Anion-exchange chromatographic behavior of recombinant rat cytochrome b_5

Thermodynamic driving forces and temperature dependence of the stoichiometric displacement parameter Z

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ABSTRACT

The HPLC anion-exchange isocratic retention behavior of the recombinant soluble core of wild type rat cytochrome b_5 on Mono Q HR 5/5 was investigated as a function of temperature and sodium chloride concentration at fixed eluent flow-rates. Retention was measured over a range of eluent flow-rates at a specified temperature to determine if true adsorption equilibrium could be approximated by the HPLC method. Apparent Van 't Hoff enthalpies of adsorption obtained from the HPLC retention data were positive, indicating an entropically driven spontaneous adsorption process, and were found to decline with increasing ionic strength. The retention results were interpreted in terms of the stoichiometric displacement model to obtain the apparent number of binding sites in the contact region, Z, as a function of temperature and of protein concentration. Z was found to depend significantly on temperature, even under conditions of nearly complete protein recovery, but did not depend on protein concentration at the low loadings studied.

INTRODUCTION

Ion-exchange chromatography of proteins is based primarily on the coulombic interactions of oppositely charged groups on the protein and adsorbent surfaces. The ion-exchange adsorption of proteins has traditionally been assumed to be governed by the net charge of the protein resulting from ionizable groups on the protein surface, so that a protein will be retained on an anion exchange surface at a pH above the protein's isoelectric point. Several studies, however, have indicated that a protein may be retained on an ion-exchange surface of the *same* sign as the net charge of the protein [1,2] implying that the adsorption of a protein can be dominated by patches or clusters of charge on the protein's surface [1,3–6]. Another approach [7] has been to correlate retention data with the mean electrostatic potential as determined through electrostatic modeling of the protein surface.

The retention behavior of proteins on ion-exchange surfaces can often be successfully correlated using the non-mechanistic stoichiometric displacement model (SDM) originally proposed by Boardman and Partridge [8] and first applied to HPLC by Kopaciewicz *et al.* [1]. This model of protein adsorption is based on a mass-action

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description consisting of an exchange equilibrium between the counterions associated with the protein and displacing ions associated with the ion-exchange surface, and an equilibrium between the protein in the bound and free forms. The regression parameter Z, the apparent number of contacts between the protein and the ion-exchange surface participating in the adsorption–desorption process, can be determined by measuring the retention of the protein over a range of ionic strengths as described by these workers [1]. The SDM has been applied by other researchers to the ion-exchange adsorption of proteins in both batch [9,10] and HPLC [1,11–15] modes, and of nucleic acids [16]. Fractional Z values have been obtained, and these have been observed to change as a function of loading [4,9,10]. Differences between values of Z observed under isocratic and gradient elution conditions for the same system have been previously noted [17], and probably arise from incomplete equilibration of counterions and displacing ions during gradient elution. The difficulties associated with calculating isocratic elution parameters for proteins from gradient elution data have been documented by other researchers [17] and greatly impair the derivation of accurate thermodynamic quantities from such data [17–19]. Hence, isocratic elution is necessary to meet the requirements of counterion equilibrium for the SDM and to obtain thermodynamic information from the retention data. The completeness of equilibration of the protein between bound and free forms can be assessed by analyzing isocratic retention data over a range of eluent flow-rates. Careful control of temperature is also required for valid measurement of thermodynamic parameters.

In the analysis of experimental data from ion-exchange retention, it is important to establish that ion exchange is the dominant mechanism of adsorption. Other interactions, including Van der Waals and hydrophobic interactions can also influence retention [7,20–22] depending on the nature of the protein, eluent and ion exchanger. A combination of ion-exchange and other interactions ("mixed-mode adsorption" [23]) can occur for proteins such as lysozyme, which display both hydrophobic and charged patches. This phenomenon is generally believed to occur

with most adsorbents [24], but has been best documented for polyethyleneimine-derivatized silica stationary phases with a range of hydrophobicities [11]. Hydrophobic interactions have been suggested to cause conformational changes in the protein upon adsorption [25,26] and are increased at elevated temperatures [27]. Minimization of these types of interactions is critical for characterization of ion-exchange adsorption alone, and to ensure that protein conformation is preserved during chromatography.

This study examines retention of the recombinant soluble core of wild type 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 and biochemical data available and the existence of an efficient system for its expression in *Escherichia coli*, which will allow the future use of surface charge mutant forms of cytochrome b_5 to probe the potential existence of a favored "chromatographic contact region" in this protein–adsorbent system. The protein is well-suited for use in studies on anion-exchange adsorption of proteins because of its great stability in solution, strong chromophore (see below), 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 charges (including the protoporphyrin) and 15 positive groups (allowing for partial titration of histidines) resulting in net charge −9.4 at pH 8.0. The effects of eluent flow-rate, temperature, protein concentration and ionic strength are examined in this work through application of the SDM and Van 't Hoff analyses. The goals of this investigation are to determine: (1) if a close approximation to equilibrium can be achieved in the HPLC system; (2) the apparent average number of interacting groups on the protein surface, "apparent Z"; (3) the apparent enthalpy of adsorption ΔH_{ads} as determined from Van 't Hoff analyses; and (4) any effects of temperature and eluent flow-rate on the apparent Z.

