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Molecular approach to protein–polymer interactions in ion-exchange chromatography

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Abstract

A model was developed and implemented to aid in understanding and predicting the retention behaviour of proteins in ion-exchange chromatography. The model structures chosen were calcium-loaded and -depleted α-lactalbumin (ALC) and hen egg white lysozyme (HEWL) and a comparison was made with chromatographic measurements. A characteristic charge of −3.4 was found under the experimental conditions applied for both forms of ALC, and HEWL was not retained. The model explicitly considers all of the atoms, each being assigned a set of force field parameters. Because of the computational time necessary to include them, water molecules were not taken into account, but a sigmoidal function of the dielectric permittivity was introduced in the calculations. Interaction potential energies from bulk down to the contact were evaluated for each protein. The results were in qualitative agreement with those of the chromatographic experiments. It was possible to reproduce the difference in retention between both forms of ALC and also the behaviour of HEWL.

1. Introduction

Proteins at interfaces have been an active area of research for several years because of the complexity of the systems and the interactions involved. In many areas, such as chromatography and the determination of biocompatability of implants, an in-depth understanding of the adsorption processes of proteins is essential. Extensive research has been carried out on this subject and some reviews are available [1,2]. However, in spite of the fact that kinetic models exist for describing the processes that occur during protein adsorption at interfaces, the mechanisms of adsorption are still not fully understood at a molecular level and new approaches to these systems are needed.

One of the numerous applications in this field is ion-exchange chromatography, which is one of the most useful methods for the separation and purification of biopolymers [3]. The retention behaviour of proteins on ion exchangers is generally explained on the basis of electrostatic interactions between the charged solute and the stationary phase. The pioneering work of Boardman and Partridge [4], further developed by Kopaciewicz et al. [5], is now considered as the classical theory called the stoichiometric dis-

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placement model (SDM). More recently, Stahlberg et al. [6,7] and Haggerty and Lenhoff [8] highlighted several inadequacies of the SDM and showed that this model is not suitable for predictive purposes. They proposed a non-stoichiometric treatment of the problem that is mainly based on the numerical solution of the linearized Poisson–Boltzmann law within a simplified geometry of the interacting species. They obtained a good correlation with experimental data and emphasized the importance of protein orientation in the adsorption process [9].

In this paper, we present the first results for a microscopic model based on a quantum and classical mechanics description of the system. This model is aimed at correlating molecular data on an atomic scale with the experimental retention behaviour of proteins on ion exchangers.

2. Experimental

2.1. Chemicals

Lysozyme (chicken egg white, HEWL), α-lactalbumin (milk, ALC) and calcium-depleted α-lactalbumin were purchased from Sigma (St. Louis, MO, USA). Triethanolamine (TEA) was obtained from Aldrich-Chemie (Steinheim, Germany) and silica gel (LiChrospher 100) for column packing from Merck (Darmstadt, Germany).

2.2. Chromatography

The chromatographic equipment was composed of a Model 420 solvent-delivery pump (Kontron Instruments, Zürich, Switzerland), a Model 7125 injection valve (Rheodyne, Berkeley, CA, USA) and a Spectra-100 UV detector (Spectra-Physics, San Jose, CA, USA). Anion-exchange HPLC experiments were carried out at 25°C using a column (100 × 4.5 mm I.D.) filled with porous silica of porosity 100 Å and particle diameter 5 μm. The silica was coated with polyvinylimidazole (PVI) cross-linked with butane 1,4-diglycidyl ether as described by Sébille et al. [10]. This chemical treatment is known to bring a positive unit charge on the imidazole ring and the polymer acquired an anion-exchange capability. The eluent was 20 m*M* triethanolamine buffer (pH 7.0). Samples were injected using a 20-μl injection loop and the flow-rate was held at 1.0 ml/min. Protein elution was followed by UV detection at 280 nm. The salt concentration (Na_2SO_4) was varied from 20 m*M* to 1 *M*.

3. Calculations

Basically, the model requires a molecular modelling of the two interacting systems, the protein and the support surface, and an interaction potential which should be calculated for various approaching pathways. The appropriate computational techniques used for these different tasks are briefly described in this section.

