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ARTICLE *in* INTERNATIONAL JOURNAL OF BIOLOGICAL MACROMOLECULES · OCTOBER 1979

Impact Factor: 2.86 · DOI: 10.1016/0141-8130(79)90045-X

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Protein binding to two-dimensional hydrophobic binding-site lattices: adsorption hysteresis on immobilized butyl-residues

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(Received 23 April 1979)

Long-lived metastable states involving multiple binding sites of a protein ligand with immobilized alkyl residues on a solid phase can be observed at high ionic strength between butyl agaroses (5, 21 μ mol/ml packed gel) and phosphorylase b by perturbations enforcing either the on-reaction (adsorption) or the off-reaction (desorption). These apparent equilibrium states are suggested because the adsorption isotherms of phosphorylase b on butyl agaroses are not retraced by the desorption isotherms. In this first example of macromolecular adsorption hysteresis on immobilized alkyl residues, it can be shown that the irreversible entropy ($\Delta_i S$) produced in an adsorption–desorption cycle lies between ~6 (5 μ mol/ml packed gel) and ~40 (21 μ mol/ml packed gel) J mol^{-1} K^{-1}. For the latter gel the apparent standard entropy of adsorption ($\Delta_a S_i^{0\prime}$) is 160 J mol^{-1} K^{-1}. The metastable state observed during adsorption is probably due to an energy barrier which must be overcome for the nucleation of protein binding on the matrix. Other metastable states may possibly be encountered during desorption when the adsorbed enzyme resists the breakage of hydrophobic interactions. In the transition from the adsorption branch to the desorption branch of the hysteresis loop, the apparent affinity of the enzyme–matrix interaction is enhanced. For the desorption branch, the apparent association constant of half-maximal saturation corresponds to $K'_{d,0.5} = 4.2 \times 10^9$ M^{-1} as compared to the respective constant of adsorption $K'_{a,0.5} = 1.6 \times 10^5$ M^{-1} (gel: 21μ mol/ml packed gel). Since the area of the hysteresis loops (see also $\Delta_i S$) depends strongly on the density of butyl residues on the gel, it is concluded that the number of alkyl residues interacting with the protein molecule is crucial for the metastable states and hysteresis. It is unlikely that hysteresis is due to the pore structure of the agarose or to nearest neighbour interactions of ligand molecules. Since thermodynamic irreversibility and hysteresis may be encountered when macromolecules, such as proteins, are adsorbed to cell membranes or cell organelles; an analysis and understanding of these phenomena should be of general biological significance.

Introduction

The non-covalent binding of small molecular weight ligands to single sites on peptides and proteins has been extensively studied. When a peptide or protein in itself however becomes a ligand*, in the binding to superstructures such as cell membranes or organelles (e.g. ribosomes), complex interactions are encountered. To a large extent this complexity arises from the macromolecular nature of the protein which permits simultaneous interactions of multiple, separate sites on the superstructure with the ligand (multivalence†). Such interactions have primarily been studied in the form of two model systems: (1) superstructures displaying a one-dimensional lattice of binding sites (e.g. nucleic acid model, see References 2–4); and (2) superstructures displaying a two-dimensional lattice of binding sites (e.g. alkyl–agarose matrix model, Reference 1; also for model applied to cell membranes, see Reference 5). Thermodynamic parameters on hydrophobic interactions have only been reported for the latter system[1]. Both models[1,3] may prove to be very helpful in the elucidation of the complex interactions between proteins and biological structures bearing binding site lattices.

Hysteresis loops caused by long-lived metastability (for review see Reference 6) have been described for a variety of systems, for example, as molecular (microscopic) hysteresis in acid–base titrations of nucleic acids and proteins[6,7] or, for example, as macroscopic hysteresis in the adsorption of gases to solids (adsorption hysteresis, for review see Reference 8).

Phosporylase *b* one of the most well known enzymes available was chosen as a model protein; not because its hydrophobic adsorption has been shown to be of biological significance, but for purely practical reasons and requirements which must be fulfilled if a model study of multivalent protein binding is to be undertaken. These are: (1) the enzyme is very stable and can be obtained in

This paper is dedicated to Professor Josef Schmidt-Thomé (Frankfurt) on the occasion of his 70th birthday. The first paper of this series[1] appeared under the running title 'Negative Cooperativity on Hydrophobic Agaroses'

* *Ligand*: a solute molecule, e.g. protein, capable of noncovalent interactions with the surface of the agarose matrix[1] or a biological superstructure

† *valence*: a unit of valence is defined as the noncovalent force resulting from the interaction of one lattice binding site (e.g. alkyl residue) on the matrix with a corresponding finite area (e.g. hydrophobic binding site) of the protein

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large amounts (~1–2 g from 3 kg of rabbit muscle); (2) the crystal structure has been determined by X-ray crystallography[9]; (3) the primary structure is known[10]; (4) changes in the native state can be checked by activity measurements; (5) the enzyme possesses a high solubility (concentrations of 50–60 mg/ml in 1.1 M ammonium sulphate are easily obtained); (6) radioactive labelling with tritium does not involve significant structural changes (see Experimental); and (7) the enzyme is readily adsorbed endothermically to butyl agarose[1].

Although it has been becoming increasingly clear from binding[1,5,11] and kinetic[11–13] measurements that the mechanism of the adsorption of phosphorylase *b* on butyl agarose is based on cooperative, multivalent interactions probably involving a minimum of 3–4 alkyl residues[1,5,11], the consequences of this mechanism have not been studied in detail.

Together with preliminary accounts[12–14] this paper is the first report on the adsorption hysteresis of a protein which binds to a two dimensional lattice of binding sites. The desorption isotherm of phosphorylase *b* on butyl Sepharose does not retrace the adsorption isotherm thereby exhibiting a closed loop (hysteresis). The shape of the hysteresis loop and the corresponding entropy production are a function of the lattice site density on the matrix. This strongly indicates that the number of interactions between matrix residues and protein (valence) is primarily responsible for the energy barriers encountered in this type of hysteresis.

Experimental

Preparation of butyl agarose, buffer and ligand solutions. The preparation and analysis of ^{14}C-labelled alkyl derivatives of Sepharose 4B (Pharmacia) have been previously described[15,16]. The substituted gels were stored at 5°C in 10 mM sodium β glycerophosphate pH 7.0, 0.01 mg/ml NaN_3 and aged for at least 1–2 weeks before use. On these gels the immobilized alkyl residue concentration* decreased ~10%/month. The butyl-Sepharoses employed in the experiments contained ~5 and 21 μ mol/ml packed gel (uncertainty: 5–10%). Only fresh, unregenerated gel was used. Phosphorylase *b* (third crystals) was prepared from frozen rabbit muscle[17] and freed of AMP[17], (for further details see Reference 1). A monomer, molecular weight[10,18] of 10^5, was employed for calculations[1]. Enzyme activity[19] and protein[20] were determined on an AutoAnalyzer (Technicon, Tarrytown, N.Y.). Reduced, tritium labelled phosphorylase *b* (^{3}H-phosphorylase b_r, 40–50 units/mg) was prepared according to Reference 21. The specific radioactivity was 1–3 $\times 10^5$ cpm/mg. After reduction the enzyme was extensively dialysed against buffer containing 10 mM tris (hydroxy methyl) amino methane/maleate, 5 mM dithioerythritol, 1.1 M ammonium sulphate, 20% sucrose, pH 7.0 which was employed for all binding experiments. After dialysis the enzyme was centrifuged for 30 min at 50000 *g*. For the determination of radioactivity ^{3}H-phosphorylase b_r was precipitated in 5% Cl_3CCOOH in the presence of ~2.5 mg bovine serum albumin and counted according to Reference 22, or solubilized in 1 ml 0.1 M NaOH, 1% SDS and counted in 10 ml Scintillator (Quickscint 212, W. Zinsser, Frankfurt) containing toluene and Triton X-100 and 0.1 ml 5 M HCl. All samples were corrected for quenching by employing an external standard. All reagents were of highest purity available. The water was deionized, distilled and finally passed through a Milli-Q-System (Millipore, Bedford, Mass.). All experiments were performed at 5°C.