EXPERIMENTAL

Chemicals and reagents

The protein studied, recombinant soluble tryptic core of rat cytocrome b_5, was prepared in *E.*

coli using pUC plasmids containing a synthetic gene for this protein synthesized in the laboratory of Dr. Stephen Sligar at the University of Illinois [28]. The protein was purified from *E. coli* lysate using ion-exchange chromatography (Q Sepharose Fast Flow; Pharmacia, Uppsala, Sweden), ammonium sulfate fractionation and size exclusion chromatography (Sephacryl HR 100, Pharmacia). Protein purity was verified by silver stained 8–25% gradient sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis (PhastGel, Pharmacia), by ultraviolet–visible spectroscopy as described below, and by the fact that the protein ran as a single peak in the HPLC experiments.

Protein stock solutions were stored at $-80°C$ at concentrations greater than 1.0 mg/ml in 10 mM Tris, pH 8.0 (pH adjusted at ambient temperature) and 0.1 mM EDTA. Protein stock solutions were assessed for purity and integrity after thawing by measuring the R_Z (the ratio of the absorbances at 412 and 280 nm). All protein stock solutions used displayed an R_Z greater than 5.6. Tris buffer salts of greater than 99.5% purity were obtained from ICN Biomedicals or Sigma.

Protein solutions and buffers

Eluents were prepared for each NaCl concentration by dilution of a 1.0 M Tris, pH 8.0 (at ambient temperature) stock solution and addition of the appropriate mass of NaCl. All solutions were prepared in volumetric flasks and adjusted to pH 8.0 with HCl and NaOH at the experimental temperature. Eluents were filtered through 0.2- or 0.45-μm cellulose acetate filters (Nalgene, Rochester, NY, USA) and degassed with high-purity helium (Linde, Houston, TX, USA).

Protein samples were prepared by dilution to approximately 0.25 mg/ml (0.50 mg/ml where specified) with 10 mM Tris, pH 8.0 and with 10 mM Tris, pH 8.0 + 2 M NaCl in a ratio such that the injected sample was in the same buffer concentration as the isocratic eluent. Protein samples were filtered using 0.22- or 0.45-μm cellulose acetate filters (Ultrafree-MC; Millipore, Marlborough, MA, USA) before injection. Protein concentrations were determined using a Beckman DU-64 spectrophotometer using the

molar extinction coefficient of 130 mM^{-1} cm^{-1} at 412 nm for the strong Soret band of the oxidized protein.

Equipment

The entire HPLC system, with the exception of the computer (NEC 386/25 CUP; NEC Technologies, Boxborough, 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 $\pm 0.5°C$. 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 441 ultraviolet–visible single-wavelength detector operating at 405 nm or a 481 variable-wavelength detector. The 405 nm wavelength was used for cytochrome b_5 retention measurements because it is located close to the oxidized protoporphyrin Soret band at 412 nm. For several experiments, the retention behavior of cytochrome b_5 was also monitored at 280 nm using the 481 detector in series to determine if the R_Z changed during the chromatographic process. The Zn source of the 441 detector was employed to detect triglycine at 214 nm in column efficiency determinations.

Data were analyzed using Maxima 3.0 (Dynamic Solutions) software. Conductivity measurements were performed with an Amber Science Model 1054 (Eugene, OR, USA) digital conductivity meter and dip probe model 525. Calibration of the 525 probe was referenced to a nominal 10 000 μS conductivity standard (Fisher Scientific, Fair Lawn, NJ, USA). An in-line conductivity cell (Model 529, Amber Science) was employed to confirm column equilibration, which was defined as the inlet and exit conductivities being equal to within the measurement error of 0.9%. Conductivity was observed to be stable for at least five column volumes before each injection was made. Temperature measurements were made with an Omega (Stamford, CT, USA) DP41-TC display connected to an Omega hypodermic thermocouple probe (Model 3). Mono Q (Pharmacia) prepacked strong anion-exchange columns (HR 5/5, 50 mm × 5 mm I.D.) were used in all studies. The columns

were equilibrated according to the manufacturer's protocol (Pharmacia) as follows: washing with 5 ml of low-ionic-strength buffer (10 mM Tris, pH 8.0) followed by 10 ml of high-ionic-strength buffer (10 mM Tris, pH 8.0 + 2 M NaCl) and then by washing with low-ionic-strength buffer until the baseline stabilized. Additional column equilibration was performed as needed.

pH measurements were performed using an Orion (Cambridge, MA, USA) EA940 meter equipped with a Ross combination electrode, calibrated using pH 4.01, 7.00 and 10.01 standards (25°C, Orion), and pH adjustments made at the temperature at which the eluent was to be used.

