Journal of Solid-Phase Biochemistry, Vol. 4, No. 3, 1979

MULTIVALENT ADSORPTION MECHANISMS IN HYDROPHOBIC CHROMATOGRAPHY[1]

HERBERT P. JENNISSEN

Institut für Physiologische Chemie
Ruhr-Universität Bochum
D-4630 Bochum 1 Postfach 10 24 48, W. Germany

Accepted May 22, 1979

INTRODUCTION

Hydrophobic chromatography, a form of multivalent interaction chromatography, has been the subject of a number of recent reviews (1–6), and therefore will not be extensively reviewed here.

Two types of hydrophobic matrices have mainly been employed: (*a*) agarose gels containing alkyl residues together with positive charges (2,3,5–7) in a ratio of ca. 4–8 : 1 (5,8,9), and (*b*) uncharged alkyl or benzyl derivatives of Sepharose (10,11). Both types of gels adsorb proteins at low ionic strength (1,12) as well as at high ionic strength (10,11,13,14).

Therefore, two types of chromatography (salting-in and salting-out chromatography) can be distinguished on these materials (14). They are based on the two different effects of salts on hydrophobic interactions (see presentation by W. G. Hanstein in this issue): (*a*) salting-in, a decrease in hydrophobic interactions by salts (8,14–16); and (*b*) salting-out, an increase in hydrophobic interactions by salts (10,11,13,17). Since additional factors are involved in salting-out chromatography, an exothermic interaction type must be distinguished from an endothermic interaction type (3,9). There is probably a continuous transition from exothermic salting-out chromatography on substituted gels to salting-out chromatography on unsubstituted gels (see presentation by F. von der Haar in this issue and ref. 18).

As to the mechanism of adsorption, it appears that the model, based on the structural concept of a *planar lattice* of homogeneous or heterogeneous binding sites on the agarose gel and the functional concept of *cooperative, multivalent protein binding* on this lattice put forward by Jennissen in 1976 (9,19,20), is being recognized as a plausible alternative to other models.

[1]This paper was presented at the symposium on "Hydrophobic and Biospecific Affinity Chromatography" held at Ruhr-Universität, Bochum, West Germany, October 4, 1978.

0146-0641/79/0900-0151$03.00/0

The first direct, experimental evidence for the adsorption of proteins on more than one alkyl residue was presented by us in the form of the nonhyperbolic, sigmoidal curves of protein binding as a function of the immobilized alkyl residue concentration at low (8,19) and at high (20) ionic strength. These experiments allowed the calculation of a minimum of 3–5 binding sites for the adsorption of phosphorylase *b* and phosphorylase kinase on butyl-Sepharose (3,20). In agreement with these results, non-hyperbolic curves of phosphorylase binding versus the concentration of immobilized alkyl residues have recently been published by Shaltiel (5).

This short survey of some late developments (21–24) in our laboratory on the adsorption mechanism of proteins on hydrophobic agaroses serves two ends: (*a*) the development of more efficient gels for the purification of proteins, and (*b*) the understanding of multivalent protein binding on two-dimensional binding-site lattices in general. Specifically in this paper, sorption kinetics and adsorption hysteresis will be discussed.

METHODS

Phosphorylase *b* was prepared according to ref. (25). For further details see ref. (9). Enzyme activity (26) and protein (27) were determined on an Autoanalyzer. Reduced, tritium labeled (^{3}H-phosphorylase b_r) and unlabeled enzyme (40–50 U/mg) were prepared according to ref. (28). After reduction the enzyme was extensively dialyzed against buffer containing 10 mM tris (hydroxymethyl) amino methane/maleate, 5 mM dithioerythritol, 1.1 M ammonium sulfate, 20% sucrose, pH 7.0 (buffer A), which was employed for all experiments. For the determination of radioactivity, ^{3}H-phosphorylase b_r was precipitated in 5% Cl_3CCOOH in the presence of ca. 2.5 mg bovine serum albumin and counted according to ref. (29), or solubilized in 1 ml 0.1 M NaOH, 1% sodium dodecylsulfate and counted in 10 ml scintillator (Quickscint 212, W. Zinsser, Frankfurt). Quenching was corrected by employing an external standard.

The preparation and analysis of ^{14}C-labeled alkyl derivatives of Sepharose 4B have been previously described (8,14). The stability of such gels is shown in Fig. 1. Under storage conditions in 0.01% NaN_3 at 5°C, the highest alkyl residue leakage (20–30% of the total amount immobilized) occurs in the first week after activation and coupling of the gels. At ca. 1 month the gels are quite stable (loss: 5–10%/month). After ca. 2 months the gels were transferred to 10 mM sodium β-glycerophosphate, 0.001% NaN_3 at pH 7.0 (buffer B). Neither this change in medium nor the removal of dissociated alkyl amine had a large effect on the decay rate. For quantitative work, gels stored as above should be analyzed at short intervals during the