The chosen building blocks of the modelled chromatographic support were syndiotactic PVI dimers. In order to characterize the conformational and electrostatic properties of the polymer we used semi-empirical methods of quantum chemistry. We used the MOPAC 6.0 package [11] and the MNDO hamiltonian [12] to optimize fully the structure of the PVI dimer. We also calculated the partial charges on each atom of the polymer chain.

Given the size of the protein (the largest dimension is 70 Å) relative to the chromatographic adsorbent, we can reduce the real process to the interaction between a planar two-dimensional polymer surface and one isolated protein molecule. To create the macromolecular surface, each PVI dimer was oriented along the *x*-axis with imidazole rings pointing towards the *z*-axis and duplicated along *x*- and *y*-axes (Fig. 1). The spacing between successive strands of quaternized PVI was chosen to be 20 Å in order to obtain a charge density of 0.15 C/m^2, which is a common value for an ion-exchange chromatographic support. For an optimum balance between computational time requirements and the precision of energy values, we calculated that the

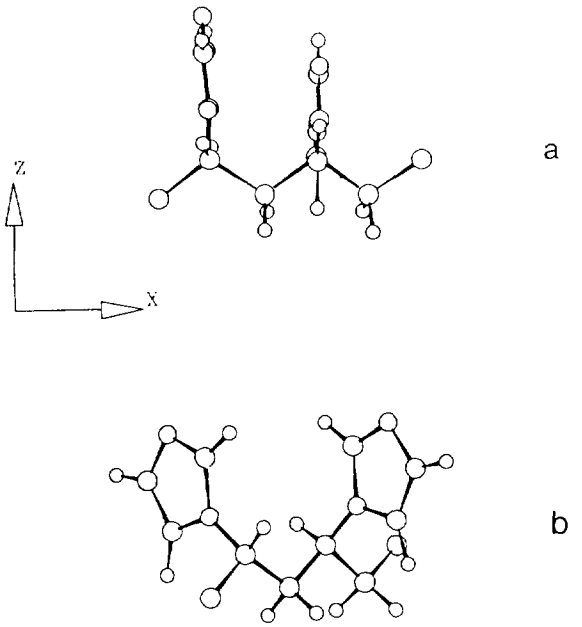

Fig. 1. Ball-and-stick representation of the PVI dimer. (a) Side view of the dimer. X is the strand direction and Z represents the direction of approach for the proteins. The coordinate system is orthonormal. The large spheres beginning and ending the polyvinyl chain are not real atoms but represent the direction of propagation for building the strand. (b) Same as (a) with a counter-clockwise rotation of 90° around Z. The imidazole rings are pointing toward the positive Z direction.

adsorbent surface size should be at least 200 Å^2. Eventually, calculations were done for a surface composed of eleven strands of 41 residues each, giving a total area of 210×210 Å^2.

Electrostatic interactions are the driving force in ion-exchange chromatography but other forces play an important role in the interfacial processes. The interaction energy was thus obtained by adding pairwise contributions including electrostatic, dispersive and repulsive forces. We used a modified version of the AMBER [13,14] force field and the total interaction energy E_{tot} within our system was evaluated using:

$$E_{tot} = \sum_{i,j} \frac{e^2 q_i q_j \, e^{-\kappa R_{ij}}}{4\pi\varepsilon_0 \varepsilon(R_{ij}) R_{ij}} + \sum_{i,j} \left(\frac{A_{ij}}{R_{ij}^{12}} - \frac{B_{ij}}{R_{ij}^{6}} \right) \quad (1)$$

where R_{ij} is the distance between atoms i and j of the protein and surface respectively, q_i is the partial charge on atom i, ε_0 is the vacuum dielectric permittivity and $\varepsilon(R_{ij})$ the relative permittivity function. The 6–12 Lennard–Jones potential for Van der Waals interactions was computed following exactly the AMBER force field.

Two modifications to the classical coulombic law were introduced. The first was intended to compensate the lack of explicit solvent in our simulations by including a distance-dependent relative permittivity function $\varepsilon(R_{ij})$ introduced by Mehler and Solmajer [15]. The second modification added an ionic strength dependence of the electrostatic term using an exponential screening factor that was a function of the inverse Debye length κ. All calculations reported in this paper were performed at a 50 mM ion concentration, which is within the usual experimental range.

