutants display reductions in retention (with respect to the wild type protein) of 75% and 72%, respectively, while the double charge reduction mutant E48,52Q displays approximately a 90% reduction in retention. The results for E48,52Q must be viewed with some caution since NMR data on this mutant [18] indicated that a significant local conformational change results from this double mutation. Even taking into account the limitations of the comparison, it is clear that the electrostatic interactions between the protein and the ion-exchange surface are fractional in nature and that the summation of these interactions is not linearly additive.

The relative contributions of various residues are further explored by the data analyses presented in Fig. 4, which presents a plot of the relative retention, k'_r versus relative Z_r which is equal to $Z_{\text{mutant}}/Z_{\text{wild type}}$. These results indicate that the deletion of a single charged residue can have dramatic effects on retention behavior, while resulting in only a small percentage reduction in the apparent number of contacts. The retention results for the site-directed mutants E73Q and E48Q clearly illustrate this phenomenon. E73Q and E48Q have retentions reduced by 45% and 74% but Z reduced by 6% and 19%, respectively. Changing a contact residue from a negative to a positive charge in the case of D70K results in virtually a complete loss of retention (87% reduction) with a decrease in Z of only 27%. A related example is E41Q, which has a relative retention (k'_r) of 0.38 while retaining 85% of the apparent number of contacts (Z_r of 0.85) of the wild type protein. For the extreme case of E48,52Q the relative retention (k'_r) is 0.098 with an associated Z_r value of 0.75. These results imply, therefore, that under the conditions examined in this study, only a small fraction (less than 30%) of the apparent contacts between the protein and the ion-exchange sur-

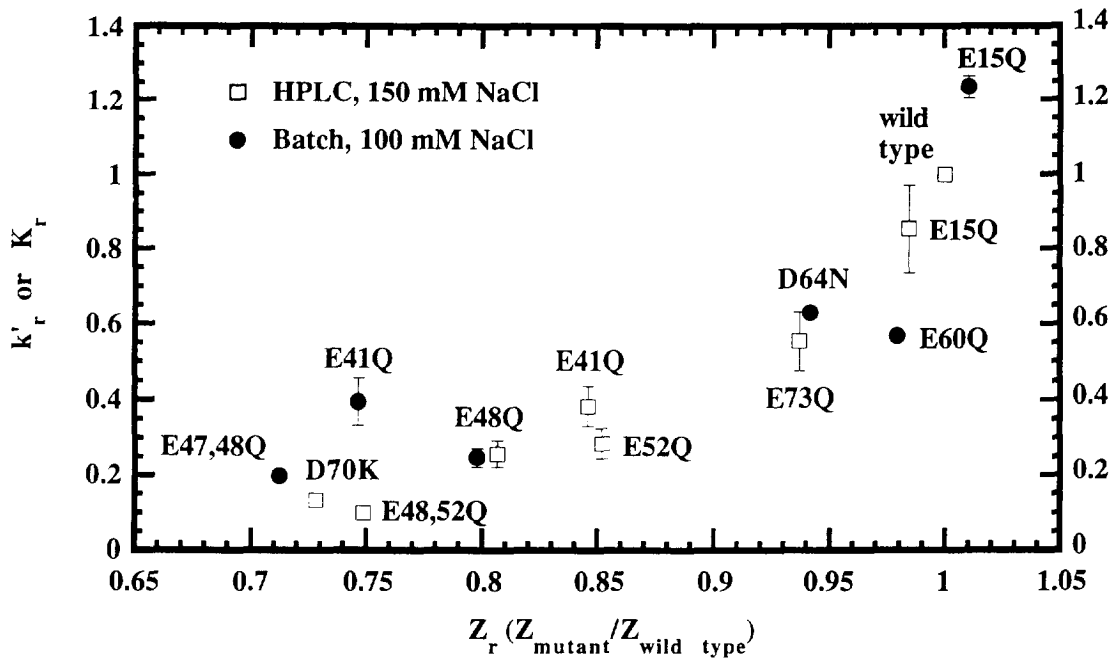

Fig. 4. A plot of the relative retention, k'_r, versus relative Z, Z_r, ($Z_r = Z_{mutant}/Z_{wild\ type}$). Error bars represent standard deviations estimated from replicate experiments.

face are responsible for the vast majority of the overall anion-exchange retention behavior of rat cytochrome b_5. The remainder of the electrostatic interactions make only a minor contribution to the observed retention behavior.