Column performance (plate count) was verified using the retention behavior at 25°C of a 20-μl sample of 0.05 mg/ml triglycine [Sigma or United States Biochemicals (Cleveland, OH, USA)] in 10 mM Tris, pH 8.0 monitored at 214 nm. 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 retired when the plate count fell below the Pharmacia specification of 25 000 plates/m.

Chromatography

Protein samples were prepared in 10 mM 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 mM Tris, pH 8.0 and NaCl in the range of NaCl concentration studied, 150 to 700 mM, matching the injection buffer. Protein sample concentrations were 0.25 ± 0.03 mg/m and 0.54 ± 0.01 mg/ml for the experiments designated 0.25 mg/ml and 0.50 mg/ml, respectively. The upper concentration range used was limited to approximately 0.60 mg/ml protein by the linearity of the 441 detector.

Retention was studied over the temperature range 4.7 to 36.2°C with a nominal eluent flow-rate of 0.5 ml/min. Retention behavior was also measured at 25°C over a range of eluent flow-rates between 0.1 and 1.0 ml/min. The evaluation of completeness of equilibration in these

HPLC experiments is based on the independence of the capacity factor to changes in eluent flow-rate, as discussed below. For each eluent flow-rate, the protein was assigned a non-retained volume corresponding to the elution volume at 700 mM NaCl, corrected for non-column system volume, based on control experiments which showed that retention does not vary significantly in the range 600 to 700 mM NaCl.

Protein recovery calculations were based on detector calibration determined by direct injection into the absorbance detector of duplicate protein samples, separated by eluent blanks, for each retention measurement. Recoveries were determined using the same eluent and conditions as the protein retention experiment as it was observed that detector sensitivity [peak area (in μV·s) per μg protein] varied as a function of eluent flow-rate and temperature. The ratio of peak area to baseline noise integrated over a period equal to the peak width at baseline was typically *ca.* 80 under conditions of strong retention. The recoveries of cytochrome b_5 at temperatures below 37°C were 100% within experimental error, (±10%), except for the most strongly retained protein at 150 mM NaCl where recoveries were in excess of 85%. Protein recoveries for 37°C experiments at 175 and 150 mM NaCl were 70 and 35%, respectively, presumably due to structural rearrangements or denaturation which may occur under these conditions of strong retention (the retention time at 150 mM NaCl was 420 min). A gradient cleaning run, employing 10 mM Tris, pH 8.0 and 2 M NaCl, was performed after isocratic runs of less than 300 mM NaCl. The protein was not able to be quantitatively recovered from the most strongly retained conditions (150 mM and 175 mM NaCl at 37°C) during extensive cleaning runs, typically resulting in loss of separation efficiency of the column after experiments at 37°C. Values of Z and Van 't Hoff enthalpies calculated without including the results of experiments showing recoveries of below 85%, were not significantly different from those calculated from the entire data set.

The accuracy and precision of the HPLC system were validated prior to acquisition of the experimental data, and control experiments were

performed to examine the effects of experimental parameters on retention. The conductivity detector and batch cell were found to give a linear response for sodium chloride concentrations of 100 to 1000 mM. The conductivity detector accuracy was 0.3% at 25°C and 1.0% at 4°C.

The eluent feed pumps were calibrated gravimetrically to 0.50 ml/min ± 2%. Eluent flow-rates ranged from 0.46 to 0.50 ml/min as determined gravimetrically for each chromatographic run, using the measured eluent density. Eluent flow-rates for the nominal 1.0 ml/min experiments ranged from 0.97 to 0.99 ml/min. The minimum flow-rate which can be commanded by software for the HPLC pumps is 0.1 ml/min, but accurate control is difficult at this low flow-rate, as the exit check valves do not close reliably at the low system pressure (less than 689 kPa). Experiments performed at a nominal flow-rate of 0.1 ml/min represent a range of flow rates from 0.083 to 0.105 ml/min. Given the strong dependence of k' on ionic strength, the accuracy of the pumps was inadequate to allow for mixing of the two eluents to achieve a given sodium chloride concentration. Hence a separate eluent was prepared for each isocratic retention experiment as described under Materials.

As discussed previously by other authors [29], the k' value can be a strong function of temperature, therefore the variation in the mean temperature of the system must be minimized in order to determine the effects of eluent flow-rate on retention. The protein samples and eluents were pre-equilibrated at the experimental temperature. Temperature measurements were conducted with the hypodermic thermocouple in the eluent reservoir, and also at the column outlet. The temperature at the column outlet was taken as that at which the reading stabilized to within 0.1°C, which occurred within several minutes. The average of the inlet and outlet temperatures is reported as the average temperature for a given run. The average difference in inlet and outlet temperatures for all experiments was 0.9 ± 0.7°C. Retention data were collected at mean system temperatures of 4.7 ± 0.2°C, 10.5 ± 0.2°C, 25.3 ± 0.2°C, 29.8 ± 0.1°C, and 36.2 ±

0.4°C at a nominal flow-rate of 0.50 ml/min, to assess the effect of temperature on retention and Z, and to allow for an approximate Van 't Hoff analysis.