Adsorption experiments. The ligand ^{3}H-phosphorylase b_r was adsorbed to butyl-Sepharose as previously described[1] either in a plexiglas beaker of 20 ml volume (beaker 2.5 cm i.d. × 9 cm, stirring bar 1.5 cm, stirring velocity 700 rev/min) or of 60 ml volume (beaker 3.5 cm i.d × 9 cm, stirring bar 3 cm, stirring velocity 450 rev/min). Samples of 0.5 ml gel free buffer solution were obtained with a steel grid as described in Reference 1. The absorbed ligand concentration† was calculated from the difference of the initial (before adsorption) and final (after adsorption) free ^{3}H-phosphorylase b_r concentration in the bulk corrected for amounts of enzyme adsorbed to unsubstituted Sepharose 4B as a control[1,11]. This calculated amount of enzyme corresponds to the amount which can be released from the gel in the presence of 0.1 M NaOH, 1% SDS. Gel and protein were incubated for 60 to 90 min; due to apparent time independence of the corrected adsorption to butyl-Sepharose after this time interval practically constant values (apparent equilibrium) for the concentration of bound ligand correlated to the free ligand concentration were obtained.

Desorption Experiments. At the apparent equilibrium the gel loaded with protein was isolated from the bulk buffer solution (time, 10–20 min) by removing the buffer with a steel grid[1] and applying the gel slurry to a small thermostated, graduated column which was allowed to run dry by gravity[1]. This loaded gel was then employed for the desorption experiments.

In the 'method of separate dilutions' freshly loaded gel (0.5–2.0 ml packed gel) was blown into fresh buffer solution[11] stirred in a thermostated beaker of 2.5 cm i.d. and 3.5 cm i.d. as described previously. For the high dilutions (e.g. 1:500 and 1:1000) a thermostated 500 ml beaker (10 cm i.d. × 14 cm, stirring bar 8 cm, 200 rev/min) was employed. An equally efficient mixing of the agarose–buffer suspension was obtained at 700 rev/min (beaker 2.5 cm i.d.) 450 rev/min (beaker 3.5 cm i.d.) and 200 rev/min (beaker 10 cm i.d.). Under these conditions the desorption rate was independent of the stirring velocity[12,13]. The enzyme was in the adsorbed state on the gel prior to dilution for a maximal time of ~70–100 min (adsorption procedures). The loaded gel was diluted, incubated for 90–120 min (time-independence) and was then discarded. Samples of 1–3 ml were taken for assay at the indicated times (see Figures) by the steel grid method[1]. Unsubstituted Sepharose 4B was incubated for 90 min prior to dilution with a concentration of free enzyme, so chosen for adsorption that a time-independent concentration of free ligand, comparable to that of the substituted gel, was obtained (see caption to *Figure* 2).

In the 'method of serial dilutions' one volume loaded packed gel was diluted ~30–100 fold and regained after ~30 min for 10 further dilutions respectively in a series of experiments. Such a series takes ~8 h.

* *Immobilized residue concentration*: concentration of alkyl residues covalently linked to the surface of the agarose matrix expressed in μ mol/ml packed gel or in mol residue/mol anhydrodisaccharide[11]

† *Adsorbed ligand concentration*: concentration of ligand non-covalently adsorbed to the surface of the agarose matrix expressed in mg/ml packed gel or mol ligand/mol anhydrodisaccharide[11]

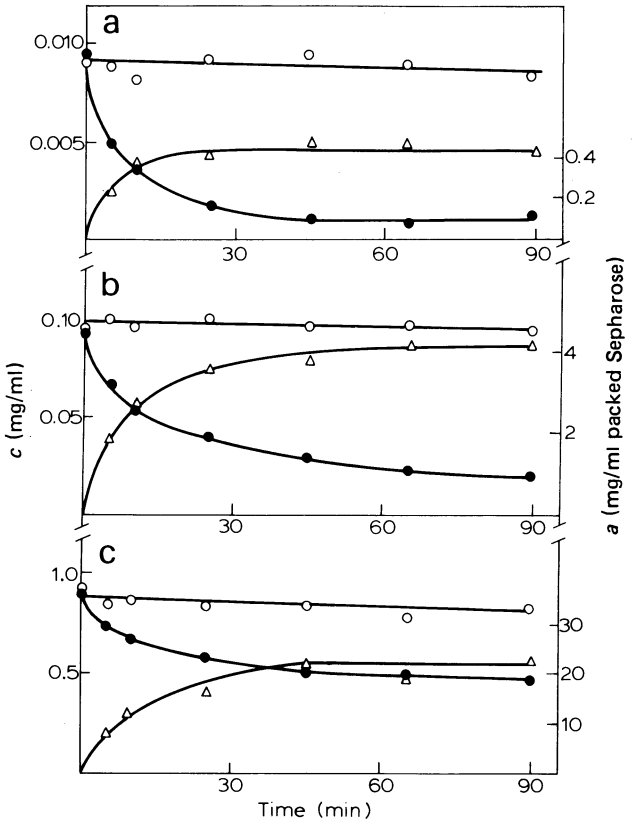

Figure 1 Progress curves of the adsorption of ^{3}H-phosphorylase b_r on butyl-Sepharose (21 μ mol/ml packed gel). (a) initial free ligand concentration, $c_0 \sim 0.009$ mg/ml; (b) $c_0 \sim 0.09$ mg/ml; (c) $c_0 \sim 0.9$ mg/ml. The incubation mixture consisted of ~ 1 ml packed gel incubated in a total volume of ~ 60 ml stirred at 450 rev/min. The sample volume was ~ 0.5 ml. For further details and the calculation of the adsorbed ligand concentration see Experimental and the text. Free enzyme (*c*) ○, unsubstituted Sepharose; ●, butyl-Sepharose, bound enzyme (*a*) △, butyl-Sepharose

Results

Progress curves of adsorption

Initial free enzyme concentrations (c_0) between ~ 0.01–1.0 mg/ml decrease rapidly after addition of butyl-Sepharose (21 μ mol/ml packed gel) to the incubation mixture (*Figure* 1). Incubation of unsubstituted Sepharose 4B in a corresponding mixture also leads to a significant but low decrease in enzyme concentration. The adsorbed ^{3}H-phosphorylase b_r concentration on butyl agarose as calculated from the difference in the concentration progress curves of the two gels (see Experimental and References 1, 11) reaches a practically time-independent plateau (apparent equilibrium) after 30–60 min. Similar kinetics are found at low immobilized residue concentrations (e.g. 5 μ mol/ml packed gel, not shown).

Progress curves of desorption

Dilution of the ^{3}H-phosphorylase b_r–butyl agarose complex at the time zero (a_0), ~ 10–1000 fold (vol. packed gel/vol. buffer) leads to a desorption of enzyme from the gel (21 μ mol/ml packed gel; *Figure* 2). Enzyme is also carried over and released by unsubstituted Sepharose 4B necessitating corresponding corrections. The adsorbed ligand concentration on the gel, as calculated from the corrected amount of enzyme released, decreases and reaches time-independent values (apparent equilibrium) after a time of ~ 10 (10-fold dilution)–90 (1000-fold dilution) min respectively. Incubation at 1000-fold dilution (*Figure* 2*c*) for 180 min (not shown) does not lead to a further significant decrease in the adsorbed ligand concentration. Although the initial, free ligand concentration (c_0) before 1000-fold dilution is decreased ~ 40-fold at time-independence the corresponding adsorbed ligand concentration is reduced by only $\sim 20\%$. Similar kinetics are found at low immobilized residue concentrations (e.g. 5 μ mol/ml packed gel, not shown).

