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Microcalorimetric characterization of the anion-exchange adsorption of recombinant cytochrome b_5 and its surface-charge mutants

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Abstract

The adsorption of recombinant soluble tryptic fragment of rat cytochrome b_5 on the strong anion exchanger Mono Q was studied using isothermal titration calorimetry and differential scanning calorimetry (DSC). Titration calorimetry results obtained at low levels of adsorbed protein show increasingly endothermic (unfavorable) enthalpies of binding with increasing surface coverage, confirming the heterogeneous nature of binding. The enthalpy of adsorption declines toward zero at higher loadings. At low surface coverage, enthalpies increase linearly with temperature, giving rise to a positive value of ΔC_p. Enthalpies of adsorption depend strongly on the history of the adsorbent. DSC is used to show that cytochrome b_5 is stable in both free and adsorbed states at all temperatures used in the titration calorimetric experiments. Site-directed mutants of recombinant cytochrome b_5 carrying single charge-neutralizing substitutions are used to test the contributions of particular residues to the thermodynamics of adsorption. Like those derived from van 't Hoff analysis of equilibrium adsorption isotherms and HPLC retention data, calorimetric enthalpies of adsorption are positive, confirming the dominant role of entropic effects in ion-exchange adsorption in this system.

1. Introduction

The adsorption of proteins at solid–liquid interfaces has been the subject of active interest because of its biomedical and technological importance [1,2]. Much attention has been focused on protein adsorption on hydrophobic surfaces [3–13], particularly those used in biomedical applications [1,2]. It is well established that protein molecules often undergo irreversible adsorption on hydrophobic surfaces, generally associated with conformational changes in the protein upon binding. The degree of conformational change increases with increasing hydrophobicity of the adsorbent and the protein [13–17].

The interactions of proteins with charged hydrophilic surfaces (e.g., ion-exchange adsorbents) have been less well characterized on a fundamental level. There is an extensive body of

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literature concerning the use of hydrophilic polyelectrolyte surfaces in the chromatographic separation of proteins, but equilibrium studies of the thermodynamics of protein adsorption on chromatographic adsorbents are uncommon. The resolution with which variations in thermodynamic behavior as a function of protein loading and temperature can be detected by batch equilibrium experiments is limited, and the indirect method of van 't Hoff analysis may be confounded by the presence of multiple subprocesses associated with adsorption. The recent advent of highly sensitive titration microcalorimeters has opened new opportunities for detailed investigation of the thermodynamics of adsorption.

Microcalorimetry has been previously used to examine the interfacial behavior of two human serum proteins–γ-globulin and fibrinogen–on silica surfaces [18,19], of human serum albumin and bovine pancreatic ribonuclease on negatively charged hydrophobic polystyrene lattices [5–7], and of human serum albumin on hematite [20]. Reported enthalpies of adsorption were negative for γ-globulin and fibrinogen (indicative of enthalpically promoted adsorption) and positive for adsorption of human serum albumin and bovine pancreatic ribonuclease on polystyrene and hematite (entropically driven adsorption). To our knowledge, only one report other than the present contains calorimetric data for protein adsorption on ion-exchange surfaces [21]. This work reported enthalpies of adsorption of bovine serum albumin on two kinds of strong anion exchangers–a cellulose-based fibrous material and a polyethyleneimine-based macroporous resin. Values of adsorption enthalpies were mostly positive (entropically driven adsorption) for the former and mostly negative (enthalpically driven adsorption) for the latter. In addition, the authors reported a characteristic, surface coverage dependent change in adsorption enthalpies which was also observed in the present work.

Our ongoing interest has been in characterizing the structural and thermodynamic contributions to adsorption of the well-characterized recombinant soluble tryptic fragment of rat cytochrome b_5 on the monodisperse, hydrophilic quaternary amine-based strong anion exchanger, Mono Q. To this end, we have used site-directed surface-charge mutants and titration calorimetry as mutually complementary strategies. Our previous work on the cytochrome b_5–Mono Q system [22–24] has established that adsorption on Mono Q is reversible (through exchange) and that batch and HPLC van 't Hoff enthalpies of adsorption are positive. Scatchard analyses of adsorption isotherms established that adsorption was heterogeneous, with the apparent adsorption affinity declining with protein loading. This result implied that enthalpies derived from van 't Hoff analysis of adsorption data might represent averages over a range of enthalpies associated with adsorption at different binding sites or different binding orientations. In the present work, we describe the use of isothermal titration microcalorimetry for direct quantitation of the enthalpy of adsorption at specific temperatures, as a function of loading. We complement these measurements with studies involving site-directed charge mutants of cytochrome b_5.

2. Experimental

2.1. Sample preparation

A synthetic gene directing the expression of the soluble tryptic fragment of rat cytochrome b_5 in *Escherichia coli* was synthesized by von Bodman et al. [25]. Bacteria were grown in batches of 5 l, as described previously [26]. Cytochrome b_5 and its mutants were purified from *E. coli* lysates by ion-exchange and size exclusion chromatography, and were characterized as homogeneous preparations using SDS-PAGE, spectrophotometry, and HPLC as described previously [22–24]. Protein samples were concentrated to 5–10 mg/ml using Centriprep YM 10 cartridges (Amicon) in a Beckman J2-21 centrifuge (Beckman Instruments) at 3800 *g* at 4°C, in two steps of one hour each, using the same cartridge.