In our studies of the effect of flow-rate on retention behavior, the mean system temperatures were 25 ± 0.4°C for the entire range of eluent flow-rates studied. As shown under Results, at 150 mM, 25°C, variations in temperature of this magnitude would produce a change in k' and Z of ca. 5% and 0.4%, respectively.

DATA ANALYSIS

The capacity factor for each isocratic retention run was calculated from the following expression:

$$k' = (V_R - V_0)/V_0 \qquad (1)$$

where k' is the capacity factor, V_R is the protein retention volume and V_0 is the protein non-retained volume. Retained and non-retained volumes were obtained from the retention time and the eluent volumetric flow-rate determined gravimetrically by eluent density for each run. The eluent flow-rate must be determined for each run as the pump delivery rate varied by up to 8% over the range of salt concentration used.

Thermodynamic information can be obtained from HPLC retention data if one can relate the measured parameter, k', to the ratio of the protein concentration in the bound and the free phase. Once this relationship is defined, the application of an approximate Van 't Hoff analysis of the logarithm of k' *versus* reciprocal absolute temperature is straightforward. The following description of the relationship of k' to protein concentrations is adapted from Kopaciewicz *et al.* [1].

The capacity factor can be also defined as

$$k' = K_i y \qquad (2)$$

where K_i is the partition coefficient in liquid chromatography, y is phase ratio equal to A_s / V_m, A_s is the available surface area (cm^2) and V_m is the mobile phase volume (ml). The partition coefficient can also be defined as

$$K_i = C_s / C_m \qquad (3)$$

where C_s is the protein concentration in the stationary phase (mol/cm^2) and C_m is the protein concentration in the mobile phase (mol/ml).

The influence of temperature on chromatographic retention has been addressed for microcapillary chromatography for reversed- and normal-phase, and cation-exchange chromatography by Takeuchi et al. [29], and a relation between k' and ΔH^0 derived. In this derivation [29], it is assumed thermodynamic equilibrium is achieved in the column to allow for computation of thermodynamic parameters from a plot of ln k' versus reciprocal absolute temperature, or an approximate Van 't Hoff analysis. A modified form of the derived expression is:

$$\ln k' = -\Delta H^0/RT + \Delta S^0/R + \ln(A_s/V_m) \qquad (4)$$

where A_s = total area of the stationary phase (cm^2), V_m = mobile phase volume of the column (ml), ΔH^0 = enthalpy of transfer of a solute from the mobile phase to the stationary phase and ΔS^0 = entropy of transfer of a solute from the mobile phase to the stationary phase.

For dilute solutions, the enthalpy of adsorption ΔH_{ads} can then be obtained by multiplying the slope of the plot of ln k' vs. $1/T$ by the ideal gas constant, R (1.978 cal/mol K).

Retention data can also be analyzed through application of the SDM [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', were calculated for representative runs from both the peak maximum and from the mean retention time and were in agreement within experimental error. The k' data presented below were determined from the peak maxima.

The validity of several underlying assumptions must be confirmed to allow for a Van 't Hoff analysis of the HPLC data. One of these is the use of protein activity coefficients of unity, which is valid for the range of protein concentrations (15 to 45 μM) in sodium chloride concentrations (150 to 700 mM) used in this study [30]. Another assumption to be confirmed is that HPLC operation approximates adsorption equilibrium. As Z

appears to be essentially independent of eluent flow-rate for flow-rates between 0.2 and 1.0 ml/min (see Discussion section), the assumption of mass flux equilibrium appears to be justified for this experimental system under these operating conditions. It should be noted, however, that slow migration of protein molecules on the adsorbent surface and/or conformational changes of bound protein may only occur over time scales longer than typically sampled in column chromatographic experiments.

RESULTS

A representative plot of k' vs. [NaCl] for an average system temperature of 25.3°C, presented in Fig. 1, illustrates that the protein retention covers several orders of magnitude from completely unretained (700 mM NaCl eluent, $k' = 0$) to very strongly retained (150 mM NaCl, $k' = 177$). This broad sampling of the retention behavior was obtained for the entire temperature range studied using NaCl concentrations of 150 to 400 mM. Control experiments with 10 mM Tris, pH 8.0 + 600 mM NaCl eluents gave retention behavior statistically indistinguishable from the 700 mM NaCl data. As is well known, the increased retention at the lower ionic strengths could be explained simply by increased competition by counterions and/or reduced electrostatic screening as compared with higher ionic strengths.