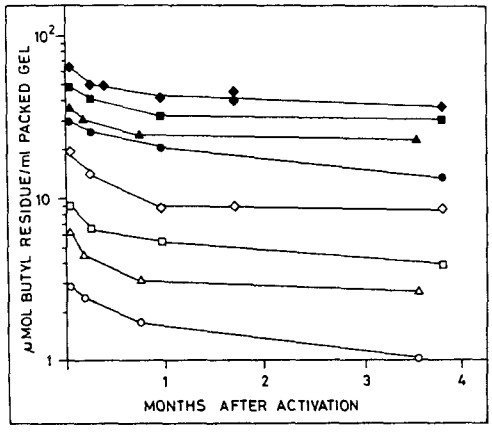

FIG. 1. Semilogarithmic plot of the time dependent decrease in immobilized residue concentration of butyl-Sepharose labeled with ^{14}C-ethyl amine and prepared by the cyanogen bromide method. The immobilized residue concentrations were determined according to ref. (8).

first few weeks or aged correspondingly. Similar observations on residue leakage have been made by others (30,31).

Substituted Sepharose gels are very rigid structures. Only at high immobilized alkyl residue concentrations (e.g., 44 μmol/ml packed gel) does a permanent gel contraction of ca. 10% occur (3). Other gel volume changes on highly substituted gels, e.g., after addition of 1.1 M ammonium sulfate (+6 to 7%) or after a temperature change of 5–34° (−2 to 3%), are close to or within experimental error.

The kinetics of desorption were measured in the form of dilution experiments (3,21–24), which correspond to kinetic systems far from equilibrium (24):

$$-d(\text{E-Aga})\,dt = k_{-1}(\text{E-Aga}) - k_{+1}(E)(\text{Aga}) \qquad (1)$$

where k_{-1} and k_{+1} are the rate constants of dissociation and association, respectively. (E), (Aga), and (E-Aga) denote, respectively, the concentration of enzyme, of alkyl agarose matrix (i.e., binding units; refs. 3,22), of enzyme-agarose complex (i.e., adsorbed protein concentration), and t denotes the time. Under conditions where the dissociation rate is independent of dilution (22,24), the second term of Eq. (1) (reassociation) becomes negligible, and the equation reduces to a first-order rate equation for the decay of the E-Aga complex.

For further details on the measurement of adsorption and desorption isotherms, see refs. (3,9,24).

RESULTS

Kinetics of Adsorption

For the calculation of the initial adsorption rates, the differential method of van't Hoff (32) was employed. The inserts in Fig. 2 show that in the first 2 min linear progress curves are obtained. If the progress curves are corrected for nonspecific adsorption on unsubstituted Sepharose, they pass through the origin (not shown) without significant change in increment. Under these conditions there is no dependence of adsorption on the stirring rate. In the first 2 min the initial free enzyme concentration (E_0) changes ca. 10–15%. From the initial rates it can be calculated that in the mean, the initial concentration of free enzyme decreases to 50% in ca. 8 min.

If the initial rates are plotted versus the initial free enzyme concentration, saturation kinetics are obtained (Fig. 2a). In the double reciprocal

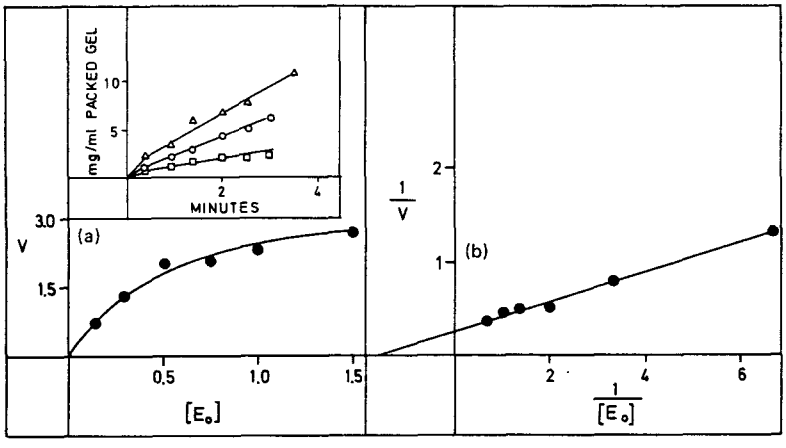

FIG. 2. Initial adsorption rate of ^{3}H-phosphorylase b_r on butyl-Sepharose as a function of the initial free enzyme concentration. (a) Saturation curve of the initial adsorption rate versus the initial free ligand concentration in the bulk. Insert: Progress curves at different initial free enzyme concentrations (E_0): (□), 0.15 mg/ml; (○), 0.75 mg/ml; (△), 1.5 mg/ml. (b) Double reciprocal plot of the initial adsorption rate versus the initial ligand concentration in the bulk. One ml packed gel (40 μmol/ml packed gel) was incubated at 5° in a total volume of 40 ml buffer A stirred at 450 rpm. The reaction was initiated with gel and samples of 0.5 ml were taken at the indicated times (see insert). The initial concentration of free enzyme (E_0) was varied between 0.15 and 1.5 mg/ml. For further details and the extrapolated values of the double reciprocal plot, see the text.

plot (Fig. 2b), it can be shown that the saturation curve corresponds to a rectangular hyperbola. From the intercept with the ordinate, a maximal adsorption rate of 3.7 mg/(min × ml packed gel) is extrapolated, and on the abscissa, a free ligand concentration for a half-maximal velocity of 0.54 mg/ml is extrapolated.

