ELSEVIER

Journal of Chromatography A, 691 (1995) 263–271

JOURNAL OF CHROMATOGRAPHY A

High-performance liquid chromatography of amino acids, peptides and proteins CXXXVIII[☆]. Adsorption of horse heart cytochrome *c* onto a tentacle-type cation exchanger

Jianrong Xie, Marie-Isabel Aguilar, Milton T.W. Hearn*

Centre for Bioprocess Technology and Department of Biochemistry, Monash University, Clayton, Victoria 3168, Australia

Abstract

Determination of the change in the Gibb's free energy from the adsorption isotherm associated with the interaction between a biomolecule and an ion-exchange resin is often achieved by assuming that a Langmuirean model prevails. However, the adsorption of horse heart cytochrome *c* onto the tentacle-type cation exchanger LiChrospher 1000 SO_3^- at pH 4.00 showed an isotherm of rectangular form. In this case the Langmuirean model is not applicable. In this paper, we propose an alternative way to deal with this situation, whereby the adsorption capacity of the adsorbent with a defined protein sample is studied as a function of displacing-ion concentration. The experimental conditions over defined ranges are then selected in order to relate this function to the change in the Gibb's free energy for the interaction between the protein and the ion exchanger. Additional comments about the general utility of the on-line adsorption vessel system employed to determine the adsorption isotherms are also made.

1. Introduction

Adsorption isotherms are widely used to study the thermodynamics of the interaction between a biomolecule and an ion exchanger. Interpretation of the molecular basis of these isotherms represents a key requirement in developing a detailed understanding of the adsorption/desorption processes which occur in high-performance ion-exchange chromatography (HPIEC) of proteins and peptides [1–5]. In liquid–solid systems of a non-competitive type, the shape of the isotherm is often assumed to conform to the Langmuir type equation [1]:

$$q^* = \frac{q_m C^*}{K_d + C^*} \quad (1)$$

where q_m is the maximum adsorption capacity of the biomolecule on the ion-exchanger particles (which in most cases does not increase continuously but comes to a limiting value manifested in the plateau), K_d represents the dissociation constant of adsorption equilibrium, and q^* is the adsorption capacity at equilibrium of the biomolecular sample at a concentration C^*. A double reciprocal plot of $1/q^*$ against $1/C^*$ permits q_m and K_d to be evaluated. Moreover, the related thermodynamic parameters, ΔG_{assoc} and ΔH_{assoc} can be obtained by applying the well-known Van 't Hoff equation. In the same

* Corresponding author.

☆ For Part CXXXVII, see Ref. [24].

0021-9673/95/$09.50 © 1995 Elsevier Science B.V. All rights reserved
SSDI 0021-9673(94)00632-6

manner, a quantitative interpretation of the protein–ion-exchange ligand binding can then be achieved in terms of the chromatographic retention parameters (capacity factors) [6,7]. From these procedures information can then be derived on the nature of the protein surface, protein conformation and related interactive processes with ion-exchange ligands [8].

Recently, a novel type of tentacle-type silica-based ion exchanger has been developed by Muller [9], and claimed to provide a better performance and maintenance of biological activity than conventional types of ion exchangers [10,11]. A previous study on the adsorption properties of these so-called tentacular adsorbents has already demonstrated their very high affinity for various proteins [12] with the chromatographic behaviour of biomolecules described mainly in terms of an on–off mechanism. This behaviour means that under certain conditions the adsorption of the biomolecule is independent of sample concentration but depends mainly on the concentration of the displacing ions. The sample components are desorbed and eluted without further interaction if the concentration of the displacing ions exceeds a specific value. Moreover, the isotherm for such an interaction can be predicted to assume a rectangular form in the absence of displacing ions. In this case, the Langmuir model cannot be applied. A critical adsorbate concentration (C_r) for the sample protein must exist in order for this adsorption isotherm to be generated, with no additional adsorption taking place above this critical concentration. However, below this critical concentration the dissociation constant is very small and the assumption of reversible interaction implicit to the Van 't Hoff equation is no longer applicable.