Sorption isotherms

From adsorption and desorption experiments as depicted in *Figures* 1 and 2 respectively, isotherms at apparent time independence were calculated and are shown in *Figures* 3 and 4. For a quantitative description of the isotherms the data have been plotted according to the empirical equation of Freundlich[1,2,3]:

$$\log a = \log \alpha + 1/n_F \log \bar{c} \qquad (1)$$

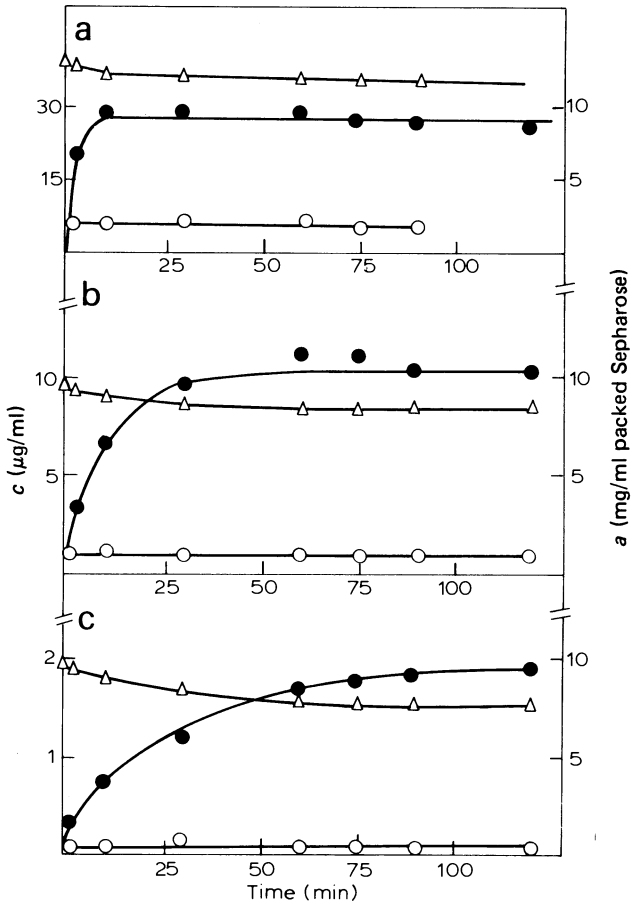

Figure 2 Progress curves of the desorption of ^{3}H-phosphorylase b_r from butyl-Sepharose (21 μ mol/ml packed gel). (a) Dilution 1:10, 1.7 ml packed gel was incubated in a total volume of 16.9 ml stirred at 700 rev/min. The sample volume was ~ 0.5 ml; (b) Dilution 1:100, 0.6 ml packed gel was incubated in a total volume of 60 ml stirred at 450 rev/min. The sample volume was ~ 1.5 ml; (c) Dilution 1:1000, 0.55 ml packed gel was incubated in a total volume of 500 ml stirred at 200 rev/min. The sample volume was ~ 3.5 ml. Prior to dilution the butyl-Sepharose was loaded with ~ 9.6 mg ^{3}H-phosphorylase b_r/ml packed gel (apparent equilibrium concentration ~ 0.06–0.07 mg/ml). The control Sepharose 4B was incubated prior to dilution with an initial concentration of 0.06–0.07 mg/ml enzyme. This initial concentration decreased to ~ 0.058–0.06 mg/ml after 90 min. For further details and the calculation of the adsorbed ligand concentration see Experimental and the text. Free enzyme (*c*) ○, unsubstituted Sepharose; ●, butyl-Sepharose; bound enzyme (*a*) △, butyl-Sepharose

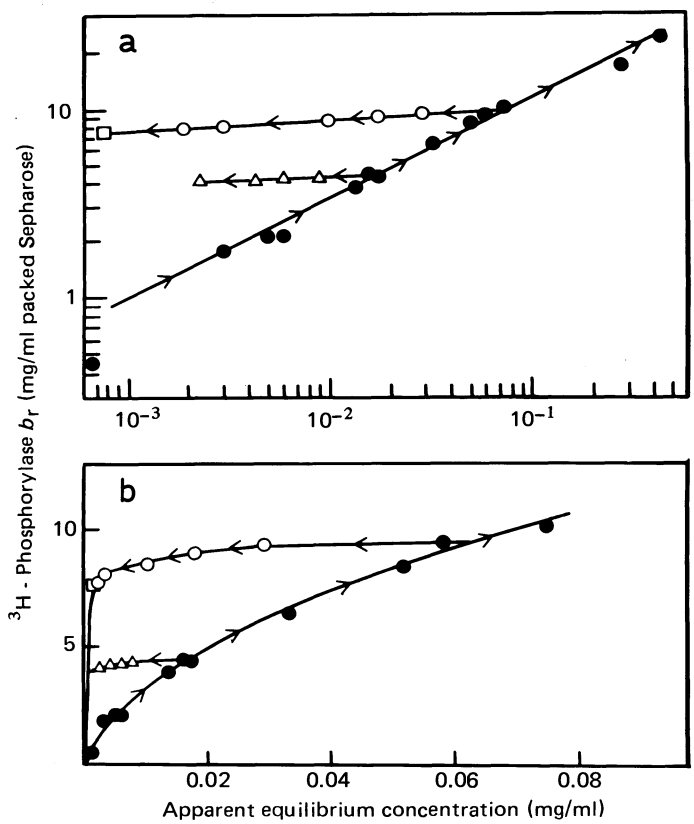

Figure 3 Adsorption and desorption isotherms (loops) of phosphorase b_r on butyl-Sepharose containing 21 μ mol/ml packed gel. (a) Double logarithmic plot of the isotherms; (b) Adsorption-desorption loops of the isotherms in Cartesian coordinates, the two uppermost values of the adsorption isotherm (see a) being omitted for clarity. Arrows indicate the direction of concentration changes imposed by adsorption and desorption. For further details see captions to *Figures* 1 and 2, Experimental and the text. ●, adsorption isotherm; ○, desorption isotherm, initial adsorbed ligand concentration, a_0 = 9.6 mg/ml packed gel; △, desorption isotherm, a_0 = 4.5 mg/ml packed gel; □, data point derived from serial dilution experiment (see Experimental, text and *Table* 1)

where a denotes the adsorbed ligand concentration (mg/ml packed gel), α the sorption constant (mg/ml packed gel), $1/n_F$ the Freundlich sorption exponent and $\bar{c}$ the free ligand concentration of apparent time-independence (apparent equilibrium) in mg/ml. Within certain limits linear isotherms are obtained for adsorption and desorption (*Figures* 3*a* and 4*a*). However the adsorption isotherm cannot be retraced by the desorption isotherm. The adsorption and desorption data plotted in Cartesian coordinates (*Figures* 3*b* and 4*b*) demonstrate that the isotherms may be closed to adsorption-desorption loops which can only be traced in one direction (see arrows) as imposed by adsorption or desorption respectively.

In the example of *Figure* 3 one concentration of free ligand (e.g. $\bar{c}$ = 0.005 mg/ml) is associated with three adsorbed enzyme concentrations (a): 2.1 (adsorption), 4.3 (desorption) and 8.5 (desorption) mg/ml packed gel respectively. Similarly at one constant adsorbed ligand concentration (e.g. a = 3.8 mg/ml packed gel) two different free ligand concentrations ($\bar{c}$) can be obtained: 0.0135 (adsorption) and 0.0021 (desorption) mg/ml respectively.

The size and form of the adsorption-desorption loop are greatly altered if the immobilized butyl-residue concentration is decreased from 21 to 5 μ mol/ml packed gel (*Figure* 4). A 60-fold dilution of the enzyme-butyl agarose complex now releases ~75% of the adsorbed enzyme (lowest point of the desorption isotherm) as compared to ~5-10% on the highly substituted gel (*Figure* 3).