Kinetics of Desorption

Figure 3 shows a typical dilution experiment at two different initial concentrations (a_0) of adsorbed protein on the gel. In the absence of unlabeled enzyme on the bulk, a rapid phase of desorption occurs in the first

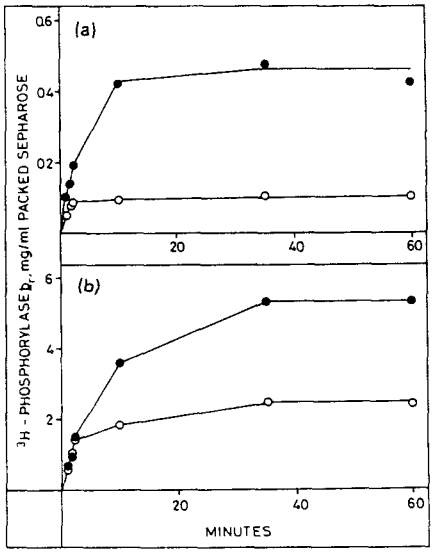

FIG. 3. Desorption of ^{3}H-phosphorylase b_r from butyl-Sepharose in the presence and absence of unlabeled phosphorylase b_r in the bulk. Ca. 1 ml packed butyl-Sepharose (ca. 30 μmol/ml packed gel) loaded with ^{3}H-phosphorylase b_r as indicated below (adsorption time for loading ca. 60–90 min) was diluted in 20 ml of buffer A at 5° (stirring rate 700–1,200 rpm) in the absence (○) and presence (●) of 1 mg/ml unlabeled enzyme in the bulk. (a) Initial immobilized ligand concentration 4.5 mg/ml packed gel; (b) initial immobilized ligand concentration 32 mg/ml packed gel. For further details, see refs. (9,22,24) and the text.

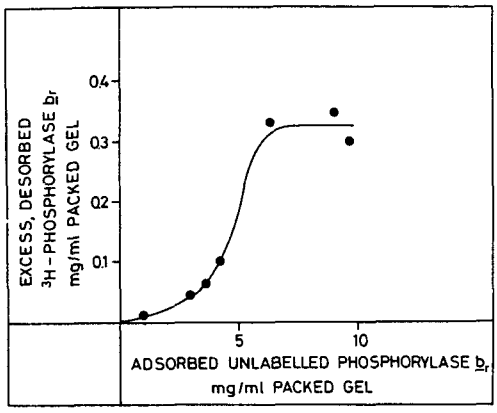

FIG. 4. Displacement of ^{3}H-phosphorylase b_r from butyl-Sepharose by unlabeled enzyme in a time span of 60 min. The data are taken from Fig. 3a by plotting the amount of desorbed excess ^{3}H-phosphorylase *b* (excess = desorbed amount of enzyme in the presence of unlabeled phosphorylase *b* minus the amount spontaneously desorbed by dilution alone) versus the amount of adsorbed unlabeled enzyme. The amount of desorbed labeled enzyme was determined by radioactivity measurements. The amount of adsorbed unlabeled enzyme was calculated from the decrease in protein concentration (measured according to ref. 9). Beginning from the origin of the coordinates, samples were taken at the following times: 0.38, 0.88, 1.38, 2.0, 10, 35, and 60 min after initiation of desorption. For further details, see legend to Fig. 3 and the text.

2 min. During this rapid phase a desorption rate of 11.8 μg/(min × ml packed gel) is observed at $a_0 = 4.5$ mg/ml packed gel, in comparison to 712 μg/(min × ml packed gel) at $a_0 = 32$ mg/ml packed gel. If the dilution of the gel is performed in the presence of 1 mg/ml unlabeled enzyme in the bulk, the initial rate of labeled enzyme release is increased to 62.9 μg/min at low fractional saturation (Fig. 3a), but remains unchanged (748 μg/min ml packed gel) at high fractional saturation (Fig. 3b). The apparent first-order off-rate constants of similar experiments have been reported in ref. (22). In the presence of unlabeled enzyme, the total amount of enzyme released is ca. 2.5–5-fold higher than in the absence of unlabeled enzyme in the bulk.