We propose here an alternative way to study this type of more complex adsorption behaviour through variation of the displacing-ion concentration and evaluation of the change in the Gibb's free energy. At low displacing-ion concentrations, e.g. in the range of 0–0.1 M, the adsorption capacity of the target biomolecule onto the ion exchanger is expected to be inversely proportional to the concentration of the displacing ion. This proportionality is directly related to the available binding sites on the ion-exchange ligand(s) and also to the conformational status of the sample protein in solution. Consequently, the study of the change in Gibb's free energy through variation of the displacing-ion concentration represents one approach to study biomolecule conformation and its interaction with the ion-exchange ligand under these circumstances. In this paper, we present an adsorption study of horse heart cytochrome *c* onto a tentacle-type cation exchanger (LiChrospher 1000 SO_3^-) over the temperature range 2–44°C with NaCl as the displacing salt. The results confirm that the change in Gibb's free energy can be derived in this manner for rectangular isotherms such as found with cytochrome *c* and this cation exchanger.

2. Experimental

2.1. Materials and chemicals

Horse heart cytochrome *c* was purchased from Sigma (St. Louis, MO, USA). The tentacle-type cation exchanger LiChrospher 1000 SO_3^- (particle size, 5 μm) was provided by E. Merck (Darmstadt, Germany). Further information about the physical characteristics of the LiChrospher series of ion exchangers can be found in Ref. [13]. The cation-exchanger particles were dried under vacuum before use. Sodium chloride was obtained from Ajax Chemicals (analytical reagent; Australia). Glacial acetic acid ($d = 1.05$ mg/ml at 20°C) was obtained from May & Baker (a Division of Rhone-Poulenc Australia).

An Orion pH meter (Model SA520) calibrated against a standard buffer solution of pH 4.00 was employed to prepare the buffer solutions. A UV–visible spectrophotometer (type 4050, LKB/Biochrom, UK) was used to determine the concentration of cytochrome *c* in solution. The adsorption measurements were carried out as static experiments in a temperature-controlled

incubator (Thermline, Australia) which covers all the experimental temperatures (± 0.1°C).

2.2. Preparation of buffer solution

The experimental buffer solution was freshly prepared each time. Glacial acetic acid (1.50 ml) was added to distilled water (500 ml) to make a solution concentration [HAc] = 0.05 *M*. The pH value of this solution was then adjusted with a 10 *M* NaOH solution to 4.00. The concentration of sodium ions in this final buffer solution was calculated to be 0.0076 *M* which was taken into account as part of the displacing-ion concentration in the experiments.

2.3. Determination of cytochrome c concentration in solution

The cytochrome *c* concentration was determined by the UV–visible detector at wavelength 215 nm. All measurements were carried out at room temperature. The concentration calibration and determination were achieved by comparing the optical absorbance of the sample solution with the initial buffer solution as a blank reference. The linear range of absorbance versus cytochrome *c* concentration in solution was found experimentally to encompass the range 0–0.08 mg/ml. The calibration factor was 15.09 ± 0.24 AU · ml/mg obtained following extensive measurements.

2.4. Adsorption isotherm and adsorption capacity

The adsorption isotherms were obtained with the on-line adsorption vessel system reported previously [1]. In the present study, about 50 mg LiChrospher 1000 SO_3^- ion-exchange particles and 30 ml of the appropriate buffer solution were introduced into the vessel. The porosity of the sintered glass filter used in the vessel was between 1.0–1.7 μm. The cytochrome *c* concentration in the vessel was determined by recycling the supernatant solution directly through the UV–visible detector cell. This solution was then returned to the adsorption vessel by a reciprocal pulse pump. The equilibrium time needed for each addition of cytochrome *c* and its concentration sampling was between 1.5–2.0 h which was found to be sufficient for this high-affinity adsorption process.