Mono Q (Pharmacia, average particle size 10 ± 0.1 μm, pore diameter approximately 1000 Å) is based on a hydrophobic, crosslinked, styrene divinyl benzene base derivatized with a

hydrophilic layer bearing quaternary amine groups. Mono Q used in this work was the generous gift of Prof. J.-C. Janson of Pharmacia LKB, and was supplied as a suspension in a 20% ethanol–water solution with sulfate as the counterion. The adsorbent was first equilibrated with 1 *M* NaCl to allow exchange of chloride for sulfate counterions, and then washed five times (15 min each at room temperature) with 10 m*M* Tris, pH 8.0 + 0.1 m*M* EDTA. The completeness of equilibration by this procedure was confirmed by a control experiment in which the conductivity of the supernatant liquid was found to be equal to that of 10 m*M* Tris buffer. Equilibrium adsorption isotherms for wild-type cytochrome b_5 on fresh Mono Q were measured as described previously [23] at 25°C, 10 m*M* Tris + 0.1 m*M* EDTA, pH 8.0. Mass balances calculated for each experiment routinely closed with recoveries of 90% or more, except where noted.

For calorimetric experiments the Mono Q bead density was adjusted to $5 \cdot 10^6$ ml^{-1} using a hemacytometer [23]. This adsorbent concentration was found to maintain a high signal-to-noise ratio (> 100) in the experiments. Careful sample preparation was required to eliminate any mismatch in pH or buffer concentration between the protein and Mono Q samples, both of which were adjusted to pH 8.0 at the experimental temperature. Small pH mismatches could potentially produce dilution or buffer titration enthalpy artifacts. The concentrated protein sample and the Mono Q suspension used in each experiment were codialyzed at 4°C against the same 10 l reservoir of 10 m*M* Tris + 0.1 m*M* EDTA, pH 8.0 buffer in separate dialysis tubes (molecular mass cutoff of 7000; Spectrum Industries) with constant stirring for at least 16 h.

2.2. Adsorption isotherm measurement

Equilibrium adsorption isotherms were measured as described previously [22]. Experiments were performed at a density of 10^7 ml^{-1} to match the Mono Q concentration used in the titration calorimetry experiments described below. Isotherms were measured using fresh Mono Q.

2.3. Isothermal titration calorimetry

Calorimetric measurements were made on an Omega titration microcalorimeter (Microcal) interfaced with a 386/25 personal computer (IST, OH, USA) and equipped with a Keithley Model 181 nanovolt pre-amplifier. The instrument was protected from electrical noise by an on-line voltage conditioner (Tripp Lite) and a ferroresonant transformer (General Signal). Isothermal titration calorimetry has been reviewed by Friere et al. [27]. The design and operation of the Omega instrument have been described by Wiseman et al. [28]. The calorimeter was connected to a Haake A81 external water bath for temperature stabilization. For the measurements at 25°C, the external bath was set at 21.0°C and the sample cell warmed to 25°C before equilibration was initiated. Temperature was routinely controlled within ± 0.3°C over the course of an experiment. The reaction cell and the loading and injection syringes were rinsed with dialysis buffer prior to filling them with the dialyzed protein or adsorbent. Samples were degassed by warming to room temperature prior to loading, and care was taken not to introduce air bubbles into the cell or the injection syringe. An amount of 2 ml (ca. 10^7) dialyzed Mono Q beads was placed in the reaction cell, which was agitated at 600 rpm to ensure efficient mixing. Protein samples (0.36–0.72 m*M*) were loaded into the 250 μl stirrer–syringe assembly, which was then installed into the calorimeter. A partial vacuum (residual pressure about 100 Torr) was applied to the compartment around the sample and reference cells to eliminate any condensation on the exterior walls of the cells, and the vacuum line was kept sealed until the end of the experiment. The instrument was regularly calibrated by filling the sample cell with buffer alone and measuring the response to standard heat pulses generated by electrical dissipation, as suggested by the manufacturer. Injection of pure buffer into the cell according to the injection schedule used in the experiments resulted in a flat baseline.

Equilibration typically required 30–60 min to reach a baseline rms noise of less than 15 ncal/s (often <10 ncal/s). A 2- or 5-μl pre-injection was made to account for any syringe leakage that might have occurred during equilibration, as recommended by the manufacturer. Power associated with such pre-injections was negligibly small compared to that observed for the experimental injections. For this reason, enthalpies of adsorption were calculated based on the experimental injections alone. Protein samples were titrated onto the adsorbent typically in twelve 10-μl injections, each lasting 15 s. Mono Q was titrated with protein and not vice versa because the high affinity of adsorption in this system would cause the injected beads to be immediately loaded with a significant amount of protein. Incremental addition of limiting amounts of protein allowed study of the surface-coverage dependence of the enthalpy of adsorption. A 5-min interval was allowed between injections for equilibration of the adsorbed protein; this period was more than sufficient for complete return of the peaks to baseline. Data were collected and analyzed using Microcal's OMEGA and ORIGIN algorithms, respectively.

At the end of each experiment, the protein-loaded adsorbent particles were withdrawn from the calorimeter cell and centrifuged at 2700 *g* for 15 min in a centrifuge with a swinging-bucket-type rotor (PR-6000, IEC) to allow the supernatant to be accurately separated from the ion-exchange beads. The adsorbed protein was eluted from the recovered beads by incubation with 2 ml of a 0.5 *M* NaCl solution in 10 m*M* Tris, pH 8.0, followed by centrifugation. This procedure had been shown to give essentially complete recovery of the adsorbed protein, and not to alter its optical properties [23]. The absorbances of the original supernatant and of the liquid containing protein eluted from the beads were measured at 412 nm using a DU-64 spectrophotometer (Beckman). Cytochrome b_5 concentration was calculated using an extinction coefficient of 130 $mM^{-1}\,cm^{-1}$ for the strong Soret band of the oxidized protein at this wavelength [25]. The spectra of protein samples were also measured from 240 to 700 nm. This allowed for detection of any major change in the tertiary structure of the free or the desorbed protein, since the spectral properties of cytochrome b_5 are altered upon unfolding. Mass balances were calculated to control for any loss of protein by proteolysis, denaturation, or adsorption on the calorimeter cell surfaces, and these normally closed within 10%. Eluted protein was discarded at the end of each experiment; the cell was cleaned with 500 ml of an 0.2% solution of the surfactant Tween 20, followed by 2 l of deionized water. When required, the cell was also rinsed with 500 ml 1 *M* NaOH solution to hydrolyze any proteinaceous material deposited in the cell, followed by rinsing with deionized water until the pH returned to neutral.