Few experimental examples of protein separations on PVI columns are available. Moreover, among the proteins studied, there are few for which the three-dimensional structure is available. These considerations determined our choice of ALC and HEWL as model proteins. We used the three-dimensional structure of baboon (*Papio cynocephalus*) ALC determined by X-ray crystallography at 1.7 Å resolution [16] and of HEWL at 2.5 Å resolution [17], respectively, extracted from the 1ALC and 7LYZ entries of the Protein Data Bank [18]. It is important to note that HEWL and ALC have very similar three-dimensional structures but opposite net charges calculated (+8 and −8, respectively) at pH 7.0, which makes them good test structures. Moreover, ALC is a calcium-binding protein that is also known to lose its calcium ion and in turn to exhibit a −10 net charge at pH 7.0. We deleted the calcium ion from the structure to obtain the calcium-depleted form of ALC.

Using the protein and polymer surface models and the analytical expression of the interaction potential, we simulated the first steps of the chromatographic process. Two main distinct steps may be considered: the progressive approach of the highly mobile solute molecules towards the relatively rigid polymer surface and

the final, quasi-equilibrium state, which can be considered as a super-molecular complex. Both steps provide useful information for the understanding of chromatographic selectivity. For gap distances ranging from 100.0 to 1.0 Å, interaction energies of each protein with the surface were calculated. All protein orientations were considered for each gap distance.

The programs implementing this model were written in Fortran 77 and the calculations were performed on a Silicon Graphics Indigo Workstation.

4. Results and discussion

The elution behaviour of calcium-loaded and calcium-depleted ALC and HEWL was studied as a function of salt concentration. Fig. 2 shows typical elution profiles of the three proteins. The results showed that calcium-depleted ALC has slightly but constantly higher retention times than the calcium-loaded protein at all salt concentrations studied. Calcium depletion is responsible for a greater negative net charge of the protein and is also expected to increase the negative charge density at the calcium binding site. Calcium removal frees some aspartate side-chains which repel each other and become exposed to the solvent. A local conformational change of the protein caused by the calcium depletion could also contribute to the observed results.

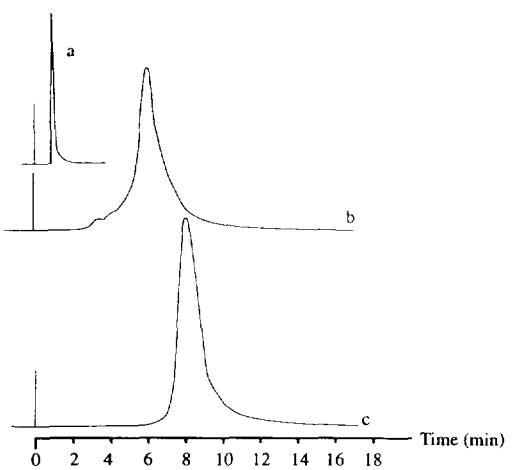

Fig. 2. Typical elution profiles obtained for (a) HEWL, (b) calcium-loaded ALC and (c) calcium-depleted ALC. HEWL is positively charged at pH 7.0 and is eluted in the void volume. Samples concentrations, 2 g/l; flow-rate, 1.0 ml/min; temperature, 25°C; eluting salt, 0.05 *M* Na_2SO_4.

The experimental data were analysed according to the SDM. From the mass action law, the capacity factor k' is related to the salt counterion concentration S according to the following relationship:

$$\log k' = \log A - \frac{Z_P}{Z_S} \cdot \log |S| \qquad (2)$$

where A is a constant, Z_P the characteristic charge of the protein in the elution buffer and Z_S is the displacer salt valency. Data representation as $\log k' = f[\log (1/|S|)]$ enabled the effective charge of the solute (Z_p) to be calculated from the slope of a line fitted to the experimental curve. We thus obtained a value of −3.4, which is in good agreement with earlier experiments from our laboratory where NaCl was used as the displacing salt [19]. Despite a high structural similarity, HEWL was not retained on such a column (Fig. 2a) owing to its positive charge at pH 7.0. It should be noted that HEWL retention on anion exchangers is only observed at a pH closer to its p*I* value [20].