The adsorption capacity required for the free energy calculation was measured by a static method. Based on the experimental results, the displacing-salt concentration was kept below 0.1 *M*. Experimental data corresponding to 4–6 concentration points were taken within this range. The concentration of the displacing ion was estimated on the basis of the sum of the added quantity of sodium chloride and the initial concentration of sodium ion in the buffer solution. A precisely known mass of about 10 mg LiChrospher 1000 SO_3^- particles was weighed in a plastic centrifuge tube. The desired amount of sodium chloride in solid form was then introduced into the tube. The cytochrome *c* in the appropriate buffer solution was directly prepared at a concentration of 0.2 mg/ml. According to the adsorption capacity and mass of the adsorbent particles in the tube, an estimated volume (ranged from 8–10 ml in the experiments) of this cytochrome *c* solution was put into each tube. This estimation was made in order to obtain a final supernatant solution after the adsorption, whereby the cytochrome *c* concentration was larger than the critical concentration C_r, but within the linear range of the concentration calibration. After all components were added into the tubes, the tubes were rapidly put into the incubator at the desired temperature. Continuous gentle agitation was started 1 h later for at least 15 h to ensure that the adsorption process took place under the desired temperature conditions. Centrifugation under the same temperature conditions was used to separate the particles from the supernatant. The cytochrome *c* concentration in the supernatant was determined as described above. The adsorbed quantity of cytochrome *c* on the particles was deduced from the total amount of cytochrome *c* added to the tube.

3. Results and discussion

3.1. Theory to evaluate the change in Gibb's free energy

In the case under study, the components in solution can be considered to form a dilute aqueous electrolyte mixture. At equilibrium, the ion-exchange interaction may thus be regarded as a ternary mixture consisting of the biomolecule sample (p), the displacing ions (i) and water (w) associated with the ion-exchange adsorbent. As the relative composition of the ion exchanger changes upon adsorption or desorption of the protein, displacing ions or water content, the variation in the Gibb's free energy will then be given by [14,15] :

$$dG = RT \ln \alpha_p dn_p + RT \ln \alpha_i dn_i + RT \ln \alpha_w dn_w \qquad (2)$$

where α is the adsorbate activity on the ion exchanger, dn is the differential quantity of the adsorbate involved in the process, T is the temperature in K and R is the gas constant. For a system where the adsorption of the biomolecule sample is thermodynamically favoured and the ion-exchange adsorbent becomes saturated (which means the biomolecule sample concentration in the solution is greater than the critical concentration C_r in the case of a rectangular adsorption isotherm), the variation in the Gibb's free energy can be rewritten in the following form if only the change in the concentration of the displacing ion is considered, i.e. the change in the concentration of water is assumed to be very small, approaching zero:

$$dG = -(RT \ln \alpha_p dn_p + RT \ln \alpha_i dn_i) \qquad (3)$$

If it is assumed that the activity coefficients involved are independent of the displacing-ion concentration and if the quantity of displacing ion adsorbed by the ion exchanger is proportional to its concentration in the solution (these are assumptions expected to approximate the condition of a loading buffer with a low displacing-ion concentration), the free energy change brought about by the change in the displacing-ion concentration can be evaluated from the following equation, obtained by integration of Eq. 3 with regard to the ionic concentration:

$$-\Delta G^0 = RT \ln [A - q_m] - RT \ln C_i = RT \ln [(A - q_m)/C_i] \qquad (4)$$

where A is the adsorption capacity in the absence of the displacing ion, and q_m is the maximum adsorption capacity with a displacing-ion concentration C_i. The combined term $(A - q_m)/C_i$ represents the slope of the plot of maximum adsorption capacity q_m against displacing-ion concentration C_i and can be evaluated under defined experimental conditions according to:

$$q_m = A - BC_i \qquad (5)$$

where the slope B is $(A - q_m)/C_i$. The change in the Gibb's free energy at a particular temperature for the protein–ion-exchange interaction can thus be determined for the specific system from the experimental data utilising Eqs. 4 and 5. It can be seen from these equations that the success of the experimental approach depends on the extent of linearity between the adsorption capacity and the displacing-ion concentration.

In order to obtain the relevant thermodynamic parameters, the relationship between the experimentally derived critical concentration and the displacing-salt concentration was determined from the adsorption isotherms. As explained above, under conditions where the adsorption is independent of target protein concentration in the solution, linearity between the adsorption capacity and the displacing-ion concentration was anticipated.