2.4. Titration calorimetry data analysis

Experimental data were collected as power, P ($\mu cal\,s^{-1}$) vs. time, and were integrated and scaled by the amount of protein adsorbed to give the apparent enthalpy of adsorption, ΔH_{ads} (kcal/mol) associated with each injection. Typically, several hundred micrograms of the protein were injected in 12 equal volumes. Since a negligible amount of protein (<25 μg) was found to remain unbound at the end of each experiment, it was assumed that all protein in each injection was adsorbed. Thus, the total amount of adsorbed protein divided by the number of injections made gave the amount adsorbed per injection. A correction for heat of dilution (ΔH_{dil}) of the protein was calculated from the results of control experiments, identical to the adsorption experiments but with the adsorbent omitted. Corrected heats of adsorption were calculated according to the relation $\Delta H_{ads} = \Delta H_{obs} - \Delta H_{dil}$. The variation in adsorption enthalpies with surface loading necessitated adoption of a standard protocol to obtain average values of ΔH_{ads}, so that data between different experiments could be directly compared. For this reason, it was decided to plot the cumulative heat of adsorption ($\sum[\Delta H_{ads}]_i$, where i is the injection number, $i = 1,2,\ldots,n$) against increasing adsorbed-protein concentration. A linear least squares regression of these data was used to

interpolate an average ΔH_{ads} for 500 μg (0.037 μmol) of protein adsorbed per 10^7 beads. The value of 500 μg per 10^7 beads represents relatively low surface loading, allowing the resulting data to be free from effects of lateral interactions. Also, this level of surface coverage approximates the loadings at which the adsorption isotherm measurements were made, allowing direct comparison with calorimetric results. A value of 13 603 was used as the relative molecular mass of recombinant soluble cytochrome b_5 [25].

2.5. Differential scanning calorimetry

Protein samples were prepared and concentrated as described above. Samples were extensively dialyzed against 10 l of 10 m*M* sodium phosphate, pH 8.0. DSC was performed on an MC-2 (MicroCal) instrument located in the Biochemical Laboratory, Baylor College of Medicine, Houston, TX, USA. The instrument was interfaced with an IBM AT computer running the Microcal DA-2 software package for data acquisition and analysis (for reviews of DSC, refer to Refs. [29,30]). The calorimeter was connected to a recirculating, refrigerated bath for optimal temperature control. Experiments were conducted at scan rates of 0.5°C/min under a nitrogen atmosphere of about 138 kPa to prevent sample outgassing at elevated temperatures. Only heating scans were recorded because irreversible denaturation was expected. As the purpose of these experiments was to establish the thermal stability of cytochrome b_5 under the conditions of the titration calorimetric experiments, no attempt was made to calculate the enthalpies of unfolding.

For DSC of the unbound protein, a baseline scan was first recorded by filling both the reference and the sample cells with dialysis buffer and scanning from 8 to 90°C. No transitions were observed on the baseline. The solution from the sample cell was then replaced with a 10 mg/ml solution of cytochrome b_5 and the scan repeated. For DSC of the bound protein, about 10^7 fresh Mono Q beads were loaded with a large excess (0.75 μmol) of cytochrome b_5 in 10 m*M* sodium phosphate, pH 8.0. After equilibration with gentle agitation for one hour (a period found previously to be sufficient for adsorption equilibration) the supernatant containing unadsorbed protein was removed. Thereafter, the beads were washed once with 10 m*M* phosphate buffer and resuspended in 20% w/v dextran (Sigma, average molecular mass 168 000) to maintain them in suspension over the period of the experiment. For protection of the DSC cell, it was verified in advance that such a solution did not congeal when heated to 100°C. The stability of soluble cytochrome b_5 which had never contacted Mono Q was also measured separately in the presence and absence of 20% dextran. Control scans on Mono Q and dextran alone were also performed.

3. Results

3.1. Adsorption isotherms and Scatchard plots

Equilibrium isotherms for adsorption of wild-type cytochrome b_5 on fresh Mono Q at the low ionic strength used in the calorimeter experiments (10 m*M* Tris + 0.1 m*M* EDTA, pH 8.0) at 25°C are shown in Fig. 1. The different symbols represent the results of independent experiments. The inset emphasizes the high affinity of adsorption, as shown by the close proximity of the initial slope of the isotherm to the *y*-axis

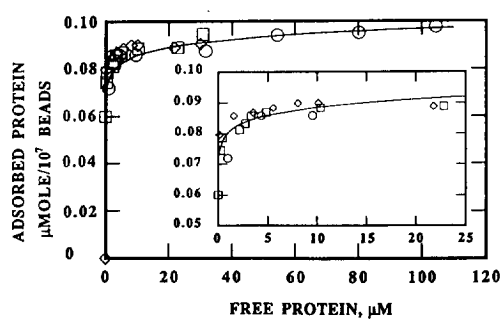

Fig. 1. Isotherms for adsorption of cytochrome b_5 on Mono Q, 25°C in 10m*M* Tris + 0.1 m*M* EDTA, pH 8.0 (three independent measurements). Inset shows data for lower surface coverage. Initial slopes virtually coincide with the *y*-axis depicting the high affinity of adsorption.

even at low free protein concentration. Because of such high affinity, nearly all protein injected into the calorimeter cell is adsorbed. A representative set of isotherm data from Fig. 1 is plotted in Scatchard form in Fig. 2 to illustrate the heterogeneous nature of adsorption. This result is in agreement with our previous observations of heterogeneity of adsorption at higher ionic strengths in this system [23,24].