5. Discussion

In this work, the anion-exchange retention of wild type cytochrome b_5 and its site-directed charge mutants has been characterized by measuring the effects of ionic strength on retention in isocratic HPLC. The best-characterized influence on the chromatographic capacity factor k' is that of ionic strength, as presented in Fig. 1. Based on results presented in Fig. 1, the resolution of the charge mutant proteins declines with increasing NaCl concentration and is virtually lost for NaCl concentrations greater than 300 m*M*. The protein purification implications of this loss of resolution with increasing NaCl concentration are important. If one were attempting to purify a desired wild type protein from closely related contaminants (e.g., deamidation products, charge mutant forms, translational variants), a high degree of resolution would best be achieved through operation at low ionic strengths. The use of a low ionic strength gradient would result in extended protein retention (process time) and reduced throughput for the purification system. In contrast, high throughput would require operation under high ionic strength conditions, with degraded resolution. Therefore, a compromise in the choice of ionic strength must be made in order to optimize the tradeoff between selectivity and productivity generally observed in separations processes.

The observation that only a small fraction of the charged amino acid residues on the protein surface participate in ion-exchange adsorption has been discussed in the literature [25] and has been demonstrated experimentally for the ion-exchange adsorption of other proteins. Chicz and Regnier [5] noted that the apparent number of contacts between subtilisin and an ion-exchange

surface fell into two distinct domains, with a sharp transition with increasing ionic strength of the eluent. Chicz and Regnier [6] also observed that when compared with the wild type subtilisin, the apparent number of contacts between the mutant proteins and ion-exchanger could increase while the retention times of the mutant proteins actually decreased, indicating possibly that electrostatic interactions between a protein and an ion-exchanger have varying interaction energies. The authors concluded that the protein tertiary structure sterically limits the number of contacts between the protein and the ion-exchange surface and that the local environment and position of each amino acid residue is important in determining its contribution to retention. The results obtained for the site-directed mutants of rat cytochrome b_5 concur with the conclusions of Chicz and Regnier [6] regarding the variable contributions of a single amino acid residue to retention. A good example of the effect of local environment is the retention result for the cytochrome b_5 mutant E15Q, which was observed to have a value of Z indistinguishable from the wild type protein, even though its net charge was reduced by one unit. Although the residue is sterically accessible for interaction with the anion-exchange surface, the location of this negative charge in an area of predominantly positive charge limits its influence on retention behavior.

Previous batch adsorption experiments had shown participation of a single major contact region [12] mediating ion-exchange retention of cytochrome b_5. This result was corroborated and extended by this investigation to reveal the presence of an additional secondary region contributing to ion-exchange retention. The two negatively charged patches on the surface of rat cytochrome b_5 which dominate anion-exchange retention (as determined from retention studies of site-directed charged mutants) can be compared with the cation binding sites on the surface of bovine cytochrome b_5 identified by Whitford et al. [13] using NMR techniques. The following description of the sites located by Whitford et al. [13] identifies homologous residues in the rat form of the protein with the rat amino acid sequence number based on the method of Ozols [26]. Three cation-binding sites were located, including site I which was composed of the heme propionates and amino acid residues E41, E42, E47, E48, E52. Site II was found to be located near the histidine residue H30 and to contain the residues E60, D57 and D64 while site III was located near the histidine residue H84 and contained residues E82, D86, and D87. D87, located in the patch of negative residues identified by Whitford et al. [13] as site III, is involved in a salt bridge with K90. E14, located near site III, is involved in a salt bridge with K18. The salt-bridging of these two negatively charged residues probably reduces the contributions of the residues in site III to the anion-exchange retention of rat cytochrome b_5. The two patches which dominate anion-exchange adsorption behavior of rat cytochrome b_5 coincide with Whitford's sites I and II. This agreement suggests that NMR may be useful in identifying surface accessible clusters of negative charge which can participate in anion-exchange adsorption.

The contribution of the heme propionates was not investigated in this study, but the location of these negatively charged species would allow for their interaction with the anion-exchange adsorbent concurrent with either of the two charged patches. The potential interaction of the residues in the third cluster identified by Whitford were not examined in these ion-exchange retention behavior experiments, but the contribution of residue E15 was examined and it was determined not to contribute substantially to the anion-exchange retention behavior. The electrostatic interactions between the soluble tryptic fragment of rat cytochrome b_5 and a model anion-exchange adsorbent have been previously investigated using molecular electrostatic modeling [24]. Computational modeling revealed the presence of three major clusters of negative potential surrounding the heme prosthetic group including (a) the cluster containing Glu47, Glu48 and Glu52; (b) the cluster containing Glu41 and Glu42; and (c) the cluster containing Asp70 and Glu73. The negative potential associated with the heme functional groups is also evident from electrostatic equipotential plots. The relative

contributions of the residues in these three clusters to the overall electrostatic interaction energy between the protein and the anion-exchange surface is also in agreement with those obtained from chromatographic retention results.