3.2. Adsorption isotherms

The adsorption isotherms for the horse heart cytochrome c and LiChrospher 1000 SO_3^- system, presented in Fig. 1, were obtained with the on-line adsorption vessel system. One of the advantages of this method is that a large number

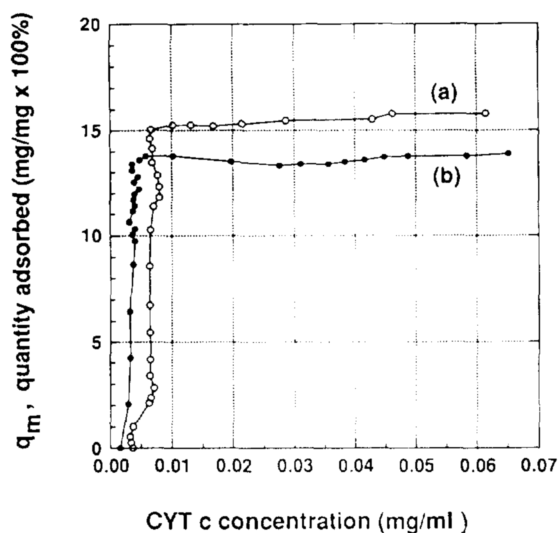

Fig. 1. Adsorption isotherms obtained with horse heart cytochrome *c* (CYT c) and the LiChrospher 1000 SO_3 tentacle-type cation exchanger in 0.05 *M* acetic acid at pH 4.00, 25°C. (a) No added displacing salt; (b) displacing salt NaCl added at a concentration of 0.1 *M*.

of data points can be obtained continuously within a relatively short time, offering a very flexible experimental approach to the measurement of isotherms and the acquisition of detailed information about the adsorption process. However, in the case of the adsorption of horse heart cytochrome *c* to the LiChrospher 1000 SO_3 particles, the experiment lasted several days for the determination of a single complete isotherm, resulting in some minor variation occurring in the day-to-day experimental measurements due to the evaporation of water when an open recycling unit was used. As a result, some of the experimental data points do not coincide with the theoretical profile expected for an "irreversible" adsorption. This problem can be circumvented through the use of a closed recycling system.

The differences between the isotherms shown in Fig. 1 and the conventional Langmuir model are very evident. The experimental isotherms clearly show a saturated plateau of protein adsorption and its critical concentration (C_r). The uptake of the protein is higher in the absence of displacing salt (isotherm a) and is consistent with a higher adsorption capacity. In addition, the critical sample concentration was found to be higher when no displacing salt was present. The estimated critical concentrations of cytochrome *c* are about $C_r = 0.003$ mg/ml and 0.006 mg/ml in the presence and absence of 0.1 *M* NaCl, respectively.

As the isoelectric point (p*I*) of horse heart cytochrome *c* is about 10, the protein will be positively charged under the chosen experimental conditions. The displacing salt here plays a dual role in solution, i.e. the sodium ions act as the displacing ions and the chloride ion as the counter-ion for the protein molecules. Compared to the case where the displacing salt NaCl is absent, the microenvironment of the tentacle chains within the ion-exchange particles will manifest a smaller negative electrical potential in the presence of 0.1 *M* NaCl due to ion suppression by the adsorbed Na^+ ions. On the other hand, the molecular surface of the horse heart cytochrome *c* molecule will become slightly more negative in the presence of 0.1 *M* NaCl, compared to when the displacing salt is absent, because of the preferential adsorption by the protein of the Cl^- counter-ions. The consequence of these ion–protein and ion–adsorbent interactions will be reflected as a lower critical concentration of the protein when the displacing salt is present.