During dilution of the enzyme-butyl agarose complex in the presence of an initial concentration of 1 mg/ml free, unlabeled enzyme in the bulk, the

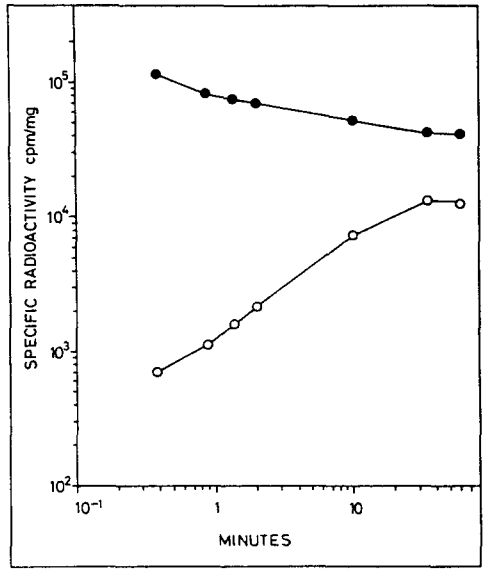

FIG. 5. Progress curves of the specific radioactivity of ^{3}H-phosphorylase b_r on the solid phase and in the bulk after dilution of the labeled enzyme-matrix complex in the presence of unlabeled enzyme (in double logarithmic coordinates). The data are taken from Fig. 3a. The initial specific radioactivity of the labeled enzyme was 1.41×10^5 cpm/mg. The expected specific radioactivity after equilibration of labeled and unlabeled enzyme is 3.5×10^4 cpm/mg. For further details see legend to Fig. 3 and the text. (●), Solid phase bound enzyme; (○), free enzyme in bulk phase.

concentration of this enzyme in the bulk decreases due to adsorption to the gel. As the unlabeled enzyme is adsorbed, the initial desorption of labeled enzyme is enhanced (Fig. 3a). Figure 4 shows the correlation of unlabeled enzyme adsorption to the amount of labeled enzyme released in excess to spontaneously desorbed enzyme. Figure 4 was derived from the kinetic experiment in Fig. 3a with the inclusion of a further data point at 0.38 min. Initially in the sigmoid portion of the curve, the amount of released excess ^{3}H-labeled enzyme rises exponentially as a function of the adsorbed unlabeled enzyme. Finally, a plateau is reached where unlabeled enzyme is adsorbed without the excess release of labeled enzyme. The sigmoidicity may indicate that more than one molecule of unlabeled enzyme is involved in the displacement of an adsorbed molecule.

On dilution of the labeled enzyme-matrix complex in the presence of unlabeled enzyme, it could be expected that the specific radioactivity (cpm/mg) equilibrates, reaching a value determined by the ratio of labeled to unlabeled enzyme. If, however, the specific radioactivity of adsorbed and free enzyme is determined simultaneously (the experiment in Fig. 3a) as a function of time (Fig. 5), a true equilibration is not found. In Fig. 5 the expected specific radioactivity for the free and bound form of the enzyme) at equilibrium is ca. 3.5×10^4 cpm/mg. However, after 60 min (apparent equilibrium in Fig. 3a) the specific radioactivity of the adsorbed enzyme is 4.1×10^4 cpm/mg and that of the free enzyme 1.2×10^4 cpm/mg. This can only be explained by the fact that a large proportion of the labeled enzyme on the matrix does not exchange with unlabeled bulk enzyme in the time span of the experiment.

Hysteresis

As has been reported previously (22–24), the desorption isotherms of physphorylase *b* on butyl-Sepharose do not retrace the adsorption isotherms. At low (5 μmol/ml packed gel, Fig. 6a) and at high (21 μmol/ml packed gel, Fig. 6b) immobilized residue concentrations, the adsorption and desorption isotherms can be closed to a loop with the lower closure point lying in the origin of the coordinates (33). The area enclosed by the hysteresis loop increases as the residue concentration is enhanced. The irreversible entropy ($\Delta_i S$) as calculated according to ref. (34) is ca. 6 $J \cdot mol^{-1} \cdot K^{-1}$ for the loop in Fig. 6a, and over 30 $J \cdot mol^{-1} \cdot K^{-1}$ for the loop in Fig. 6b. The Freundlich constants in Fig. 6a are for adsorption, $\alpha = 1.4$ mg/ml packed gel, $1/n = 0.82$, and for desorption, $\alpha = 1.2$ mg/ml packed gel, $1/n = 0.39$. From the Hill plots (9), the apparent association constants of half-maximal saturation ($K'_{0.5}$) can be calculated to be $1.8 \times 10^4\ M^{-1}$ for adsorption and $2.2 \times 10^5\ M^{-1}$ for desorption. Similarly, at 21 μmol/ml packed gel (Fig. 6b) the Freundlich constants of $\alpha =$ 35.5 mg/ml packed gel, $1/n = 0.52$, for adsorption, and $\alpha = 4.9$ mg/ml packed gel, $1/n = 0.027$, for desorption are obtained. The corresponding value for the apparent association constant of half-maximal saturation is $1.5 \times 10^5\ M^{-1}$ for adsorption and over $10^9\ M^{-1}$ for desorption. It could be argued that the enzyme is adsorbed in a form that cannot be desorbed at all at high immobilized residue concentrations (Fig. 6b). This is, however, not the case. If the loaded gel is diluted in the presence of 1 mg/ml unlabeled enzyme in the bulk, ca. fivefold more enzyme is released (Fig. 6b) than by dilution in the absence of unlabeled enzyme in the bulk (see also Fig. 3).