3.2. Titration calorimetry

Fig. 3 shows isothermal titration calorimetric data for the adsorption of wild-type cytochrome b_5 on fresh Mono Q measured at 25°C in 10 m*M* Tris buffer + 0.1 m*M* EDTA, pH 8.0. The results shown are representative of multiple replicate experiments. Twelve 10-μl injections of cytochrome b_5 (6 mg/ml) were made into a suspension of Mono Q. The top panel in Fig. 3 shows the raw power corresponding to the apparent heats of adsorption (upward peaks) as well as heats of dilution (smaller, downward peaks) derived from separate experiments, depicted as μcal s^{-1} versus time (in minutes). Manual peak-by-peak determination of the resting baseline resulted in a better fit than the automatic estimation provided by the calorimeter software; the two procedures gave similar average results. Integrated heats of adsorption

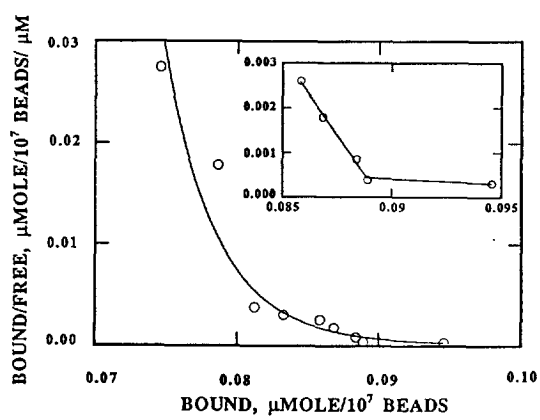

Fig. 2. Scatchard plot of a representative data set from Fig. 1. Concave upward plot suggests negative cooperativity or the presence of heterogeneous binding sites. Inset shows curvature for low surface coverage.

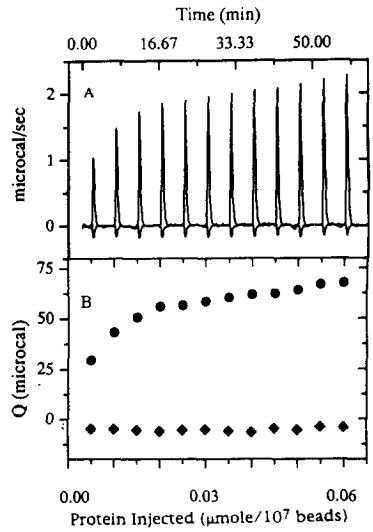

Fig. 3. Titration microcalorimetric data collected on a MicroCal Omega instrument for the adsorption of wild-type cytochrome b_5 on Mono Q at 25°C in 10 m*M* Tris + 0.1 m*M* EDTA, pH 8.0. Twelve 10-μl injections were made into the cell containing a suspension of about 10^7 Mono Q beads in the same buffer. Panel A shows raw data; upward peaks correspond to apparent heats of adsorption, smaller, downward peaks are the heats of dilution measured in a separate experiment in which the adsorbent was omitted. Panel B shows the integrated heats of adsorption (●) and heats of dilution (◆) in microcalories as a function of injection number.

and dilution for each peak are shown in the lower panel of Fig. 3. The kinetics of heat evolution observed in the raw data suggest that adsorption is complete well within the time between injections (5 min). The effect of surface coverage on heat of adsorption was further studied by making a larger number of injections (25); the results obtained are shown in Fig. 4. The amount of protein adsorbed per injection was used to calculate the incremental heat of adsorption as a function of protein adsorbed, by dividing the integrated heats of adsorption by the moles of protein adsorbed in each injection (Fig. 4, lower panel). Because of the heterogeneous nature of the adsorbent surface no attempt was made to extract an equilibrium binding constant (or Gibbs free energy of binding, ΔG_{ads}) from the calorimetric data. In the past, attempts have been made to calculate the ΔG_{ads} from adsorption isotherm measurements [31–33]. Because of

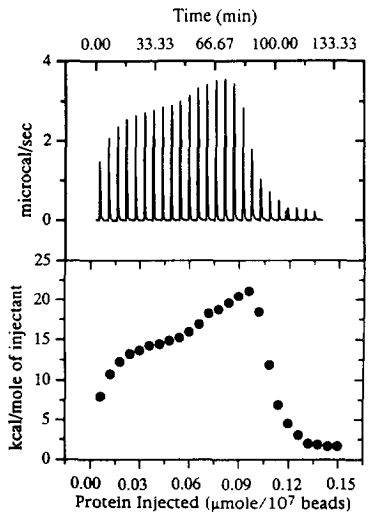

Fig. 4. Titration microcalorimetric data for the adsorption of wild-type cytochrome b_5 on Mono Q at 25°C in 10 m*M* Tris + 0.1 m*M* EDTA, pH 8.0. Twenty-five 10-μl injections of the protein were made into a suspension of about 10^7 Mono Q beads in the same buffer. The top panel shows corrected data for adsorption and the bottom panel shows the integrated heats of adsorption in kcal/mol.

the uncertain validity of such calculations (primarily, lack of assured reversibility of adsorption), numbers derived for ΔG_{ads} through such analyses must be viewed with caution [34].