The adsorption isotherm with a displacing-salt concentration of 0.4 *M* is presented in Fig. 2. Two regions of the isotherm are discernable. A steep change in q_m versus cytochrome *c* concentration is evident up to the protein concentration of 0.005 mg/ml, followed by an asymptotic shape reminiscent of the Langmuir-type isotherm. Despite the high concentration of displacing salt, the normally strong electrostatic interaction associated with this cation exchanger [16] may primarily dominate the first part of the adsorption. When the protein concentration increases and the electrostatic binding sites become gradually occupied, the "multilayer dissolution" mechanism proposed for the tentacle-type ion exchanger [12] may become more dominant, making the second part of the adsorption process of relatively lower affinity.

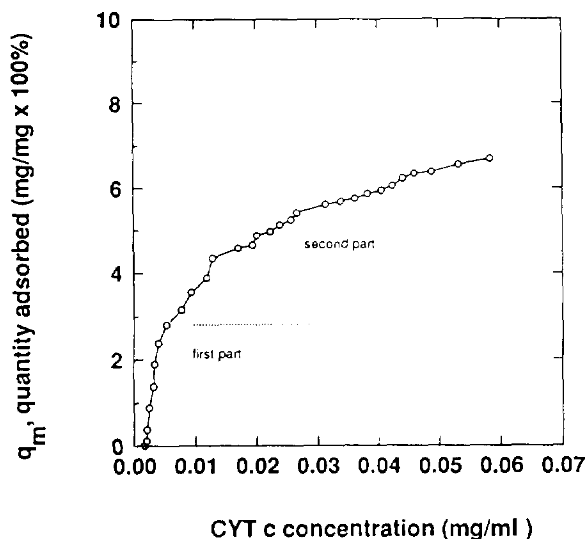

Fig. 2. Adsorption isotherm obtained with horse heart cytochrome *c* (CYT c) and the LiChrospher 1000 SO_3^- tentacle-type cation exchanger in 0.05 *M* acetic acid at pH 4.00, 25°C. The displacing-salt (NaCl) concentration was 0.4 *M*.

It can be noted that the isotherms presented in Fig. 1, where the displacing-salt concentrations were significantly lower, did not show the same curve pattern as evident in Fig. 2 over the same protein concentration range. It is known [17,18] that one of the major effects of salts on the electrostatic interactions between polyelectrolytes such as proteins and charged surfaces is the ability of salts to influence the structure of the polymer backbone through ion–dipole interactions. Under conditions of low ionic strength it is thus unlikely that the "multilayer dissolution" mechanism will make a significant contribution to the adsorption/desorption process due to the large number of protein molecules already bound on-site. Under these low-ionic-strength conditions with few positive charged small ions, like Na^+ present, the tentacle chains probably will curve around the globular-shaped cytochrome *c* molecules, giving little possibility for the protein to be adsorbed by a "multilayer dissolution" mechanism.

With buffers of low ionic strength, the sulphonic acid-modified tentacular ligands will most probably exist as self-repelling extended chain entities due to their high charge density. When a positively charged biopolymer such as horse heart cytochrome *c* is present, charge complementarity between the ligand and the adsorbate could result in these extended tentacular ligands collapsing around the biopolymer. Similarly, with buffers of higher ionic strength, the intrinsic molarity of the displacing ion (in this case Na^+) could reach significant levels, possibly above 4 *M*, within the Donnan double layer due to the repetitive nature of the sulphonic acid group linked to each monomer unit of the graft linear polymer within the tentacular adsorbent. Depending on the extent of molecular entrapment of the protein by the tentacular ligands, adsorption capacities exceeding monolayer coverage could occur with an affinity distribution in binding sites. As the ionic strength increases, the adsorption capacities for the protein will decrease dramatically, with narrowing of the affinity distribution. The differences between the isotherms, in the absence of NaCl, and when 0.1 and 0.4 *M* NaCl are present, could thus have their origin in such a phenomenon. Similar conclusions have been reached for the adsorption of bovine serum albumin to the N-trimethylammoniummethyl (TMAE)-LiChrospher 1000 anion exchanger [12], an anion exchanger with similar isothermal behaviour.