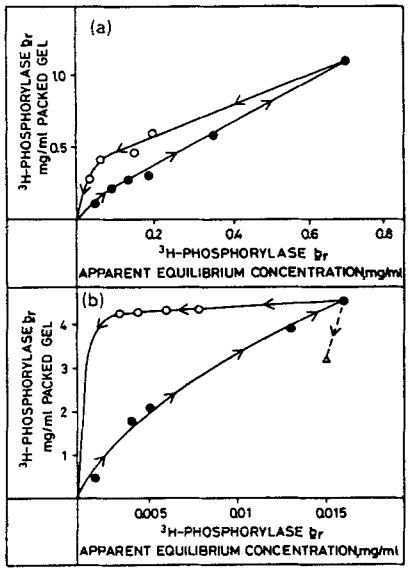

FIG. 6. Adsorption hysteresis of ^{3}H-phosphorylase b_r on butyl Sepharose. Adsorption was performed for 60–90 min in a buffer volume of ca. 60 ml, containing ca. 1 ml packed gel stirred at 450 rpm with a 3-cm stirring bar. Desorption was performed for 60–120 min according to refs. (3,33) by adding ca. 0.2–2.0 ml packed gel to 10–20 ml buffer stirred at 700 rpm (1.5 cm stirring bar). (a) 5 μmol/ml packed gel; (b) 21 μmol/ml packed gel. (●), Adsorption isotherm; (○), desorption isotherm. (△): Part (b) shows desorption in the presence of 1 mg/ml unlabeled enzyme in the bulk. The concentration on the abscissa corresponds to the labeled, released enzyme at apparent equilibrium and not to the total free enzyme concentration (i.e., labeled + unlabeled; from ref. 33).

DISCUSSION

The hyperbolic saturation kinetics (Fig. 2) show that during adsorption the collision rate between ligand and matrix is not the rate-limiting step, but that some alternative step, e.g., a specific orientation of the ligand on the matrix for multivalent nucleation, may be rate limiting. The following equation can be employed in analogy to enzyme kinetics (32) to describe the adsorption rate:

$$v = \frac{k[\mathrm{Aga}]_0[\mathrm{E}]}{K_m^* + [\mathrm{E}]} \tag{2}$$

In this equation v is the adsorption rate, $[\mathrm{Aga}]_0$ the total concentration of butyl Sepharose, and [E] the free concentration of enzyme; k is the rate constant of the rate limiting step, and K_m^* the free ligand concentration corresponding to the half-maximal adsorption rate. This result indicates that the adsorption reaction is similar to an enzyme-substrate or carrier-mediated transport reaction, which involve rate limiting steps other than the collision rate. On hydrophobic gels this rate limiting step could correspond to an orientation step of the enzyme that precedes or accompanies multivalent nucleation on, e.g., four alkyl residues (3). This reaction may also

pose an energy barrier leading to metastability (see model of hysteresis below).

The saturation kinetics are not due to the pore structure of the agarose, as can be shown in the following way. The pore radius of Sepharose 6B has been reported to be ca. 40 nm (35). From this value the pore radius of Sepharose 4B can be estimated by employing the exclusion limits of the gels for globular proteins. Since the exclusion limit for Sepharose 6B corresponds to a molecular weight of 4×10^6 and for Sepharose 4B to a molecular weight of 20×10^6 (36), and since the effective molecular radius is proportional to the 0.33–0.5 power of the molecular weight (37), 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 in reasonable agreement with parameters obtained from the chromatography of protein-SDS complexes on controlled pore glass (38). 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 ca. 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, which is in agreement with the calculations performed above on the agarose gels.

From the dimensions of the phosphorylase *b* molecule ($6.3 \times 6.3 \times 11.6$ nm, refs. 39, 40) and the calculated mean pore radius of ca. 80 nm for Sepharose 4B (see above), a ratio of effective ligand radius to pore radius of 0.072 can be calculated. From this ratio it can be estimated (41) that the diffusivity of phosphorylase *b* in the pores of Sepharose 4B is ca. 25% less than in water.

The above ratio of solute to pore radius is also the basis for the planar lattice concept (9) proposed for the adsorption of proteins to hydrophobic agarose gels. On the inside of a Sepharose 4B pore a planar lattice can be constructed in a similar manner, as has been devised for the outer surface of spheres (42,43). At a ca. 14-fold larger pore radius versus solute radius, the curvature of the pore can be practically neglected in a first approximation (see model in Fig. 7).