The majority of the fresh Mono Q used in this study was received as a liquid suspension. The average ΔH_{ads} (to a loading of 500 μg protein/10^7 beads) for this material was 11.4 kcal/mol. We wished to compare the behavior of this material with that of Mono Q recovered from packed FPLC columns, which was used in our previous work [23,24]. The ΔH_{ads} value obtained with Mono Q extracted from a fresh FPLC column and prepared as described above was 10.4 kcal/mol, in reasonable agreement with the material received unpacked. Practical use of ion-exchange adsorbents involves many cycles of adsorption and regeneration, which could affect binding characteristics. We therefore examined the enthalpy of adsorption of cytochrome b_5 onto Mono Q that had been regenerated after isotherm measurements in which the adsorbent was loaded to 20–25% of its capacity [23] with cytochrome b_5. Mono Q was regenerated by extensive elution with 1 *M* NaCl until the supernatant A_{280} fell to values approximately equal to that of the buffer alone ($\leq$0.05 AU), and then used in calorimetric experiments under conditions identical to those of Fig. 4. Adsorption of wild-type cytochrome b_5 on regenerated Mono Q gave an enthalpy of adsorption of only 0.3 kcal/mol (up to 500 μg/10^7 beads) at 25°C in 10 m*M* Tris, pH 8.0, well below the values obtained with the two types of fresh Mono Q. In the presence of high ionic strength ($>$200 m*M* NaCl), no detectable adsorption or enthalpic signal was observed for fresh or regenerated Mono Q. These results suggest that microcalorimetry may offer a particularly sensitive method of characterizing the surface properties of chromatographic media, as regenerated columns give similar chromatographic separation [35]. It is likely that traces of uneluted protein remain after regeneration, and/or that cycles of loading and elution change the character of the ion-exchange surface on Mono Q. This question is the subject of further investigation. For the present work, fresh Mono Q was used in all experiments reported hereafter.

As a check for potential contributions of buffer titration enthalpy to the observed heats of adsorption, a control experiment was performed with the use of sodium phosphate buffer instead of Tris. The heat of ionization (ΔH_{ion}) of sodium phosphate buffer at 25°C ($\Delta H_{ion} = 1.22$ kcal/mol) is one-tenth that of Tris ($\Delta H_{ion} = 11.51$ kcal/mol; Ref. [36]). The average value obtained for ΔH_{ads} in phosphate buffer was 0.5 kcal/mol, in agreement with results using Tris, allaying any concern of a buffer contribution to the observed enthalpies of adsorption. This result also establishes that the adsorption of cytochrome b_5 is not accompanied by significant proton uptake or liberation.

Experiments were also performed at 12 and 37°C to examine the temperature dependence of the adsorption enthalpy (Fig. 5). These experiments were conducted in 10 m*M* Tris adjusted to pH 8.0 at the experimental temperature. All samples were pre-equilibrated to the experimental temperature to allow for rapid equilibration of the calorimeter, and to avoid degassing of samples as temperature increased. Experiments

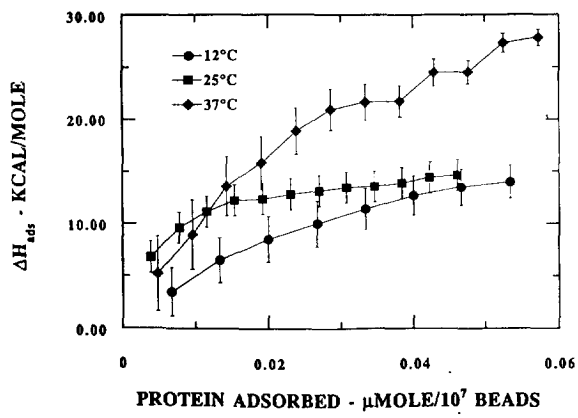

Fig. 5. Enthalpies of adsorption of cytochrome b_5 on Mono Q as a function of extent of surface coverage, at three different temperatures.

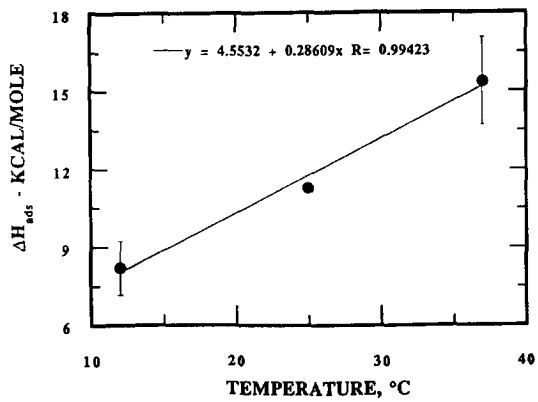

Fig. 6. ΔH_{ads} calculated for a loading of 500 μg protein adsorbed per 10^7 beads (see text for details) as a function of temperature, derived from averages of duplicate experiments. Line represents linear least squares fit with slope corresponding to $\Delta C_p = 286 \pm 27$ cal $°C^{-1}$ mol^{-1}.

at subambient conditions required cooling of the reaction cell by an external water bath to well below the desired temperature combined with calorimeter-controlled warming. For this reason, the external bath circulator was set at 2.0°C for the 12°C experiment and 37.0°C for the 37°C experiment. For accurate quantitation of the extent of adsorption, the contents of the sample cell were recovered at the end of each experiment and promptly centrifuged to allow assessment of the extent of adsorption under the experimental conditions. These experiments were conducted under conditions of low surface coverage to allow for comparison with van 't Hoff enthalpies derived from measurements at similar loadings. Average ΔH_{ads} up to 500 μg of protein adsorbed per 10^7 Mono Q beads (0.037 μmol/10^7 beads) are plotted in Fig. 6. These values increase linearly with temperature from 12 to 37°C, giving a correlation coefficient of 0.99 and a positive ΔC_p at this loading of 286 ± 27 cal mol^{-1} $°C^{-1}$ (Fig. 6). It is clear from the results presented above, however, that the magnitude (and even sign) of ΔC_p depends of the extent of loading. At higher loadings the dependence of ΔH_{ads} on temperature is no longer linear, suggesting a more complex relationship between the two.