The application of the SDM to the data in Fig. 1 is illustrated in Fig. 2. For the salt employed in this study, a one-to-one electrolyte, the slope of the plot log k' vs. log (1/[NaCl]) equals twice Z. For this example, a linear least squares regression of the data yields a value of Z equal to 3.41.

The values for Z obtained over the range of eluent flow-rate 0.1 to 1.0 ml/min are presented in Fig. 3. The values obtained for Z are relatively independent of eluent flow-rate for flow-rates greater than 0.2 ml/min, and may be ca. 5% lower at the lowest flow-rate tested, 0.1 ml/min. The lowest eluent flow-rate may allow the protein to sample the ion-exchange medium for sufficient time to reach "true" adsorption equilibrium as would be determined by batch experiments. The average Z value of 3.41 ± 0.09
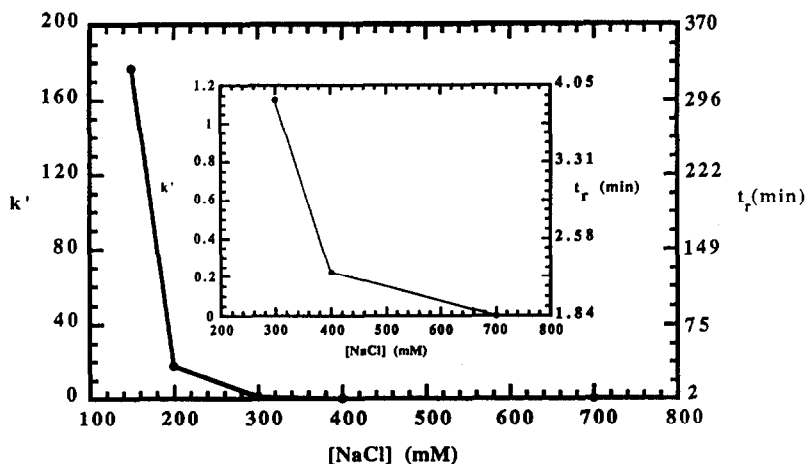
Fig. 1. Retention of cytochrome b_5 (k') on Mono Q HR 5/5 as a function of ionic strength of NaCl at a mean system temperature of 25.3°C and an eluent flow-rate of 0.5 ml/min. The figure illustrates the wide range of k' sampled during a representative set of retention experiments.

obtained over the range of flow-rates tested is in fair agreement with the value of 2.79 ± 0.22 obtained for batch equilibrium adsorption experiments [10] at 25°C with the value obtained at a flow-rate of 0.1 ml/min (3.22 ± 0.19) being in closest agreement with the batch result. The results obtained at the lowest flow-rates used must be interpreted with caution, however, because of poor pump precision at these flow-rates. The remainder of the experimental data, therefore, were collected at nominal eluent flow-rates of 0.5 ml/min.
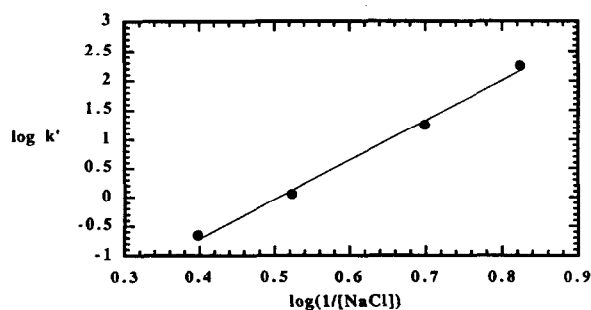
The experimental data clearly indicate the strong dependence of k' on temperature. Over the range of temperatures studied (4.7–36.2°C), k' changes from 38.3 to 298 at 150 mM NaCl, and from 0.100 to 0.267 at 400 mM NaCl. As the ionic strength decreases, the sensitivity of k' to temperature increases.

Another aspect of the influence of temperature on retention behavior is revealed by calculation of the SDM parameter Z, which is found to vary with temperature. Averaged Z values as a function of mean system temperature from replicate experiments, with the exception of the single data point at 10.5°C, are presented in Table I; the individual measurements are displaced in Fig. 4, which clearly demonstrates a
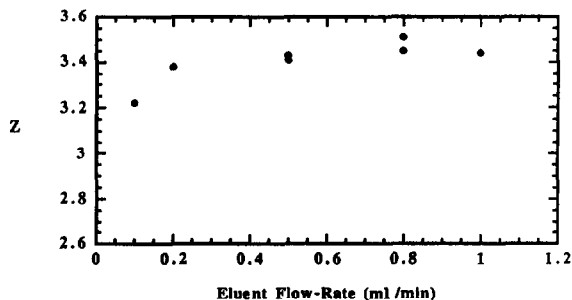

Fig. 2. Plot of log k' vs. log ($1/[NaCl]$) or Z plot of the data presented in Fig. 1. The value of Z derived from the least squares linear analysis of the data equals one half the slope or 3.41. The data were acquired at a mean system temperature of 25.3°C and a nominal eluent flow-rate of 0.5 ml/min over the [NaCl] range 150 to 700 mM. The linear least squares fit is $y = -3.439 \pm 0.202 + 6.811 \pm 0.32x$ with $R = 0.998$.