Since the desorption rate is independent of dilution and the stirring rate (22,24), and since readsorption can be neglected quantitatively (22,24), the effect of initial rate enhancement by unlabeled enzyme in the bulk, which is dependent on the fractional saturation (Fig. 3), can be interpreted on the basis of nonindependent binding. This is shown in the model of Fig. 7. The following simplifications are made: (*a*) a one-dimensional instead of a two-dimensional lattice is employed; (*b*) the pore curvature is omitted; (*c*) the phosphorylase *b* molecule is depicted as a rectangle (rectangular prism, ref. 39); and (*d*) the alkyl residues are drawn at a greater thickness than that

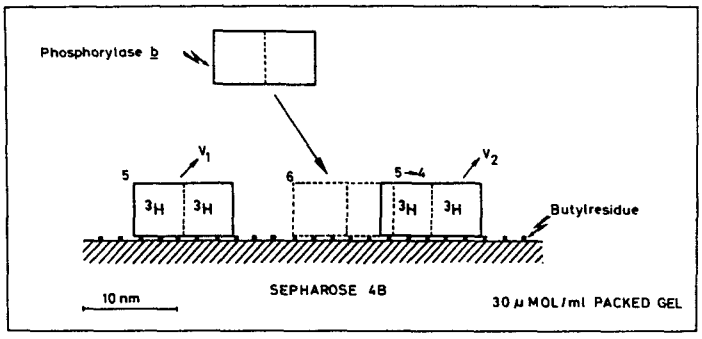

FIG. 7. Tentative scale model in schematic form of the postulated kinetics of nonindependent protein binding on butyl-Sepharose as exemplified by the displacement of a labeled (^{3}H) by an unlabeled molecule. Conformational changes are not considered. The numbers indicating the residues involved in binding the enzyme are hypothetical. See also ref. 46 and the text.

which corresponds to the scale. If, for example, a labeled phosphorylase *b* molecule is adsorbed on 5-alkyl residues, it has a dissociation rate of v_1. Subsequently, an unlabeled molecule of phosphorylase *b* can be adsorbed in such a manner that the binding units of the labeled and unlabeled molecules overlap (3). During the propagation step of binding (see hysteresis below), the number of binding alkyl residues increase, and a force may be exerted by the unlabeled enzyme molecule on the previously bound labeled molecule, leading to the loss of one binding site and to a dissociation rate v_2 ($v_2 > v_1$). The sigmoid curve in Fig. 4 indicates that possibly more than one molecule of unlabeled enzyme may be necessary for such a displacement of a bound labeled molecule. The experiments showing that a true equilibration of radioactivity between the bound and bulk enzyme does not occur (Fig. 5) provide strong evidence against the argument that the desorption experiments in the presence of unlabeled bulk enzyme are simple exchange experiments. Aside from possible conformational changes, which remain to be shown, binding site displacement after cooperative multivalent binding of an enzyme on a heterogenous lattice of binding sites can also explain the phenomenon of biospecific elution (44,45). Similar to "protein-protein elution" (23,24), in which case a newly adsorbed protein molecule reduces the number of binding sites of the previously adsorbed molecule (Fig. 7), a substrate could also displace an interacting active site of an enzyme from a lattice binding site, leading to a higher off-rate, i.e., elution of the protein.

The closed adsorption-desorption loops of Fig. 6 are an indication of long-lived metastable states and thermodynamic irreversibility in the

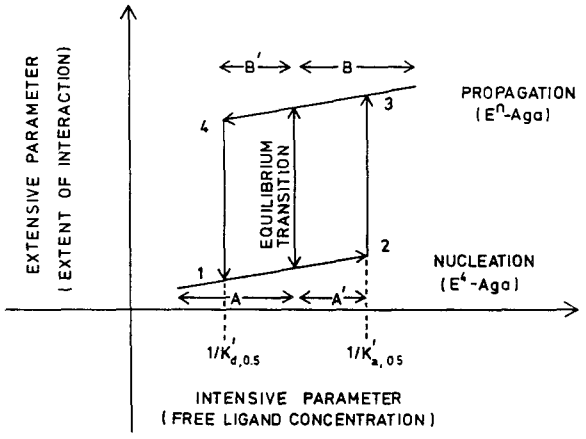

FIG. 8. Schematic adsorption-desorption loop of a domain in the adsorption and desorption of phosphorylase *b* on butyl-Sepharose. An increase in the extent of the ligand-matrix interaction (extensive parameter) is taken to correspond to an increase in the valence of the interaction (33). On the abscissa $K'_{d,0.5}$ and $K'_{a,0.5}$ denote the apparent association constant of half-maximal saturation as derived from the desorption and adsorption isotherms, respectively. E is the enzyme and Aga the substituted agarose matrix. The number in the superscript (E^4, E^n, n is a positive integer, $n>4$) denotes the valence involved per binding unit (3). *A* denotes a first stable state, e.g., an unnucleated form of phosphorylase *b*. *A'* is the metastable state of *A*, e.g., a nucleated form of the enzyme (E^4-Aga). *B* denotes a second stable state, e.g., phosphorylase *b* after propagation (E^n-Aga). *B'* is the metastable state of *B*. For further details, see refs. (24,47) and the text (from ref. 33).