Finally, enthalpies of adsorption were measured for three site-directed mutants of cytochrome b_5 with single amino-acid substitutions on different regions of the protein surface. We have previously identified [24] a preferred chromatographic contact region for the anion-exchange adsorption of cytochrome b_5 based on the pronounced effect of mutations in the dominant cluster of negatively charged groups on the protein surface (Glu 41, 47, 48, and 52) on the stoichiometry and affinity of binding. Mutant E48Q belongs to the preferred cluster of negative groups, D64N lies in the negatively charged domain opposite the E48Q cluster, and E15Q lies away from the dominant negative regions around the heme-binding cleft. Data for the enthalpies of adsorption as a function of surface coverage for the wild-type protein along with these three mutant forms are shown in Fig. 7.

3.3. Differential scanning calorimetry

The DSC thermograms of phosphate buffer or phosphate-buffered 20% dextran solution containing Mono Q alone were featureless. DSC of soluble wild-type cytochrome b_5 in phosphate buffer produced a single, rather broad transition around 75°C, while adsorption of cytochrome b_5 to Mono Q in the presence of 20% dextran reduced the protein's transition temperature to about 50°C (Fig. 8). In the presence of a phosphate-buffered 20% dextran solution, DSC of

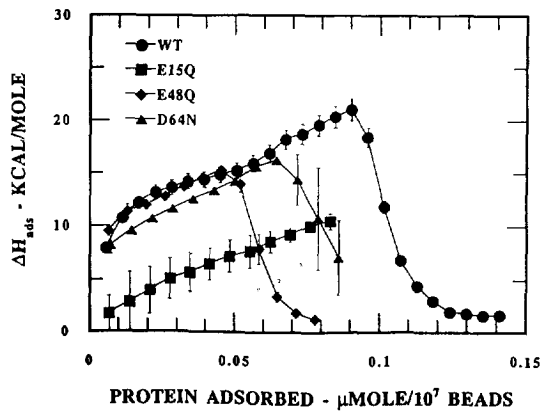

Fig. 7. ΔH_{ads} for adsorption of mutant forms of cytochrome b_5 listed in Table 1 onto Mono Q at 25°C in 10 m*M* Tris + 0.1 m*M* EDTA, pH 8.0. WT represents wild-type cytochrome b_5, E15Q represents the mutation Glu15→Gln, E48Q the mutation Glu48→Gln, and D64N represents Asp64→Asn.

soluble cytochrome b_5 produced a major transition at 78°C. The need to include dextran in the DSC samples and its apparent effect on cytochrome b_5 stability prevents the DSC measurements from being conducted under conditions completely representative of those of the titration calorimetric experiments, but in all cases cytochrome b_5 is stable to temperatures significantly above the highest temperature used in our

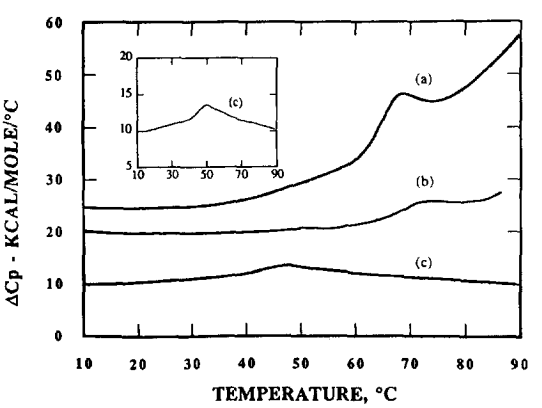

Fig. 8. Differential scanning calorimetric data for (a) free cytochrome b_5 in 10 m*M* phosphate buffer, (b) free cytochrome b_5 in 10 m*M* phosphate buffer + 20% dextran, and (c) cytochrome b_5 bound to Mono Q in the presence of 10 m*M* phosphate buffer + 20% dextran. Measurements were made on a MicroCal MC-2 scanning calorimeter using scan rates of 30°C/h. Inset shows the thermal transition for the bound protein (T_m ca. 50°C) in greater detail.

adsorption studies (37°C). It should also be noted that mass balances on the free and adsorbed protein after titration calorimetry closed within 2% for experiments at 12 and 25°C, and within 18% for experiments at 37°C, implying that significant irreversible denaturation may have occurred only at 37°C or above. Denaturation under such conditions could make some contribution to the observed enthalpy of adsorption. Reduced recoveries of cytochrome b_5 bound to Mono Q under conditions of low ionic strengths and high retention times have also been observed in our independent HPLC studies [22]. Further investigation of these issues is in progress.

4. Discussion

The high affinity of the adsorption isotherm even at significant loadings implies that ΔG_{ads} is negative under the conditions used. The heterogeneous nature of the adsorbent surface and the ill-characterized surface-charge density and distribution, however, preclude calculation of a meaningful value of ΔG_{ads} [34]. Furthermore, the van 't Hoff analysis often used for calculation of ΔH_{ads} is based on the assumption that a reversible equilibrium exists between the bound and the free protein. It lumps together all subprocesses accompanying protein adsorption and is therefore only a qualitative indicator of adsorption thermodynamics. Direct microcalorimetry does not resolve the difficulties in estimating ΔG_{ads}, but does allow for a more robust quantitation of the enthalpy of adsorption than is achievable by van 't Hoff analysis. It also allows for examination of the potential variation of ΔH_{ads} with loading and with temperature in a more direct manner than is possible by any other means.