Fig. 3. Plot of Z vs. eluent flow-rate at a mean system temperature of 25.2°C for the soluble tryptic core of rat cytochrome b_5. This figure illustrates the approximation to equilibrium for the flow-rate of 0.5 ml/min.

TABLE I

APPARENT NUMBER OF CONTACTS, Z, VS. TEMPERATURE

System temperatures (°C)			Z
Eluent	Column effluent	Mean	
4.0	5.5	4.7	3.04 ± 0.05
9.9	11.2	10.5	3.19 ± 0.08
14.3	15.6	14.9	3.23 ± 0.10
24.8	25.8	25.3	3.42 ± 0.01
29.4	30.1	29.8	3.40 ± 0.07
36.1	36.4	36.2	3.58 ± 0.04

steady increase in Z with increasing temperature. The change in Z over the temperature range studied (0.54) is significantly larger than the maximum error associated with duplicate Z measurements at a given temperature (0.20) and well above the standard deviation of replicate measurements (0.10).

Two other parameters, protein sample concentration and definition of the protein retention time, which could potentially affect the interpretation of the adsorption behavior were investigated through use of the SDM. For isocratic elution, the potential for peak asymmetry or skewness exists and would be expected to increase with increasing protein sample concentration or loading. The effect on Z of calculating k' based on mean retention time or peak maximum was explored at two temperatures at a
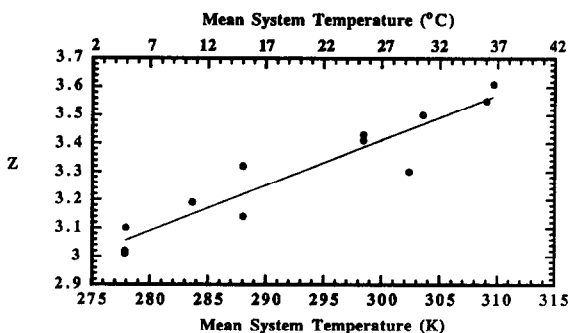

Fig. 4. A plot of Z vs. mean system temperature for cytochrome b_5 for the range of temperatures 4.7 to 36.2°C and an eluent flow-rate of 0.5 ml/min. This plot demonstrates that Z is a function of temperature for this protein-ion exchanger system. The linear least squares fit yields the equation with the standard deviation of the slope and intercept: $y = -1.41 \pm 0.52 + 1.61 \cdot 10^{-2} \pm 1.789 \cdot 10^{-3}\ T$.

nominal eluent flow-rate of 0.5 ml/min. Application of the two methods to data collected at a mean temperature of 30.4°C and an average protein concentration of 0.21 mg/ml yielded Z values of 3.50 and 3.37 for peak maximum and mean retention time analyses, respectively, which compares favorably with the average value of Z obtained from peak maxima for replicate experiments at a mean temperature of 29.8°C of 3.40.

The influence of protein loading was determined by comparison of Z values obtained from retention data at 25.1°C and at a higher average protein concentration, 0.54 mg/ml, which yielded values of Z of 3.45 (by peak maximum) and 3.44 (by mean retention time). The skewness would be expected to be even larger for this higher protein concentration. Little effect of skewness on calculated Z values was observed, however, possibly because the amount of protein loaded even at the higher protein concentration is significantly less than the capacity of the Mono Q HR 5/5 column. As applied to the results of this study, the two methods of data analysis give indistinguishable results, independent of temperature and protein concentration. Therefore, the remainder of the data are presented based on peak maxima.

As described above, an estimate of the enthalpy of adsorption, ΔH_{ads}, can be obtained from the approximate Van 't Hoff plot for each NaCl concentration. Representative analyses for given ionic strengths are presented in Fig. 5, which clearly indicate the strong dependence of k' on temperature. Average values for ΔH_{ads} from duplicate experiments as a function of ionic strength are presented in Fig. 6.