adsorption and desorption of phosphorylase *b* on butyl Sepharose. The increase in the apparent association constant of half-maximal saturation (see results above) in the transition from adsorption to desorption indicates that the number of alkyl residue-protein interactions increases. Furthermore, these two apparent binding constants in a hysteresis loop may correspond to a binding constant of nucleation and propagation, respectively (two-step mechanism). A model (see also ref. 47) of the postulated mechanism of hysteresis is given in Fig. 8. Binding of the ligand begins in a nucleation step (24,33), which probably involves a metastable state (A' of Fig. 8). After nucleation on a critical number of ca. four alkyl residues (3), the reaction proceeds (24,33) to n (n is a positive integer, $n>4$) alkyl residues by way of propagation. The nucleation step can be taken to correspond to the impera-

tive, multivalent (all or none) binding step (3,20) of cooperative adsorption (9,19,20). During desorption probably another metastable state (B′ in Fig. 8) is passed, due to the resistance of the adsorbed molecule to the rupture of hydrophobic bonds (33).

It should, however, be emphasized that there is no evidence for a de facto "irreversible" adsorption of phosphorylase *b* on butyl-Sepharose. The reaction is only thermodynamically irreversible (hysteresis). This is illustrated by the fact that under conditions where only ca. 3–5% of the adsorbed enzyme can be released by dilution alone (Fig. 6b; see also ref. 24), up to 40% of the adsorbed enzyme can be desorbed by very mild means, e.g., addition of ca. 8 mg/ml unlabeled enzyme to the bulk (24).

If the mechanism of nonindependent desorption (Fig. 7) is combined with the mechanism of adsorption hysteresis (Fig. 8) it can be postulated that the action of an unlabeled molecule on the adsorbed molecule may reverse the hysteresis loop (Fig. 8), e.g., E^n-Aga → E^4-Aga → free enzyme (23,24).

The phenomenon of hysteresis (i.e., two-step mechanism of adsorption) also has a number of consequences for chromatographic procedures. Due to the relatively low binding constant of nucleation, the protein concentrations for adsorption must be relatively high (ca. 10^{-5} M). As soon as the protein is nucleated, however, the binding constant rises by magnitudes due to propagation. This accounts for the advantage of being able to wash the gel column extensively without losing significant amounts of the adsorbed protein. These extremely high binding constants, e.g., over $10^9\ M^{-1}$, also explain why hydrophobic agaroses can be employed for the immobilization of enzymes (48).

ACKNOWLEDGMENTS

The excellent technical assistance of Mrs. G. Botzet is gratefully acknowledged. This work was supported by a grant (Je 84/4) from the Deutsche Forschungsgemeinschaft.

REFERENCES

1. HJERTEN, S., and PAHLMANN, S. (1978) Hydrophobic interaction chromatography on non-charged Sepharose derivatives. *In* Chromatography of Synthetic and Biological Polymers, Vol. 2, EPTON, R. (ed.), Ellis Horwood Ltd., Chichester, U.K., pp. 53–59.
2. HOFSTEE, B. H. J., and OTILLIO, N. F. (1978) J. Chromatogr. 159 : 57.
3. JENNISSEN, H. P. (1978) J. Chromatogr. 159 : 71.
4. OCHOA, J. L. (1978) Biochimie 60 : 1.