A comparison of the Freundlich constants in *Table* 1 for the butyl-Sepharose containing 21 μ mol/ml packed gel illustrates that the desorption exponents (0.027-0.064) are 8-20 fold lower than the adsorption exponent (0.52) depending on a_0. The sorption constants (α) also differ significantly for adsorption and desorption being a function of the initial adsorbed ligand concentration (a_0). The desorption isotherm obtained by separate dilution experiments (duration ~90 min, see *Figure* 2) cannot be distinguished from the desorption isotherm determined by serial dilutions of one initial enzyme agarose complex (duration ~8 h, see Experimental and *Table* 1). For the interaction of the enzyme with butyl agarose containing 5 μ mol/ml packed gel (*Table* 1) the desorption exponent (0.39) is only ~2-fold lower than the adsorption exponent (0.82). This desorption exponent is ~5-10 fold higher than the corresponding exponents of the gel containing 21 μ mol/ml packed gel.

As was shown previously[1] an estimate of the value for gel saturation with protein (v_{sat}) of adsorption isotherms can be extrapolated from Scatchard plots[1,24]. (For the limitations of this method when employed in conjunction with Hill plots[25] see Reference 1.) In the case of desorption, only the desorption isotherm originating at the saturation point (or range) of the adsorption isotherm (main loop) is extrapolated in the Scatchard plot to the same saturation value as the adsorption isotherm. Since a saturation of alkyl agaroses with protein is difficult to

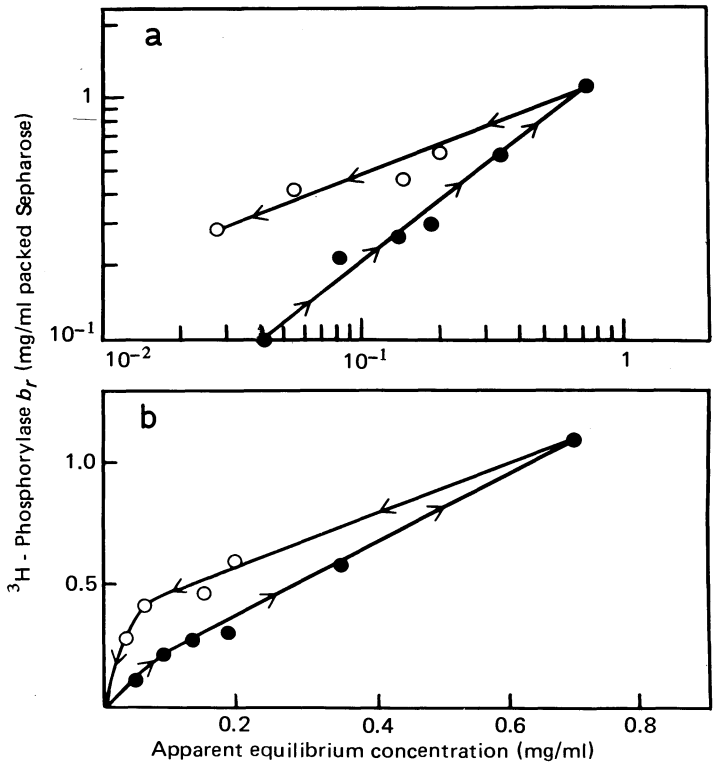

Figure 4 Adsorption and desorption isotherms (loop) of ^{3}H-phosphorylase b_r on butyl-Sepharose containing 5 μ mol/ml packed gel. (a) Adsorption and desorption isotherm in double logarithmic coordinates; (b) Adsorption-desorption loop in Cartesian coordinates. The sample volume was 0.2-0.5 ml. The highest dilution employed for the desorption isotherm was 60 fold. For further details see legends to *Figures* 1-3 and the text. ●, adsorption isotherm; ○, desorption isotherm (a_0 = 1.1 mg/ml packed gel)

Table 1 Constants of the Freundlich equation for the adsorption and desorption isotherms of ^{3}H-phosphorylase b_r on butyl-Sepharose

Isotherm	α $\left(\frac{\text{mg}}{\text{ml packed gel}}\right)$	$1/n_F$	r^2
Adsorption isotherms			
5 μ mol/ml packed gel	1.4	0.82	0.97
21 μ mol/ml packed gel	35.5	0.52	0.99
Desorption isotherms			
5 μ mol/ml packed gel	1.2	0.39	0.94
21 μ mol/ml packed gel			
a_0=4.5 mg/ml packed gel	4.9	0.027	0.99
a_0=9.6 mg/ml packed gel			
separate dilution steps	11.6	0.064	0.99
serial dilution steps	11.9	0.062	0.96

The constants were derived from a least squares analysis (see equation 1) of the isotherms (*Figures* 3 and 4) as described[1]. α, adsorption constant; $1/n_F$, adsorption exponent; r^2, the coefficient of determination; and a_0 the initial adsorbed ligand concentration of the desorption isotherms (i.e. upper closure point, see caption to *Figure* 6). For further details see the captions to *Figures* 3 and 4, the text and Reference 1

obtain[1] only desorption isotherms inside the main loop (upper closure point of loop < saturation value of the adsorption isotherm), so-called scanning curves,[8] were determined. For each scanning curve a separate lower saturation value than that of the main loop can be extrapolated. It is therefore proposed to assign a 'virtual' (intrinsic) saturation value to each desorption scanning curve. Thus virtual saturation ($\bar{v}'_{sat}$) is defined as the extrapolated point on the abscissa of the Scatchard plot corresponding to the desorption isotherm of a scanning curve. These assumptions and definitions allow Hill plots (see *Figure* 5 and the constants in *Table* 2) to be derived from Scratchard plots (not shown, for data see caption to *Figure* 5) of desorption isotherms. The reciprocal of the apparent equilibrium concentration of free protein at which half-maximal saturation of gel is reached is denoted by $K'_{0.5}$. Numerical values for $K'_{0.5}$ may be obtained from Hill plots (*Table* 2); they yield estimates of the magnitude of the affinity differences associated with the two branches of an adsorption–desorption loop. On the gel containing 5 μ mol butyl residues/ml packed gel this half-saturation constant of adsorption ($K'_{a,0.5}$) increases $\sim$1 magnitude in passing from the adsorption to the desorption branch (see half-saturation constant of desorption, $K'_{d,0.5}$ in *Table* 2). In the case of gel containing 21 μ mol/ml packed gel the value of the half-saturation constant increases by $\sim$4 magnitudes in the transition from the adsorption to the desorption branch of the loop (*Table* 2). Furthermore an increase in the immobilized residue concentration is parallelled by an increase in the corresponding half-saturation constant.

Discussion

As seen in *Figures* 1 and 2 the time course of the adsorption and desorption curves shows that apparent time-independent values are reached after $\sim$30–90 min within the accuracy of the experimental technique. It can therefore be excluded that the adsorbed ligand concentrations employed in the isotherms (*Figures* 3 and 4) are a result of insufficient equilibration. The adsorption–desorption loops however demonstrate that true, thermodynamic equilibria (see Discussion below) are not reflected in these experiments.