Microcalorimetric characterization of the adsorption of cytochrome b_5 on fresh Mono Q shows endothermic heats of adsorption which, at low loadings, increase with the amount of protein adsorbed (Fig. 3). Heats of dilution, in contrast, are constant, exothermic, and much smaller (about −1 kcal/mol). Calorimetric mea-

surements made at higher surface coverages (Fig. 4) show rapidly declining enthalpies of adsorption after the initial linear increase. A transition in adsorption enthalpies occurs at a loading of about 0.09 μmol/10^7 beads, closely corresponding to the higher-slope region of the adsorption isotherms (Fig. 1). However, mass balances performed after the last injection show that of the total protein recovered from the calorimeter cell, >95% is in the bound state. It is noteworthy that the adsorption enthalpy has a uniformly positive sign, implying an entropic driving force under these conditions. Positive values of ΔH_{ads} have also been calculated for this system from the results of HPLC and equilibrium adsorption experiments, as discussed below. The large increase in entropy may arise from liberation of bound ions from the adsorbent and protein surfaces, as well as waters of hydration from both surfaces.

Between 12 and 37°C, the average ΔH_{ads} (to 500 μg/10^7 beads) increases from about 8 kcal/mol to 15 kcal/mol, giving ΔC_p ($= \partial(\Delta H_{ads}/\partial T)_p$) equal to 286 ± 27 cal mol^{-1} $°C^{-1}$, relatively independent of temperature ($r = 0.99$). A classical enthalpy–entropy compensation effect [37,38] does not seem to be active as both ΔG_{ads} [22–24] and ΔH_{ads} increase with temperature over the range of interest. Positive ΔC_p values are usually attributed to the transfer of nonpolar moieties from a hydrophobic to a more polar environment [39]. Also, the expected sign of ΔC_p for an increase in exposure of electrostatic charges is negative, implying that for the reverse case (creation of electrostatic links, as in ion-exchange adsorption) ΔC_p might be expected to be positive [40]. This is in agreement with arguments made by Norde [34] that in aqueous media changes in ΔC_p are largely due to changes in water structure around the interacting surfaces and transfer of ions from the binding interface to the bulk solvent.

The sign of ΔC_p, however, depends on the conditions under which it is measured. Van 't Hoff analyses of the equilibrium adsorption of cytochrome b_5 gave enthalpies of +3.8 kcal/mol at 4–25°C and −0.7 kcal/mol at 25–37°C [23]. The overall change in ΔH_{ads} from 4 to 37°C corresponds to a negative average ΔC_p. In contrast, for HPLC, a small positive value of ΔC_p is implied by the linear van 't Hoff plots observed over temperatures ranging from 4 to 37°C [22]. A similar observation was made by Sigursjold and Bundle [41], who reported opposing signs of ΔC_p measured by van 't Hoff methods and by titration calorimetry. This difference was ascribed to factors such as release or uptake of ions, conformational change, or change in solvation upon association. As discussed above, the possibility of such subprocesses in the adsorption of cytochrome b_5 on Mono Q exists. It should also be noted that the characteristic times of the experimental methods differ significantly. Titration calorimetry reports the enthalpy of processes occurring on a time scale of 1–5 min, equilibrium adsorption experiments occupy 30–60 min, and isocratic HPLC retention times can range up to 900 min. The different protein loadings involved can also impair comparison of results obtained by different methods. The influence of loading on thermodynamic parameters is clearly illustrated in Figs. 4 and 5.

Both the bound and the free protein appear to be stable over the entire temperature range used in the titration calorimetry experiments, as shown by the absence of DSC transitions at these temperatures, the closure of mass balances for each experiment, and the results of previous work [42]. Although the observed destabilization upon adsorption requires further investigation, the present results suggest that the observed positive ΔC_p is not primarily due to a conformational-change dependent entropy increase. It may arise both from liberation of waters of hydration at elevated temperature and from partial exposure of buried hydrophobic side chains.

The enthalpy of adsorption varies quite strongly with the history of the adsorbent, although fresh and regenerated Mono Q produced the same chromatographic behavior. Adsorption measurements described in Ref. [23] show that the Z number (apparent number of contacts made by the protein with the adsorbent [48]) is virtually identical for fresh and regenerated Mono Q, and the apparent affinity constant (K)

is 5- to 10-fold higher for the fresh Mono Q. Titration calorimetry in 10 m*M* Tris buffer at 25°C gave ΔH_{ads} values of 11.4 and 0.3 kcal/mol for fresh and regenerated Mono Q, respectively. Van 't Hoff analysis of batch adsorption data (in 100 m*M* NaCl at 4–25°C) gave 9.0 kcal/mol for adsorption on the fresh material and 2.9 ± 0.3 kcal/mol for the regenerated material. An HPLC van 't Hoff ΔH_{ads} of 10.6 ± 0.4 kcal/mol was derived from data collected at 4–37°C at 150 m*M* NaCl on relatively fresh Mono Q; the van 't Hoff ΔH_{ads} fell with increasing ionic strength to 5.2 ± 0.4 kcal/mol at 400 m*M* NaCl [22]. Although the reproducibility of the HPLC experiments is excellent, the repeated injection of small amounts of protein relative to the total column capacity makes it uncertain at which point HPLC adsorbents should no longer be regarded as "fresh". Another unique characteristic of the HPLC experiments is that their duration varies significantly with ionic strength, potentially allowing for greater energetic contributions from slower processes such as conformational rearrangement. Despite the differences among the experimental methods, all give a larger value of ΔH_{ads} for fresh than for regenerated Mono Q. The difference between the calorimetric and the van 't Hoff enthalpies is appreciable, nonetheless it is noteworthy that both methods confirm the entropic driving force under these conditions.