DISCUSSION

In this work the anion-exchange adsorption of cytochrome b_5 has been investigated by measuring the effects of temperature, eluent flow-rate and ionic strength on retention in isocratic HPLC. The extent of approximation of adsorption equilibria by HPLC in the system was defined, allowing for application of the SDM. The influence of temperature on the SDM parameter Z could be observed, and an approxi-
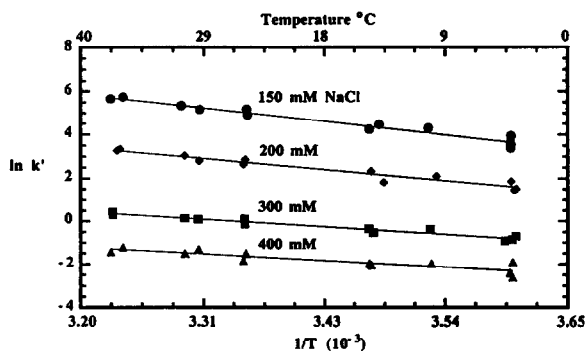
Fig. 5. Approximate Van 't Hoff plot of ln k' vs. $1/T$ for the range of NaCl concentrations studied. The slope of the least squares linear fit for a given NaCl concentration is equal to $-\Delta H_{ads}/R$. Linear regression results are as follows:

[NaCl] (mM)	ΔH_{ads} (kcal/mol)	Intercept	R
150	10.56 ± 0.36	23.56 ± 1.24	0.979
200	8.84 ± 0.36	17.96 ± 1.25	0.969
300	6.18 ± 0.25	10.57 ± 0.84	0.971
400	5.22 ± 0.44	7.46 ± 1.52	0.889

mate Van 't Hoff analysis allowed determination of ΔH_{ads} as a function of ionic strength.

Previous investigators have described the importance of satisfying the various requirements for a given chromatographic model. Several models [31–34] have been proposed to describe the criteria for equilibrium in HPLC ion-exchange and to account for the non-equilibrium behavior observed in some cases. The microscopic sub-processes involved in ion-exchange chromatography have been described by Norde [35]; the macroscopic effects of these sub-proces-
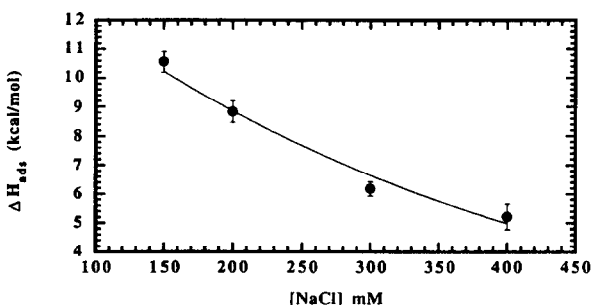

Fig. 6. Plot of the average ΔH_{ads} calculated from approximate Van 't Hoff analyses versus eluent NaCl concentration. The plot illustrates the decrease in enthalpy of adsorption with increasing NaCl concentration.

ses are most commonly manifested as variations in retention.

The best-characterized influence on the chromatographic capacity factor k' is that of ionic strength, as presented in Fig. 1. Our results are in accord with those of previous investigators in that a plot of log k' vs. log $(1/[NaCl])$ (Fig. 2) defined a straight line, supporting the application of the SDM to our data set.

Once the dominant effect of ionic strength on retention had been assessed, the influences of flow-rate and of temperature were considered. The value of Z in this experimental system was found to be essentially independent of flow-rate between 0.2 to 1.0 ml/min, as presented in Fig. 3. The average value of Z obtained over the flow-rates examined, 3.41 ± 0.09 is in close agreement with the value of 2.79 ± 0.22 obtained from batch equilibrium adsorption experiments [10]. The value obtained at 0.1 ml/min, the lowest flow-rate examined, was 3.22 ± 0.19 and was in even closer agreement with the equilibrium result.

The direct comparability of the batch and HPLC results may be reduced by differences in ionic strength and contact time. The batch experiments employ ionic strengths of 100 to 175 mM, and have been found to reach adsorption equilibrium in less than 60 min. In HPLC, the retention times for cytochrome b_5 at 0.5 ml/min ranged from approximately 2 min to greater than 300 min over the ionic strength range 400 to 150 mM, and extended to over 900 min at 0.1 ml/min. As discussed below, extended retention times may result in conformational changes in the protein, particularly at evaluated temperatures. The agreement of the HPLC data with the results of batch equilibrium adsorption experiments, along with the relative independence of Z of flow-rate, indicates that equilibrium behavior could be approximated by the HPLC system.

The value of Z was found to depend significantly on temperature, increasing from 3.04 ± 0.05 to 3.58 ± 0.04 as the temperature was varied from 4.7 to 36.2°C (Fig. 4). This increase in Z suggests the creation of a larger number of contacts between the protein and the adsorbent surface at higher temperatures. This may arise from a more efficient optimization of the orienta-

tion and/or distribution of protein molecules on the adsorbent sites, from changes in the character of the polymeric adsorbent surface at higher temperatures, or from changes in protein structure as a function of temperature or time in the adsorbed state. The change with temperature of the activity coefficients of the counterions and coions and hydration waters on the ion exchange and protein surfaces could also be a potential explanation for the observed dependence of Z on temperature. Any hydrophobic contributions to retention would also be expected to increase with temperature, possibly increasing the extent of any conformational rearrangements. The influence of these phenomena may be enhanced by the stronger retention, and hence longer contact times, observed at higher temperatures.