Pore obstruction of the agarose beads as a cause of the difference between the adsorption and desorption isotherms (*Figures* 3 and 4) can be ruled out in the following way: (1), non-retracing desorption isotherms are already found at low fractional gel saturation (e.g. θ=0.10, i.e. 10%, at a_0=4.5 mg/ml packed Sepharose, (see *Figure* 3);

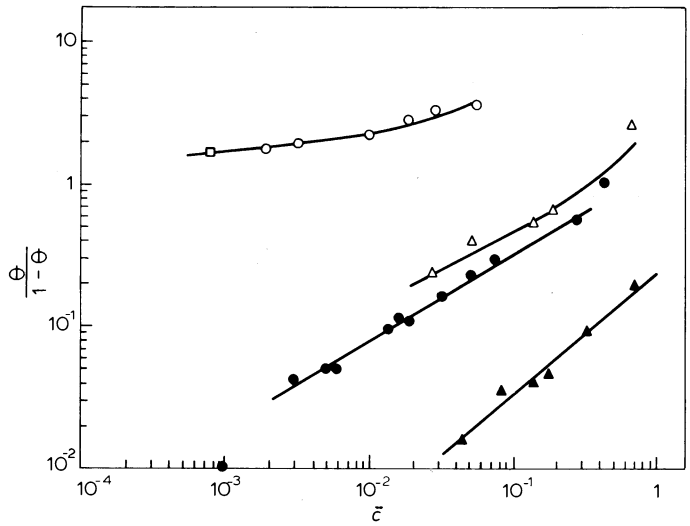

Figure 5 Hill plots of adsorption and desorption isotherms of ^{3}H-phosphorylase b_r on butyl-Sepharoses. The solid lines were calculated according to equation 1 and the constants of *Table* 1 employing the logarithmic form of the Hill equation[1,25]: $\log(\theta/1-\theta)=\log K_H+n_H \log \bar{c}$. θ, denotes fractional saturation; $\bar{c}$, is the apparent equilibrium concentration of ligand; K_H, is the Hill constant and n_H is the Hill coefficient. The apparent association constant of half-maximal saturation ($K'_{0.5}$) can be derived from the equation: $K_H=(K'_{0.5})^{n_H}$. Fractional saturation was calculated from the following apparent (adsorption, Reference 1) and virtual (desorption) saturation values ($\bar{v}_{sat}$) and ($\bar{v}'_{sat}$) respectively: ●, adsorption isotherm from *Figure* 3, $\bar{v}_{sat}\sim$45 mg/ml; ○ and □, desorption isotherm from *Figure* 3 (a_0=9.6 mg/ml packed gel), $\bar{v}'_{sat}\sim$12 mg/ml packed gel; ▲, adsorption isotherm from *Figure* 4, $\bar{v}_{sat}\sim$6.8 mg/ml packed gel; △, desorption isotherm from *Figure* 4, $\bar{v}'_{sat}\sim$1.5 mg/ml packed gel. For further details see captions to *Figures* 3 and 4, *Table* 2, Reference 1 and the text.

Table 2 Constants of the Hill plots of the adsorption and desorption of ^{3}H-phosphorylase b_r on butyl-Sepharoses

Immobilized residue concentration	n_H	$K'_{0.5}$, M^{-1}
5 μ mol/ml packed gel		
Adsorption isotherm	0.85	1.8×10^4
Desorption isotherm	0.52	2.2×10^5
21 μ mol/ml packed gel		
Adsorption isotherm	0.61	1.6×10^5
Desorption isotherm	0.14	4.2×10^9

The Hill coefficients (n_H) and the apparent association constants of half-maximal saturation ($K'_{0.5}$) which are defined in the caption to *Figure* 5 were derived from the linear portions of the Hill plots (*Figure* 5). $K'_{0.5}$ can be differentiated into a half saturation association constant of adsorption ($K'_{a,0.5}$) and desorption ($K'_{d,0.5}$). For further details see the caption to *Figure* 5 the text and Reference 1

(2), at constant pore radius (see Reference 11) the isotherms are a function of the immobilized residue concentration (*Figures* 3 and 4); and (3), the initial dissociation rate of ^{3}H-phosphorylase b_r–agarose complex can be enhanced 3–5 fold by free unlabelled enzyme in the bulk solution (see References 11–13). This latter observation strongly indicates that the adsorbed enzyme molecules must be easily accessible to free enzyme in the bulk solution.

Restricted diffusion occurs when the pore dimensions, e.g. of a membrane, are comparable to the size of the diffusing solute molecule[26,27]. Beck and Schultz[27] have shown that for molecules ranging from 0.52 to 4.3 nm in diameter, the extent of hindrance *versus* the ratio of solute to pore radius correlates well with the Renkin equation[28]. The pore radius of Sepharose 6B has been reported to be ~ 40 nm[29]. From this value the pore radius of Sepharose 4B can be estimated by employing the exclusion limits of these gels for globular proteins. Since the exclusion limit for Sepharose 6B[30] corresponds to a molecular weight of 4×10^6 and for Sepharose 4B[30] to a molecular weight of 20×10^6 and since the effective molecular radius is proportional to the 0.33–0.5 power of the molecular weight[31] it can be calculated that the effective pore radius of Sepharose 4B is 1.7–2.2 fold larger (i.e. 68–89 nm) than the pore radius of Sepharose 6B. These data are also in agreement with the parameters obtained from chromatography of protein–SDS complexes on controlled pore glass[32]. In this chromatographic system a molecular weight of 0.95×10^6 was found as the exclusion limit on controlled pore glass containing a pore radius of 33 nm. From these experiments the authors calculated that the effective radius of the protein is proportional to the 0.41 power of the molecular weight. From the dimensions of the phosphorylase *b* molecule (6.3 nm × 6.3 nm × 11.6 nm, Reference 9) and the calculated mean pore radius of ~ 80 nm for Sepharose 4B a ratio of effective ligand to pore radius of 0.072 is obtained. From this ratio it can be estimated[27] that the apparent diffusivity of phosphorylase *b* in the pores of Sepharose 4B is reduced by $\sim 25\%$ *versus* the diffusivity in water. Equilibration is therefore not significantly hindered by restrictions in diffusion within the scope of these experiments.

The above ratio of solute to pore radius is also the basis of the planar lattice concept proposed[1] for the adsorption of proteins to hydrophobic agarose gels. On the inside of a Sepharose 4B pore a planar lattice of binding sites can be constructed in a similar manner as has been devised for the outer surface of spheres[33,34].

Since insufficient equilibration and restrictions of diffusion can be excluded as a cause of adsorption–desorption loops (*Figures* 3 and 4) it can be concluded that hydrophobic agaroses exhibit *adsorption hysteresis*. The hysteresis loop originating at the saturation point (or range) of an adsorption isotherm (main upper closure point or range) is called the main hysteresis loop[8]. Of the scanning curves[8] which lie inside the main hysteresis loop (e.g. *Figure* 3) an infinite number can be thought to exist.

As true thermodynamic equilibria are not obtained in hysteretic systems[6,8] the equations according to Freundlich, Scatchard, Hill and Clausius–Clapeyron (see below) do not apply in a strict sense. However, in dealing with long-lived metastability (*Figures* 1–4) the state of apparent time-independence may be taken to manifest an apparent equilibrium. Therefore (see also the Discussion on the Clausius–Clapeyron equation) these equations can be descriptively employed to yield *apparent* parameters. The application of these equations to hysteretic systems can also be justified on the basis that the states encountered in long-lived metastability can be described by thermodynamic state functions (see below) in the same way as equilibrium states[6,35]. The novel situation of adsorption hysteresis necessitated the introduction of 'virtual' saturation (see Results to *Figure* 5) which together with Hill plots allows the evaluation of scanning curves in terms of apparent association constants of half-maximal saturation (*Table* 2) until more exact methods are developed. The half-saturation constants of adsorption ($K'_{a,0.5}$) and desorption ($K'_{d,0.5}$) may be tentatively classified into an apparent nucleation and a lattice propagation binding constant respectively, in which case $K'_{a,0.5} < K'_{d,0.5}$ (see model below).