5. SHALTIEL, S. (1978) Hydrophobic chromatography. *In* Chromatography of Synthetic and Biological Polymers, Vol. 2, EPTON, R. (ed.), Ellis Horwood Ltd., Chichester, U.K., pp. 13–41.
6. YON, R. J. (1978) Int. J. Biochem. 9:373.
7. PORATH, J. (1968) Nature 218 : 834.
8. JENNISSEN, H. P., and HEILMEYER, L. M. G., Jr. (1975) Biochemistry 14 : 754.
9. JENNISSEN, H. P. (1976) Biochemistry 15 : 5683.
10. HJERTEN, S. (1973) J. Chromatogr. 87 : 325.
11. PORATH, J., SUNDBERG, L., FORNSTEDT, L., and OLSSON, I. (1973) Nature 245 : 465.
12. ER-EL, Z., ZAIDENZAIG, Y., and SHALTIEL, S. (1972) Biochem. Biophys. Res. Commun. 49 : 383.
13. RIMERMAN, R. A., and HATFIELD, G. W. (1973) Science 182 : 1268.
14. JENNISSEN, H. P. (1976) Protides Biol. Fluids Proc. Colloq. (May 1975) 23 : 675.
15. JENNISSEN, H. P. (1978) Cooperative phenomena in the adsorption of phosphorylase kinase and phosphorylase *b* on hydrophobic agaroses. *In* Chromatography of Synthetic and Biological Polymers, Vol. 2, EPTON, R. (ed.), Ellis Horwood Ltd., Chichester, U.K., pp. 42–52.
16. HATEFI, Y., and HANSTEIN, W. G. (1969) Proc. Natl. Acad. Sci. U.S.A. 62 : 1129.
17. LEWIN, S. (1974) Displacement of Water and its Control of Biochemical Reactions, Academic, New York, pp. 85, 260.
18. MORRIS, C. J. O. R. (1978) J. Chromatogr. 159 : 33.
19. JENNISSEN, H. P. (1976) Hoppe-Seyler's Z. Physiol. Chem. 357 : 1201.
20. JENNISSEN, H. P. (1976) Hoppe-Seyler's Z. Physiol. Chem. 357 : 1727.
21. JENNISSEN, H. P. (1977) Hoppe-Seyler's Z. Physiol. Chem. 358 : 255.
22. JENNISSEN, H. P. (1978) Hoppe-Seyler's Z. Physiol. Chem. 359 : 281.
23. JENNISSEN, H. P. (1978) Hoppe-Seyler's Z. Physiol. Chem. 359 : 1101.
24. JENNISSEN, H. P., and BOTZET, G. (1978) Protides Biol. Fluids Proc. Colloq. (May 1978) 26 : 657.
25. FISCHER, E. H., KREBS, E. G. (1958) J. Biol. Chem. 231 : 65.
26. HASCHKE, R. H., HEILMEYER, L. M. G., Jr. (1972) Anal. Biochem. 47 : 451.
27. LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L., and RANDALL, R. J. (1951) J. Biol. Chem. 193 : 265.
28. STRAUSBAUCH, P. H., KENT, A. B., HEDRICK, J. L., and FISCHER, E. H. (1967) Methods Enzymol. 11 : 671.
29. HASCHKE, R. H., HEILMEYER, L. M. C., Jr., MEYER, F., and FISCHER, E. H. (1970) J. Biol. Chem. 245 : 6657.
30. PARIKH, I., MARCH, S., and CUATRECASAS, P. (1974) Methods Enzymol. 34B : 77.
31. WILCHEK, M., and MIRON, T., Methods Enzymol. 34B : 72.
32. LAIDLER, K. J. (1958) The Chemical Kinetics of Enzyme Action, Oxford University Press, London, p. 60.
33. JENNISSEN, H. P., and BOTZET, G. (1979) Int. J. Biolog. Macromolecules 1 : 171.
34. EVERETT, D. H., and WHITTON, W. I. (1955) Proc. Roy. Soc. (London) A 230 : 91.
35. LASCH, J., IWIG, M., KOELSCH, R., DAVID, H., and MARX, I. (1975) Eur. J. Biochem. 60 : 163.
36. PHARMACIA FINE CHEMICALS (1969) Sepharose 2B-4B-6B in Bead Form, Uppsala, Sweden, p. 3.
37. RODBARD, D. (1976) Estimation of molecular weight by gel filtration and gel electrophoresis. *In* Methods of Protein Separation, Vol. 2, CATSIMPOOLAS, N. (ed.), Plenum, New York, pp. 145–179.
38. COLLINS, R. C., and HALLER, W. (1973) Anal. Biochem. 54 : 47.

39. PUCHWEIN, G., KRATKY, O., GÖLKER, Ch. F., and HELMREICH, E. (1970) Biochemistry 9 : 4691.
40. JOHNSON, N. L., MADSEN, N. B., MOSELY, J., and WILSON, K. S. (1974) J. Mol. Biol. 90 : 703.
41. BECK, R. E., and SCHULTZ, J. S. (1970) Science 170 : 1302.
42. CASPAR, D. L. D., and KLUG, A. (1962) Cold Spring Harbor Symposia on Quant. Biology 27 : 1.
43. LAIKEN, N., and NÉMETHY, G. (1970) J. Phys. Chem. 74 : 4421.
44. YON, R. J. (1977) Biochem. J. 161 : 233.
45. MOSBACH, K. (1978) Immobilized adenin coenzymes in general ligand affinity chromatography. *In* Chromatography of Synthetic and Biological Polymers, Vol. 2, EPTON, R. (ed.), Ellis Horwood Ltd., Chichester, U.K., pp. 199–230.
46. JENNISSEN, H. P. (1979) Cooperative, multivalent protein binding on two-dimensional, hydrophobic binding-site lattices: a model for the mechanism of protein adsorption on hydrophobic agaroses. *In* Affinity Chromatography, INSERM editions Vol. 86, EGLY, J.-M. (ed.), INSERM Paris, pp. 253–264.
47. KATCHALSKY, A. (1969) Chemical dynamics of macromolecules and their cybernetic significance. *In* Biology and the Physical Sciences, DEVONS, S. (ed.), Columbia University Press, New York, pp. 267–298.
48. HOFSTEE, B. H. J., and OTILLIO, N. F. (1973) Biochem. Biophys. Res. Commun. 53 : 1137.