Factors other than the net charge of the protein and the adsorbent can also play a large effect in mediating protein ion-exchange retention behavior. The importance of protein tertiary structure in determining the number of interactions between the protein and the ion-exchanger has been noted by Drager and Regnier [27] for the anion-exchange adsorption of lactate dehydrogenase. Drager and Regnier concluded that multiple parts of the protein surface could potentially interact cooperatively to determine the observed protein retention behavior. The observed heterogeneous contributions of amino acid residues and the distribution of retention times for charged mutants of the same net charge for cytochrome b_5 is in accord with the ideas proposed by Drager and Regnier.

The results of these HPLC retention studies combined with the results from equilibrium batch adsorption experiments [10,12] indicate that the observed cytochrome b_5 ion-exchange retention behavior results from the summation of many fractional electrostatic interactions of varying energies. Protein ion-exchange retention is therefore governed by both the number and the character of the contacts between the protein and the adsorbent surface.

Acknowledgements

E. coli strain TB1 expressing recombinant soluble core of rat cytochrome b_5 was the generous gift of Dr. Stephen Sligar of the University of Illinois. We would like to thank Dr. Sligar and Dr. Karla Rodgers for helpful discussions. Support was provided by NSF under CTS-8910087, by the Welch Foundation, and by an NSF Presidential Young Investigator Award to RCW. Additional support was provided by the 3M Corporation, by Pharmacia and by Waters.

References

[1] W. Kopaciewicz, M.A. Rounds, J. Fausnaugh and F.E. Regnier, *J. Chromatogr.*, 266 (1983) 3.
[2] V. Lesins and E. Ruckenstein, *Colloid Polym. Sci.*, 266 (1988) 1187.
[3] J. Fausnaugh-Pollitt, G. Thevenson, L. Janis and F.E. Regnier, *J. Chromatogr.*, 443 (1988) 221.
[4] I. Mazsaroff, S. Cook and F.E. Regnier, *J. Chromatogr.*, 443 (1988) 119.
[5] R. Chicz and F.E. Regnier, *J. Chromatogr.*, 443 (1988) 193.
[6] R. Chicz and F.E. Regnier, *Anal. Chem.*, 61 (1989) 2059.
[7] N.K. Boardman and S.M. Partridge, *Biochem. J.*, 59 (1955) 543.
[8] W. Kopaciewicz, M.A. Rounds and F.E. Regnier, *J. Chromatogr.*, 318 (1985) 157.
[9] D.J. Roush, D.S. Gill and R.C. Willson, *J. Chromatogr. A*, 653 (1993) 207.
[10] D.S. Gill, D.J. Roush and R.C. Willson, *J. Colloid Interface Sci.*, 167 (1994) 1.
[11] R.D. Whitley, R. Wachter, F. Liu and N.-H.L. Wang, *J. Chromatogr.*, 465 (1989) 137.
[12] D.S. Gill, D.J. Roush and R.C. Willson, *J. Chromatogr. A*, 684 (1994) 55.
[13] D. Whitford, D.W. Concar, N.C. Veitch and R.J.P. Williams, *Eur. J. Biochem.*, 192 (1990) 715.
[14] A. Willie, P.S. Stayton, S.G. Sligar, B. Durham and F. Millett, *Biochemistry*, 31 (1992) 7237.
[15] M. Rivera, F.A. Walker, M.R. Mauk, A.G. Mauk, M.A. Cusanovich and G. Tollin, *Biochemistry*, 32 (1993) 622.
[16] J.J. Wendoloski, J.B. Matthew, P.C. Weber and F.R. Salemme, *Science*, 238 (1987) 794.
[17] D. Wu and R.R. Walters, *J. Chromatogr.*, 598 (1992) 7.
[18] K. Rodgers, T. Pochapsky and S.G. Sligar, *Science*, 240 (1988) 1657.
[19] R.D. Guiles, V.J. Basus, I.D. Kuntz and L. Waskell, *Biochemistry*, 31 (1992) 1365.
[20] R.D. Guiles, V.J. Basus, S. Sarma, S. Malpure, K.M. Fox, I.D. Kuntz and L. Waskell, *Biochemistry*, 32 (1993) 8329.
[21] D.S. Gill, D.J. Roush and R.C. Willson, *J. Biomol. Struct. Dyn.*, 11 (1994) 1003.
[22] S. Beck von Bodman, M. Schuler, D. Jollie and S. Sligar, *Proc. Natl. Acad. Sci. USA*, 83 (1986) 9443.
[23] T. Takeuchi, M. Masayoshi and D. Ishii, *J. Chromatogr.*, 235 (1982) 309.
[24] D.J. Roush, D.S. Gill and R.C. Willson, *Biophys. J.*, 66 (1994) 1290.
[25] F.E. Regnier, *Science*, 238 (1987) 319.
[26] J. Ozols, *Biochem. Biophys. Acta*, 997 (1989) 121.
[27] R.P. Drager and F.E. Regnier, *J. Chromatogr.*, 406 (1987) 237.