Previous investigators have ascribed abrupt changes in Z as a function of ionic strength to changes in protein conformation [5,23], and non-linear Van 't Hoff plots have been reported for reverse-phase chromatography [36], but there has been no previous report of a variation of Z in a protein ion-exchange system with temperature.

Comparison of the R_Z (ratio of absorbance at 405 nm to that at 280 nm) of the eluted protein with that of the loaded protein ($R_Z = 5.6$) revealed no significant change after chromatography at 25°C, but R_Z changed and multiple peaks were observed at 280 nm at 37°C, providing further evidence for a possible conformational change of the eluted protein. This result was in accord with the reduced recoveries observed at 37°C at 150 and 175 mM NaCl, which could be a result of changes in conformation as a function of time in the adsorbed state. Additional evidence for a contact time-dependent change in protein conformation is provided by the fact that doubling the eluent flow-rate at 37°C at 150 and 175 mM NaCl, resulting in reduced contact times, improved recovery with similar values of k'.

While cytochrome b_5 is stable in solution to temperatures well above those studied here [37], adsorption is known to reduce protein stability in some cases. These previous examples involved mixed-mode adsorption of lysozyme on silica [26], and the adsorption of γ-crystallins on sur-

faces of increasing hydrophobicity [25]; such a result has not previously been reported for an ion-exchange system.

The study of adsorption thermodynamics through HPLC has previously been investigated [29,36,38,39], although results for protein and peptide systems are scarce. Hancock et al. [36] observed a linear dependence of ln k' on reciprocal absolute temperature in reversed-phase chromatography of a lipid associating peptide, but a non-linear dependence for insulin, and other authors have noted a strong dependence of k' on temperature in modes other than ion-exchange [38–41]. Takeuchi et al. [29] noted a linear dependence of ln k' on reciprocal temperature for cation-exchange HPLC of adenosine and cytidine in derivatized microcapillary columns at pH 3.5.

The present work involved the determination of the enthalpy of adsorption of the protein in an isocratic anion-exchange system which had been verified to approximate equilibrium at the flow-rates employed. An entropic driving force for adsorption is implied by the fact that protein adsorption is a spontaneous process (negative ΔG_{ads}), and the Van 't Hoff enthalpy of adsorption ΔH_{ads} is positive for all NaCl concentrations tested. The same conclusion has been reached from the results of batch adsorption experiments on this system [10].

Enthalpies of adsorption were observed to decrease significantly with increasing ionic strength. There are several possible explanations for this behavior, and it is not possible to select among the potential explanations with the limited data available. The possible explanations include changes in the nature of the adsorbent surface, protein conformational changes, and activity effects. The mobility, packing and flexibility of the adsorbent's charged polymer chains would be expected to vary with ionic strength, perhaps changing the nature of their interactions with adsorbed protein molecules. Slight conformational change may be induced in protein molecules strongly bound at low ionic strengths, resulting in larger enthalpies of adsorption. The activity effect arises from the fact that counterions and waters of hydration can have different activity coefficients in the bulk solution than

when associated with the charged surfaces of the protein and ion exchanger [24]. Changes in counterion activities (and in the activities of the associated waters of hydration) upon protein adsorption results from a dilution effect associated with the release of the counterions from the surface into the bulk [42]. The observed decrease in the enthalpy of adsorption with increasing NaCl concentration is potentially consistent with a decrease in the dilution effect at higher ionic strength, potentially resulting in a reduced contribution to ΔH_{ads} with increased ionic strength.

While there are no directly comparable thermodynamic results for protein ion exchange available in the literature, data on related protein/polyelectrolyte systems may aid in interpretation of the present results. Koutsoukos *et al.* [43] inferred an entropic driving force from data on adsorption of human serum albumin to hematite. The hematite surface is relatively hydrophilic, but adsorption on hematite is not mediated solely by ion exchange, particularly near the protein's isoelectric point.

Record *et al.* [42] have reviewed the extensive literature on the involvement of counterions and waters of hydration in the association of proteins with nucleic acids. In cases of sequence-independent binding, in which the primary interactions of the protein with the nucleic acid are mediated by the backbone charges, this phenomenon which may well be comparable to ion exchange adsorption of proteins. In these systems, the release of counterions and waters of hydration upon protein binding provides a significant entropic driving force. Based on the arguments summarized by Record *et al.*, the release of counterions and waters of hydration upon the binding of cytochrome b_5 to Mono Q could be the primary mechanistic driving force for adsorption. The discussion by Fraaije and Lyklema [44] of the binding of ions by proteins suggests that the electrolyte binds preferentially in the double layer associated with the protein surface. In the double layer formulation, overlap of the double layers associated with the charged ion exchange surface and the protein surface upon binding would result in release of counterions, further supporting the arguments of Record *et al.* [42]. The thermodynamic manifestation of these sub-

processes would be an entropic driving force arising from the increased degrees of freedom associated with the liberated ions and water molecules, in agreement with our observations.

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