The adsorption (or interaction) potential for the three protein structures was computed as a sum of all atomic contributions at 50 m*M* salt concentration. For each of the sampled gap distances we calculated the mean interaction energy over all the generated protein orientations. The HPLC time-scale is significantly longer than the scale of molecular motions (the rotational correlation time of proteins is of the order of 10^{-8} s) and this justifies comparison of experimental data with the averaged values we calculated.

Fig. 3 plots the distance dependence of these values for the studied protein structures. In all instances there was no significant interaction at a distance greater than 50 Å. At lower distances, the interaction energy becomes greater than the thermal energy ($kT \approx 2.4$ kJ/mol at 25°C) and evolves specifically for the different molecular species studied. As is clearly observed from Fig.

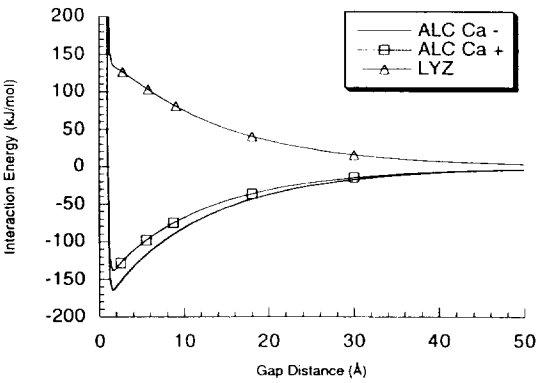

Fig. 3. Mean values of the calculated interaction energy as a function of the distance to the polymeric surface for adsorption simulations of ALC [(□) with or (no symbol) without calcium] and (△) HEWL.

3, ALC is attracted towards the polymer phase whether the calcium ion is present or not. HEWL is repelled from the surface in agreement with its elution at the void volume although its tertiary structure is very similar to that of ALC. We reproduced the difference observed in the chromatographic retention of the two forms of ALC and that of HEWL. We also note in Fig. 3 that there exists a minimum at 1.5 Å for interaction of ALC with the surface whereas no such variation can be observed for lysozyme in our calculations. The extreme values are −138.04 and −162.20 kJ/mol for calcium-loaded and -depleted forms, respectively. From a qualitative point of view, we can state that the small relative difference in retention between ALC and calcium-depleted ALC is predicted by the model. Positioned at the same gap distance, the HEWL protein exhibits a 136.62 kJ/mol interaction energy. These values could not be compared with those obtained experimentally because of the lack of general entropy treatment in such models. Nevertheless, these values are within the range of observed adsorption enthalpies for proteins on ion-exchangers [21].

These preliminary results show a good correlation with chromatographic experiments on a qualitative basis. They seem to emphasize the importance of the protein total net charge on retention times and in further studies we shall investigate the influence of local charge densities and examine the preferred orientations for adsorption and also the influence of different separation conditions.

Because the model considers the interaction of a single molecule with the adsorbent, its applicability is restricted to low surface coverages. It can be applied to explain chromatographic experiments performed in the Henry's law region. The main interest in this model lies in its explicit inclusion of all atoms but its shortcomings are the same as for most current atomic simulations, i.e., lack of explicit solvent and ions, rigid structures and use of a force field with a limited number of contributions. For example, it seems that solvation forces should be taken into account for obtaining a more realistic model but implicit inclusion of water molecules in addition to ions is computationally complex. This is the reason why most of the current research in this field resorts to macroscopic models, which have the drawback of using simplified geometries for the system.

5. Conclusions

During the movement toward the stationary phase surface, ALC molecules evolve in an attractive potential which represents the driving force for optimum adsorption which is likely to occur within a reduced set of preferred orientations. As a first approximation, we consider the protein molecule and the support as rigid structures. In addition, as suggested by Lu and Park [22], further orientation phenomena or conformational changes may occur after adsorption and during diffusion on the surface. At present, these conformational variations cannot easily be accounted for. Nevertheless, this encouraging basis prompts us to refine our model and test it further with various well characterized polymers and proteins.

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