3.3. Thermodynamic parameters

As shown by the data presented above, the adsorption isotherms for horse heart cytochrome *c* with the LiChrospher 1000 SO_3^- consist of a plateau uptake with a clearly defined critical concentration when the displacing-salt concentration is below 0.1 *M*. This adsorption process does not occur in the same way when the displacing-salt concentration is increased to 0.4 *M*. Consequently, in order to determine the thermodynamic parameters, the concentration of the displacing salt in our static experiments was limited to ionic strength values below 0.1 *M* NaCl. The experiments were designed so that the cytochrome *c* concentration in the supernatant solution was higher than 0.006 mg/ml after equilibrium adsorption was reached.

The dependence of the adsorption capacity on the displacing-ion concentration for the adsorption of horse heart cytochrome c onto the LiChrospher 1000 SO_3^- sorbent between 2 and 45°C is shown in Fig. 3. The initial concentration of sodium ions (0.0076 M) in the buffer solution has been included within the total $[Na^+]$ value. The deduced slopes per mol unit and the coefficients determined from linear regression analysis of the plots, as well as the calculated change in Gibb's free energy are listed in Table 1. It can be seen that good linearity was obtained over the entire temperature range. Janzen et al. [12] have studied the adsorption capacity of bovine serum albumin (BSA) with tentacle-type anion exchangers at different displacing-salt concentrations. Their results also showed that the adsorption capacity decreased linearly over the low range of displacing-salt concentrations (approximately up to 0.15 M) and reached zero at a concentration of displacing salt of 0.4 M.

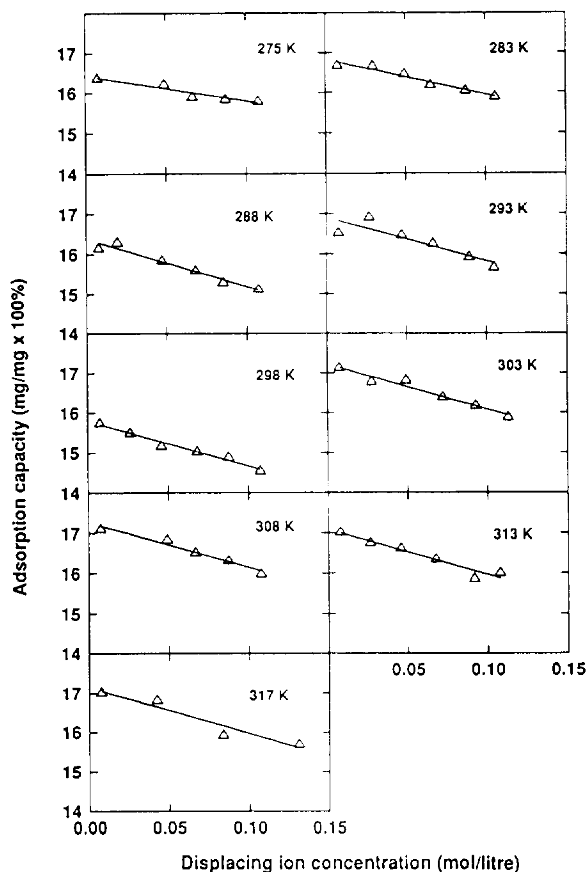

Fig. 3. Plot of the adsorption capacity (mg/mg × 100%) against the displacing-ion concentration (M) for horse heart cytochrome c adsorption onto the LiChrospher 1000 SO_3^- tentacle-type cation exchanger over the temperature range 275–317 K.

Table 1
Experimentally obtained slope of the plots of adsorption capacity versus the displacing-ion concentration, and the derived change in Gibb's free energy for the adsorption of horse heart cytochrome c onto the LiChrospher 1000 SO_3^- tentacle-type cation exchanger

Temperature (K)	Slope B (mol^{-1})	Regression coefficient	$-\Delta G^0$ (kJ/mol)
275	6.36	0.95	4.23
283	8.89	0.98	5.13
288	12.11	0.96	5.97
293	10.79	0.89	5.80
298	11.21	0.99	5.99
303	11.34	0.98	6.12
308	11.23	0.98	6.19
313	11.28	0.97	6.31
317	11.75	0.96	6.48