Thermodynamically the adsorption hysteresis of gases on solids can be expressed as a work cycle by the cyclic integral[8]:

$$\Delta W = \oint dW = -RT \oint n^a \cdot d \ln p \qquad (2)$$

where W is the work performed n^a are the moles gas adsorbed and p is the pressure. The accompanying 'loss of work'[36] i.e. irreversible entropy ($\Delta_i S$, References 8 and 37) production would be:

$$\Delta_i S = R \oint n^a \cdot d \ln p \qquad (3)$$

For an ideal solution under isothermal, isobaric conditions the molar irreversible entropy* is:

$$\Delta_i S = R \oint N \cdot d \ln c \qquad (4)$$

where N denotes the dimensionless quotient a/a_0 (see equation 1 and captions to *Figures* 3 and 6 for definitions) with a_0 symbolizing the adsorbed ligand concentration of the upper closure point (see Reference 37). The molar concentration of free ligand at apparent equilibrium is denoted by $\bar{c}$. Therefore in a plot of N *versus* $\ln \bar{c}$ the molar irreversible entropy produced will be equal to the area of the loop multiplied by R. The prerequisite is however, that the system returns to the initial state i.e. a closed loop with a lower and an upper closure point is obtained. Since the lower closure point in the hysteresis loops corresponds to the origin in *Figures* 3*b* and 4*b* this point may be extrapolated to $N = 0$ in a plot of N *versus* $\ln \bar{c}$. This is shown for butyl-Sepharose substituted with 5 μ mol/ml packed gel in *Figure* 6. The extrapolated free enzyme concentration (for $N = 0$) is 0.98×10^{-7} M. This value lies ~ 50-fold below the $(K'_{d,0.5})^{-1}$ value of the desorption isotherm and ~ 500 fold below the $(K'_{a,0.5})^{-1}$ value of the adsorption isotherm (see *Table* 2). The irreversible entropy (see equation 4) produced in this cycle per mol (a_0 = 1.1 mg/ml packed gel; $\theta = 0.16$) is $\Delta_i S = 5.9$ J mol^{-1}K^{-1} (*Table* 3). If it is argued that a much lower concentration of free ligand may be the true value for the condition $N = 0$, the area of the loop closing at 10^{-8} M was determined and yields $\Delta_i S = 6.7$ J mol^{-1} K^{-1}. Thus an error in the

* The irreversible free energy can be calculated according to the equation[6]: $\Delta_i G = -RT \oint N \cdot d \ln c$

*see note on last page

extrapolation of ~1 magnitude imposes an error of ~12% on the calculated entropy value. If the same procedure (e.g. *Figure* 6) is applied to the hysteresis loop of the gel substituted with 21 μ mol/ml packed gel (*Figure* 3, $a_0 = 9.6$ mg/ml packed gel; $\theta = 0.21$) extrapolation to $N = 0$ leads to concentrations between 10^{-11}–10^{-12} M (not shown) which lie ~25–250 fold below the $(K'_{0.5})^{-1}$ value of the desorption isotherm (see *Table* 2). The minimum value of the irreversible entropy change produced in this loop is ~33–42 J mol^{-1} K^{-1}. Comparing $\Delta_i S$ of the two gels (5 and 21 μ mol/ml packed gel) at similar fractional saturation of the upper closure point (θ ~0.16–0.21), shows that a ~6-fold higher molar irreversible entropy is produced on the gel of 21 than on the gel of 5 μ mol/ml packed gel. These calculations again strongly indicate that hysteresis described by the irreversible entropy change is correlated to the number of alkyl residues interacting with the protein ligand.

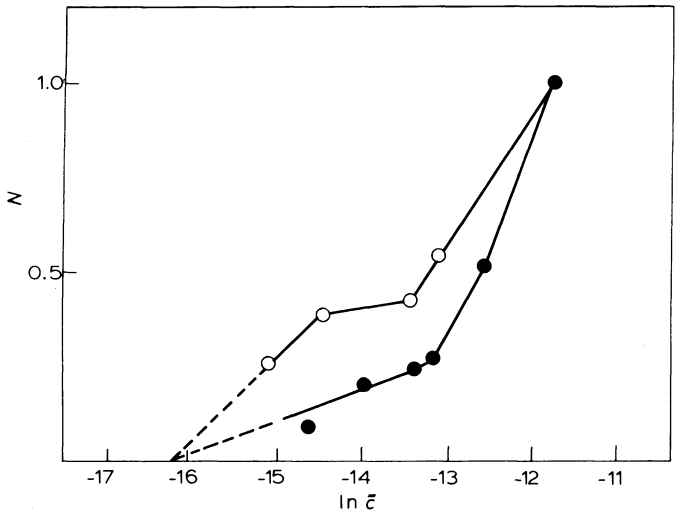

Figure 6 Semilogarithmic plot of the hysteresis loop on butyl-Sepharose containing 5 μ mol/ml packed gel for the calculation of $\Delta_i S$. N is the dimensionless quotient of a/a_0. The adsorbed ligand concentration at the upper closure point (a_0) is 1.1 mg/ml packed gel (see equation 1 and caption to *Figure* 4). The apparent free ligand equilibrium concentration $\bar{c}$ is expressed in mol/l. ---, indicate the mode of extrapolation. The area of the loop was determined planimetrically. For further details see caption to *Figure* 4, equation 4 and the Discussion.

In a previous paper[1] the Clausius–Clapeyron equation was applied to the adsorption isotherms of phosphorylase *b* on alkyl agaroses for the calculation of the isosteric, molar enthalpy (heat) of adsorption ($\Delta_a H_i$). Since however, as shown here (*Figures* 3 and 4) the interaction of phosphorylase *b* with butyl-Sepharose involves thermodynamically irreversible steps, the Clausius–Clapeyron equation is not applicable in a strict sense; it can nevertheless be employed for the analysis of hysteresis loops yielding *apparent* molar enthalpy ($\Delta_a H'_i$) values (see basic paper of Everett and Whitton, Reference 37). Since it is of interest to compare the apparent isosteric, molar entropy of adsorption ($\Delta_a S_i^{\dagger\prime}$) with the molar irreversible entropy ($\Delta_i S$) produced in a hysteresis loop, $\Delta_a S_i^{\dagger\prime}$ has been calculated from the apparent isosteric enthalpy of adsorption (see caption to *Table* 3 and References 36 and 37) for butyl-Sepharose containing 29 μ mol/ml packed gel[1]. Under standard conditions (for definitions see caption to *Table* 3) the apparent *standard* isosteric thermodynamic state functions are obtained (*Table* 3). Since at 5°C the adsorption of phosphorylase *b* to butyl-Sepharose containing 21 μ mol/ml packed gel is indistinguishable from the adsorption of the enzyme to a gel containing 29 μ mol/ml packed gel (see Reference 11 and also the $\Delta_a G_c^{0\prime}$ values in *Table* 3), the state functions of these two gels can be compared. An evaluation of the

*__Table 3__ Apparent, thermodynamic state functions of adsorption and desorption including the irreversible entropy of hysteresis for the binding of phosphorylase *b* to butyl-Sepharose at 5°

Immobilized residue concentration (μ mol/ml packed gel)	θ	$\Delta_a H'_i$ (kJ mol^{-1})	$\Delta_a S_i^{\dagger\prime}$ (J mol^{-1} K^{-1})	$\Delta_a G'_i$ (kJ mol^{-1})	$\Delta_a G_c^{0\prime}$ (kJ mol^{-1})	$\Delta_d G_c^{0\prime}$ (kJ mol^{-1})	$\Delta_i S$ (J mol^{-1} K^{-1})
5	0.16				−22.7	−28.5	5.9
21	0.21				−27.7	−51.3	33–42
29	0.11	56.1	327	−34.9			
	0.22	37.3	248	−31.8			
	0.33	24.7	197	−30.1			
	0.5	16.3	160	−28.3	−26.6		