The calorimetric behavior of surface-charge mutants of cytochrome b_5 does not correlate in a direct way with their ion-exchange chromatographic properties. As described elsewhere, equilibrium adsorption and HPLC retention of the protein are dominated by a cluster of negatively charged residues lying around the heme-binding cleft, shown in Fig. 9 [24,53]. Structurally conservative mutations neutralizing charged amino acids in these clusters exert far greater influence on the affinity and apparent number of protein/adsorbent contacts than do mutations on the opposite end of the protein. There is independent 2D NMR evidence that the mutation does not induce large conformational changes in several of the proteins of interest [49]. The calorimetric behavior of the mutant proteins,

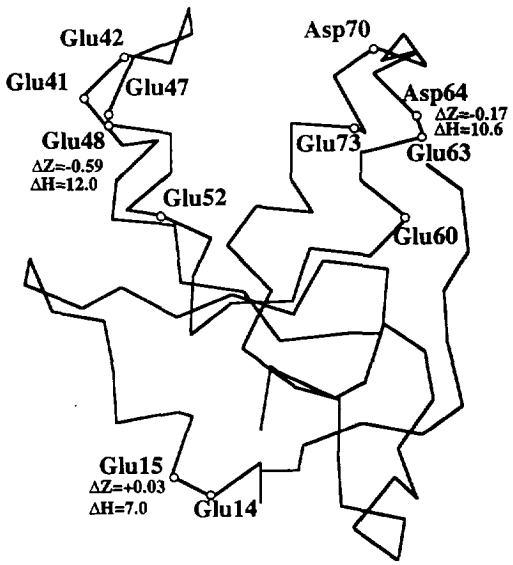

Fig. 9. Three-dimensional representation of tertiary structure of rat cytochrome b_5 [52] is shown; the C_α backbone atoms are connected by pseudo-bonds. Acidic groups which form clusters of negative charge are shown on either side of the heme-binding cleft (open pocket in the top-center of the diagram). The positions of the mutations calorimetrically characterized in this study are shown along with values of ΔH_{ads} and ΔZ, the change in the apparent number of contacts relative to the wild-type cytochrome b_5.

however, does not reflect their retention behavior. As shown in Table 1 and Fig. 9, proteins altered in residues belonging to the dominant charge cluster (e.g., E48Q) show enthalpies of adsorption quite similar to that of the wild-type protein. Mutant E15Q, which is very little altered in its adsorption and HPLC retention behavior, shows a very different pattern of enthalpy of adsorption as a function of loading. This observation is particularly striking in view of the fact that calorimetric experiments were conducted at low ionic strength at which batch adsorption and HPLC retention differences among the mutants are greatest. This result suggests that the large enthalpies of adsorption on fresh adsorbent arise from different sources than do the lower (near-zero) enthalpies associated with adsorption on regenerated adsorbent.

One source of enthalpy which may be unique to fresh adsorbent is denaturation on high-affinity sites produced by local clusters of adsorbent

Table 1
Values of ΔH_{ads} at 25°C in 10 m*M* Tris, pH 8.0, for mutant forms of cytochrome b_5 calculated for low surface coverage (500 μg protein/10^7 beads)

Protein	Q^{net}	Z^{Batch}	Z^{HPLC}	K^{Batch}	k'^{HPLC}	ΔH_{ads} (kcal/mol)
Wild type	−9.5	2.92 ± 0.32	3.42 ± 0.01	0.081 ± 0.002	155.6 ± 21.2	11.4 ± 0.2
E48Q	−8.5	2.33 ± 0.34	2.76 ± 0.07	0.020 ± 0.000	40.1 ± 1.7	12.1 ± 0.3
D64N	−8.5	2.75 ± 0.33	ND	0.051 ± 0.000	ND	10.6 ± 0.2
E15Q	−8.5	2.95 ± 0.25	3.37 ± 0.04	0.100 ± 0.002	132.8 ± 5.5	6.9 ± 0.9

Q^{net} is the net charge of the proteins [51]. *Z* and *K* values are from Refs. [24,53]. Reported values of K^{Batch} were measured at 100 m*M* NaCl and those of k'^{HPLC} were measured at 150 m*M* NaCl. The superscripts "Batch" and "HPLC" refer to equilibrium batch adsorption and isocratic, isothermal HPLC methods. ND represents not determined. Three-dimensional representation of the positions at which mutations were characterized using microcalorimetry are shown in Fig. 9.

charge, or by hydrophobic patches on the adsorbent surface. The possible role of hydrophobic interactions is supported by the fact that residue 15 lies near the two largest patches of hydrophobic surface on the cytochrome b_5 surface, in a region which we have shown to have a net positive charge [51] otherwise unfavorable for interaction with the cationic adsorbent surface. One hydrophobic patch (337 Å^2; 1.4 Å probe radius using CHARMm v. 20.1, see also Ref. [50]) comprises atoms from residues Ile91, Pro94, and Leu98. Another (323 Å^2) consists of residues Val8, Tyr10, Tyr11, His84, and Pro85. Together these patches comprise 11% of the total solvent-accessible surface area of the molecule and 50% of the hydrophobic surface area. The insensitivity of the enthalpy of adsorption to mutations which strongly affect adsorption and retention, together with insensitive van 't Hoff enthalpies of adsorption previously determined for a larger set of mutants [24], further support our conclusion that enthalpy does not play an important role in the selectivity of anion-exchange adsorption in this system. While the origins of the large enthalpies of adsorption on fresh Mono Q or of their surface-coverage dependence are not yet entirely clear, the calorimetric results support our previous conclusion that anion-exchange adsorption in this system is entropically driven, presumably by liberation of counterions or waters of hydration.

In conclusion, we have shown that titration calorimetry can be used as a sensitive probe to test the energetics of ion-exchange adsorption. We have confirmed in a direct manner our previous conclusion that adsorption in this system is entropically driven. The character of adsorption on fresh and regenerated adsorbent is quite different, in ways which are demonstrated more directly by ITC than by any other available technique.

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