The ΔG^0 values for the interaction between cytochrome c and the adsorbent were determined from the experimental data according to Eqs. 4 and 5. The plot of ΔG^0 against temperature (Fig. 4) shows that there are two regions, denoting two different patterns of binding between the horse heart cytochrome c and the cation exchange ligands. Overall, the ΔG^0 values generally increased with increasing temperature. Two sets of related thermodynamic parameters (ΔH^0_{assoc} and ΔS^0_{assoc}) could be deduced from these plots over the temperature ranges from 2 to 20°C and from 20 to 44°C and the results are listed in Table 2. These results reveal that the relative contribution of the apparent entropy and enthalpy to the overall change in the Gibb's free energy for the cytochrome c adsorption to the LiChrospher 1000 SO_3^- adsorbent varies with the operating temperature. Roush et al. [19] derived thermodynamic parameters for the adsorption of cytochrome b_5 onto an anion exchanger which were of similar magnitude range as those found in the present investigations for the adsorption of cytochrome c onto the LiChrospher 1000 SO_3^-.

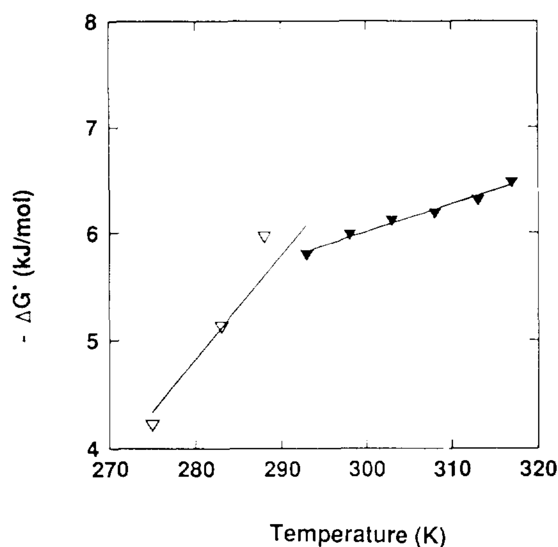

Fig. 4. Plot of the change in Gibb's free energy, ΔG^0 against temperature over the range 275–317 K.

Moreover, these investigators demonstrated that the stoichiometric displacement parameter Z, which can be directly related to $\Delta G^0_{adsorption}$, depends on temperature and showed an increase in Z value with increasing temperature. Their findings are thus in a good agreement with the results presented here.

Studies on the effect of temperature on the three-dimensional structure of horse heart cytochrome c have demonstrated [20–22] that the protein changes conformation at low pH and low temperature. The two different patterns of interaction between horse heart cytochrome c and the tentacle-type cation-exchange ligand may arise either from conformational changes of the protein molecule or from changes in the orientation and folding pattern of the tentacle chains of the ion exchanger itself. The relative contribution of these two processes in the interaction can be revealed from Hill plots of the experimental data when single step, hyperbolic isotherm of well defined saturation value are obtained at different temperature, provided the heat of adsorption does not vary with the extent of adsorbate coverage [23]. These observations warrant further examination by other independent methods such as microcalorimetry.

Table 2
Experimentally obtained thermodynamic parameters for the interaction between horse heart cytochrome c and the LiChrospher 1000 SO_3^- tentacle-type cation exchanger

Temperature range (K)	$\Delta G^0 = \Delta H^0 - T\Delta S^0$
275–293	$\Delta H^0 = 22.2$ kJ/mol $\Delta S^0 = -96$ J/mol · K
293–317	$\Delta H^0 = 1.79$ kJ/mol $\Delta S^0 = -26$ J/mol · K

4. Conclusions

The measurement of the change in Gibb's free energy associated with the adsorption of a biopolymer to an adsorbent as proposed here has provided an alternative way to study biomolecule interactions with tentacle-type ion exchangers, and other HPIEC adsorbents generating rectangular isotherms. The success of the method relies on the linearity between the adsorption capacity and the displacing-ion concentration, which can normally be observed experimentally over a narrow range of low concentrations for high-affinity ion exchangers. Employing the on-line adsorption vessel system to acquire data on the isotherm allows a large number of data points to be taken continuously, and the experimental information on the nature of the isotherm and the adsorption process directly to be obtained.