The apparent isosteric, molar enthalpy of adsorption ($\Delta_a H'_i$) as calculated from the Clausius–Clapeyron equation is taken from Reference 1. Fractional gel saturation as denoted by θ, is valid for all state functions in a line except $\Delta_a G_c^{0\prime}$ and $\Delta_d G_c^{0\prime}$ unless the fractional saturation of adsorption or desorption is 0.5. For $\Delta_i S$, θ corresponds to the upper closure point. The apparent isosteric molar entropy of adsorption ($\Delta_a S_i^{\dagger\prime}$) for the standard† condition, $c^\dagger = 1$ M, is calculated from the integrated form of the Clausius–Clapeyron equation (temperature independent ΔH, see Reference 36, 37) modified for ideal solutions and the adsorption system employed in this paper: $\ln \bar{c}_i = -(\Delta_a H'_i/RT) + (\Delta_a S_i^{\dagger\prime}/R)$. The concentration $\bar{c}_i$ is equal to the apparent free ligand equilibrium concentration in the plot $\ln \bar{c}_i$ *versus* $1/T^{-1}$ and is correlated to θ of the isostere. The apparent molar Gibbs energy change of isosteric adsorption ($\Delta_a G'_i$) was calculated from $\Delta_a H'_i$ and $\Delta_a S'_i$. Under complete standard conditions: $c_\dagger = 1$ M and $\theta = 0.5$, the apparent *standard* isosteric thermodynamic state functions are obtained: $\Delta_a H_i^{0\prime}$, $\Delta_a S_i^{0\prime}$ and $\Delta_a G_i^{0\prime}$ (see state functions for $\theta = 0.5$ in the Table above). The apparent molar standard Gibbs energy change of adsorption and desorption as calculated directly ($\Delta G_c^0 = -RT \ln K_c$) from the apparent association constants of half-maximal saturation ($K'_{a,0.5}$, $K'_{d,0.5}$, see *Table* 2 and Reference 1) are symbolized by $\Delta_a G_c^{0\prime}$ and $\Delta_d G_c^{0\prime}$ respectively. The calculation of the irreversible entropy ($\Delta_i S$) produced in a hysteresis cycle is described in the Discussion to *Figure* 6. For the nomenclature and symbols employed in the Table see the 'Recommendations for Measurement and Presentation of Biochemical Equilibrium Data, *Eur. J. Biochem.* 1977, **72**, 1 and the Appendix II, Part I (Definitions, Terminology and Symbols in Colloid and Surface Chemistry) of the 'UPAC Manual of Symbols and Terminology for Physicochemical Quantities and Units, *Pure Appl. Chem.* 1972, **31**, 579

*see note on last page

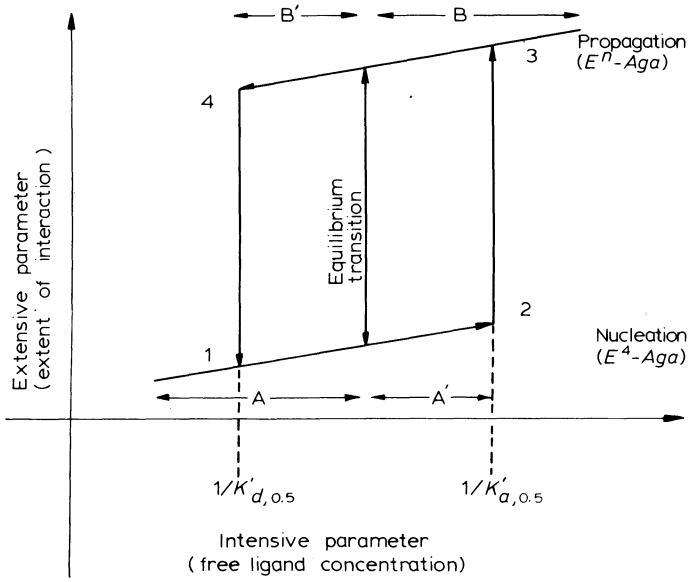

Figure 7 Schematic representation of a domain for a hysteretic adsorption-desorption cycle of phosphorylase *b* on butyl-Sepharose. *E* symbolizes the enzyme and *Aga* the substituted agarose matrix (i.e. binding unit, Reference 11). The number in superscript (E^4, E^n; *n* is a positive integer, $n>4$) denotes the assumed valence (i.e. number of butyl residues) involved in binding per binding unit. *A*, first stable state e.g. an unnucleated form, of phosphorylase *b*; *A'*, the metastable state of *A* e.g. a nucleated form of the enzyme (E^4-*Aga*); *b*, second stable state e.g. phosphorylase *b* after propagation (E^n-*Aga*); *B'* the metastable state of *B*. For further details see the Discussion and Reference 38.

entropy values at equivalent fractional saturation ($\theta \sim 0.2$) shows (*Table* 3) that the irreversible entropy ($\Delta_i S$) produced (i.e. 33–42 J mol^{-1} K^{-1}) is ~13–17% of the apparent entropy of adsorption ($\Delta_a S_i{}^{\dagger\prime} = 248$ J mol^{-1} K^{-1}). Presuming that $\Delta_i S$ for the standard condition of $\theta = 0.5$ (i.e. $\Delta_i S^0$) will be somewhat larger but of the same magnitude as $\Delta_i S$ for $\theta = 0.21$, it can be estimated that the standard irreversible entropy produced ($\Delta_i S^0$) will be ~30% of the apparent standard entropy of adsorption of $\Delta_a S_i^{0\prime} = 160$ J mol^{-1} K^{-1}. The apparent standard molar Gibbs energy change ($\Delta_a G_i^{0\prime} = -28.3$ kJ mol^{-1}) as calculated from the isosteres (*Table* 3) compares quite well with the apparent, standard Gibbs energy change ($\Delta_a G'^0_c = -26.6$ to -27.7 kJ mol^{-1}) as calculated directly from the $K'_{0.5}$ of adsorption (*Table* 3). The difference of the two values is due to the fact that $K'_{a,0.5}$ is calculated from the linear portion of the Hill plots of adsorption (*Figure* 5, Reference 1). In reality the Hill plots curve upwards at high fractional saturation ($\theta/(1-\theta) > 1$) yielding higher $K'_{0.5}$ values for extrapolations from these nonlinear portions of the plot. An interesting and as yet open question is: if and how the standard irreversible entropy ($\Delta_i S^0$) produced is correlated to the changes in water structure which entropically ($\Delta_a S_i^{0\prime}$) drive the adsorption process (*Table* 3).

As has been shown previously[11] the concentration of adsorbed phosphorylase *b* is a sigmoidal function of the immobilized butyl residue concentration. Cooperative, multivalent, all-or-none adsorption on a minimum of 4 butyl residues can be concluded from Hill plots[1,11] to be the predominant reaction in the saturation (plateau) region of the sigmoidal curve at 5 C (e.g. 21 μ mol/ml packed gel). These basic observations and adsorption-desorption loops (*Figures* 3 and 4; i.e. affinity increase in the transition from the adsorption to the desorption branch of the loop) can be employed for a model of adsorption hysteresis on butyl-Sepharose on the basis of multivalent ligand-matrix interactions. Intermolecular, nearest neighbour interactions (i.e. attraction, repulsion) during adsorption are very improbable for phosphorylase *b*[1] especially at low (e.g. 10%) fractional gel saturation, (see *Figures* 3 and 4 and caption to *Figure* 5). *Figure* 7 schematically illustrates a domain during the adsorption–desorption cycle of phosphorylase *b* on butyl-Sepharose in analogy to schemes of Katchalsky[38]. On the ordinate the extent of the ligand–matrix interaction (i.e. number of alkyl residues interacting with the ligand, valence) is depicted as a variable. The valence is correlated to the free energy of binding over the half-saturation constants ($K'_{0.5}$). On the abscissa the free ligand concentration is shown. After adsorption (nucleation) on a minimum of 4 alkyl residues (E^4-*Aga*, see $1/K'_{a,0.5}$) the cooperative reaction is propagated in a second interaction until *n* residues have reacted with the enzyme (path: 1–2–3). Subsequent to propagation (E^n-*Aga*, see $1/K'_{d,0.5}$) desorption occurs via the path 3–4–1. Two metastable states *A'* (for adsorption) and *B'* (for desorption) may be assumed, both of which could be caused by energy barriers that must be overcome for nucleation as well as for desorption respectively. It is probable that the hysteresis observed here as elsewhere[6] is a strong indication for cooperativity leading to metastability. The affinity change correlated with the transition from the adsorption to the desorption branch of the loop (*Tables* 2 and 3) is a strong indication for metastability and the existence of state *A'* with the following irreversible transition to state *B*. From the adsorption and desorption measurements shown here other irreversible steps (*Figure* 7) can however not be definitely specified. During nucleation the correct orientation of the ligand for the cooperative interaction of e.g. 4 butyl residues with the protein may be of critical importance. Besides multivalent binding (valence-model in *Figure* 7) a conformational change of the adsorbed protein (conformational model) could be additionally involved in an adsorption-desorption loop. In such a model, for which there is as yet no evidence, a strain would be imposed on the protein by the multivalent interactions of nucleation which would induce a change in the conformation and binding properties of the ligand. Then propagation could take place. Gross unfolding of phosphorylase *b* on the hydrophobic matrix is however improbable. This can be concluded from experiments which show that phosphorylase *b*[39] and other enzymes[40,41] retain their catalytic activity when adsorbed to hydrophobic agaroses. Elution of active phosphorylase *b* from such gels[15,16,38] also excludes denaturation by unfolding of the protein. Therefore phosphorylase *b* appears to behave as a quite rigid molecule in the reaction with alkyl agaroses[1] although smaller scale deformations of the protein cannot be excluded.