This study on the adsorption of horse heart cytochrome c with the tentacle-type cation exchanger, LiChrospher 1000 SO_3^-, has shown that over a temperature range from 2 to 44°C two different adsorption processes were involved depending on the ionic strength of the buffer. The experimentally derived values of the thermodynamic parameters were found to be of similar magnitude to the findings reported by other researchers for other types of protein–anion exchanger interactions.

Acknowledgements

This investigation was supported by the Australian Research Council and from funds provided by the Buckland Foundation.

References

[1] F.B. Anspach, A. Johnston, H.J. Wirth, K.K. Unger and M.T.W. Hearn, *J. Chromatogr.*, 476 (1989) 205.
[2] F.B. Anspach, A. Johnston, H.J. Wirth, K.K. Unger and M.T.W. Hearn, *J. Chromatogr.*, 499 (1990) 103.
[3] H.J. Wirth, K.K. Unger and M.T.W. Hearn, *Anal. Biochem.*, 208 (1993) 16.
[4] A. Johnston and M.T.W. Hearn, *J. Chromatogr.*, 557 (1991) 335.
[5] Q.M. Mao, A. Johnston, I.G. Prince and M.T.W. Hearn, *J. Chromatogr.*, 548 (1991) 147.
[6] W. Kopaciewicz, M.A. Rounds, J. Fausnaugh and F.E. Regnier, *J. Chromatogr.*, 266 (1983) 3.
[7] R.D. Whitley, R. Wachter, F. Liu and N.-H.L. Wang, *J. Chromatogr.*, 465 (1989) 137.
[8] M.I. Aguilar, A.N. Hodder and M.T.W. Hearn, in M.T.W. Hearn (Editor), *HPLC of Proteins, Peptides and Polynucleotides*, VCH, New York, 1991, p. 199.
[9] W. Muller, *Eur. J. Biochem.*, 155 (1986) 203.
[10] W. Muller, presented at the *8th International Symposium on HPLC of Proteins, Peptides and Polynucleotides, Copenhagen, 31 October–2 November 1988*, paper 121.
[11] K.K. Unger, K.D. Lork and H.J. Wirth, in M.T.W. Hearn (Editor), *HPLC of Proteins, Peptides and Polynucleotides*, VCH, New York, 1991, p. 72.
[12] R. Janzen, K.K. Unger, W. Muller and M.T.W. Hearn, *J. Chromatogr.*, 522 (1990) 77.
[13] G. Jilge, K.K. Unger, U. Esser, H.J. Schafer, G. Rathgeber and W. Muller, *J. Chromatogr.*, 476 (1989) 37.
[14] G.E. Myers and G.E. Boyd, *J. Phys. Chem.*, 60 (1956) 521.
[15] H.F. Walton, in E. Heftmann (Editor), *Chromatography (Chemistry Textbook Series)*, Reinhold, New York, 2nd ed., 1967, Ch. 12.
[16] G. Malmquist and N. Lundell, *J. Chromatogr.*, 627 (1992) 107.
[17] C.T. Shibata and A.M. Lenhoff, *J. Colloid Interface Sci.*, 148 (1992) 469.
[18] C.T. Shibata and A.M. Lenhoff, *J. Colloid Interface Sci.*, 148 (1992) 485.
[19] D.J. Roush, D.S. Gill and R.C. Willson, *J. Chromatogr. A*, 653 (1993) 207.
[20] M. Ohgushi and A. Wada, *FEBS Lett.*, 164 (1983) 21.
[21] S. Potekhin and W. Pfeil, *Biophys. Chem.*, 34 (1989) 55.
[22] Y. Kuroda, S. Kidokoro and A. Wada, *J. Mol. Biol.*, 223 (1991) 1139.
[23] S.J. Gregg and K.S.W. Sing, *Adsorption, Surface Area and Porosity*, Academic Press, London, 2nd ed., 1982, p. 199.
[24] M.I. Aguilar and M.T.W. Hearn, *Methods Enzymol.*, (1994) in press.