Biologically, adsorption hysteresis as a phenomenon caused by multivalent interactions in general (homogeneous and heterogeneous binding site lattice, Reference 1, 5) would be of special interest since a hysteresis loop could generate and store information e.g. on cell surfaces. Desorption data which could be interpreted as indicating a non-retracing behaviour *versus* adsorption as postulated above have been reported e.g. for the interaction of concanavalin A with fat cells[42] and recently for the interaction of growth hormone with hepatocytes[43].

Acknowledgements

For valuable technical help the authors would like to thank Miss I. Gajewski. For stimulating discussions in the preparation of the manuscript the authors thank Dr. G. Findenegg (Bochum) and Dr. H. Wieker (Dortmund). The authors are particularly grateful to Dr. E. Neumann (Martinsried) for a review of the manuscript and helpful suggestions. This work was supported by a grant from the Deutsche Forschungsgemeinschaft (Je 84/4).

References

1 Jennissen, H. P. Biochemistry, 1976, **15**, 5683; *ibid* 1977, **16**, 2312
2 Latt, S. A. and Sober, H. A. *Biochemistry* 1967, **6**, 3293
3 McGhee, J. D. and von Hippel, P. H. *J. Mol. Biol.* 1974, **86**, 469
4 Draper, D. E. and von Hippel, P. H. *J. Mol. Biol.* 1978, **122**, 321
5 Jennissen, H. P. *Hoppe-Seyler's Z. Physiol. Chem.* 1976, **357**, 1727
6 Neumann, E. *Angew. Chem. Int. Ed.* Engl. 1973, **12**, 356
7 Cox, R. A., Jones, A. S., Marsh, G. E. and Peacocke, A. R. *Biochem. Biophys. Acta* 1956, **21**, 576
8 Everett, D. H. in 'The Solid Gas Interface', (Ed. E. A. Flood) Marcel Dekker, New York, 1967, Vol 2, pp 1055–1113
9 Johnson, N. L., Madsen, N. B., Mosely, J. and Wilson, K. S. *J. Mol. Biol.* 1974, **90**, 703
10 Titani, K., Koide, A., Hermann, J., Ericsson, L. H., Kumar, S., Wade, R. D., Walsh, K. A., Neurath, H. and Fischer, E. H. *Proc. Natl. Acad. Sci. USA* 1977, **74**, 4762
11 Jennissen, H. P. *J. Chromatogr.* 1978, **159**, 71
12 Jennissen, H. P. *Hoppe-Seyler's Z. Physiol. Chem.* 1978, **359**, 281
13 Jennissen, H. P. and Botzet, G. *Protides Biol. Fluids Proc. Colloq.* (*May* 1978), 1979, **26**, 657
14 Jennissen, H. P. *Hoppe-Seyler's Z. Physiol. Chem.* 1978, **359**, 1101
15 Jennissen, H. P. and Heilmeyer, L. M. G. Jr. *Biochemistry* 1975, **14**, 754
16 Jennissen, H. P. *Protides Biol. Fluids Proc. Colloq.* (*May* 1975), 1976, **23**, 675
17 Fischer, E. H. and Krebs, E. G. *J. Biol. Chem.* 1958, **231**, 65
18 Cohen, P., Duewer, T. and Fischer, E. H. *Biochemistry* 1971, **10**, 2683
19 Haschke, R. H. and Heilmeyer, L. M. G. Jr. *Anal. Biochem.* 1972, **47**, 451
20 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. *J. Biol. Chem.* 1951, **193**, 265
21 Strausbauch, P. H., Kent, A. B., Hedrick, J. L. and Fischer, E. H. *Methods Enzymol.* 1967, **11**, 671
22 Haschke, R. H., Heilmeyer, L. M. G. Jr., Meyer, F. and Fischer, E. H. J. Biol. Chem. 1970, **245**, 6657
23 Freundlich, H. *Kapillarchemie*, 2nd Edn, Akademische Verlaganstalt Leipzig, 1922, 150
24 Scatchard, G. *Ann. N.Y. Acad. Sci.* 1949, **51**,660
25 Hill, A. V. *J. Physiol.* 1910, **40**, Proceedings IV–VII
26 Manegold, E. *Kolloid Z.* (*Colloid and Polym. Sci.*) 1929, **49**, 372
27 Beck, R. E. and Schultz, J. S. *Science* 1970, **170**, 1302
28 Renkin, E. M. *J. Gen. Physiol.* 1954, **38**, 225
29 Lasch, J., Iwig, M., Koelsch, R., David, H. and Marx, I. *Eur. J. Biochem.* 1975, **60**, 163
30 Pharmacia Fine Chemicals 'Sepharose 2B-4B-6B in Bead Form' Uppsala, 1969, 3
31 Rodbard, D. in 'Methods of Protein Separation' Vol 2 (Ed. N. Catsimpoolas) Plenum Press, New York, 1976, pp. 145–179
32 Collins, R. C. and Haller, W. *Anal. Biochem.* 1973, **54**, 47
33 Caspar, D. L. D. and Klug, A. *Cold Spring Harbor Symp. Quant. Biol.* 1962, **27**, 1
34 Laiken, N. and Nemethy, G. *J. Phys. Chem.* 1970, **74**, 4421
35 Katchalsky, A. and Spangler, R. *Quart. Rev. Biophys.* 1968, **1**, 127
36 Prigogine, I. and Defay, R. in 'Chemical Thermodynamics', (Trans. D. H. Everett), 5th Edn, Longmans, London, 1969, p 44 and 198
37 Everett, D. H. and Whitton, W. I. *Proc. Roy. Soc.* (*A*) 1955, **230**, 91
38 Katchalsky, A. in 'Biology and the Physical Sciences', (Ed. S. Devons), Columbia University Press, New York, 1969, pp 267–298
39 Shaltiel, S. *Metab. Interconvers. Enzymes Int. Symp. 3rd* 1973, 1974, 379
40 Hofstee, B. H. J. and Otillio, N. F. *Biochem. Biophys. Res. Commun.* 1973, **53**, 1137
41 Caldwell, K. D., Axen, R., Bergwall, M. and Porath, J. *Biotechnol. Bioeng.* 1976, **18**, 1573
42 Cuatrecasas, P. *Biochemistry* 1973, **12**, 1312
43 Donner, D. B., Martin, D. W. and Sonenberg, M. *Proc. Natl. Acad. Sci. USA* 1978, **75**, 672

*Note:
The somewhat unusual term "irreversible state function" (i.e. "irreversible entropy") does not indicate that the state function itself i.e. entropy is irreversible, but that this state function is