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Base-atom recognition in protein adsorption to alkyl agaroses

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ABSTRACT

The three proteins phosphorylase *b*, calmodulin and fibrinogen are adsorbed onto thioalkyl derivatives of Sepharose much more strongly than onto gels carrying the same alkyl residue coupled via a carbamate linkage. This enhancement of binding onto alkyl-S-Sepharoses compared with alkyl-N-agaroses is not primarily due to an increase in the extent of conformational changes of the proteins occurring on the gel surface. This can be shown in experiments with the tripeptide Trp–Trp–Trp. The Trp tripeptide is also adsorbed with a much higher affinity to butyl-S-Sepharose than to butyl-N-Sepharose, showing that the primary interaction between the immobilized alkyl residue and the amino acids of the protein is decisive for adsorption. A model stressing the strong influence of an atom or a group of atoms at the base of an immobilized alkyl residue is described as "base-atom recognition".

INTRODUCTION

Although affinity chromatography and hydrophobic (interaction) chromatography have been available for about 20 years [1–3] the mechanism of protein adsorption onto these substituted surfaces is still a matter of controversy and research. In biospecific affinity chromatography it is generally accepted that the protein binds via a specific site, *e.g.* the substrate binding site, onto the immobilized substrate. However, in hydrophobic chromatography binding is more complex. Initially, Er-El *et al.* [2] proposed that the basis for the binding of a protein to a hydrophobic agarose gel (Sepharose 4B) lies in the interaction of an alkyl residue with a hydrophobic pocket on the surface of the protein. It was subsequently shown that adsorption onto such gels occurs through the multivalent interaction of the immobilized alkyl residues with the hydrophobic surface sites of the protein [4,5]. The interaction of alkyl residues with hydrophobic pockets or areas of the protein could also be modified by ionic groups simultaneously immobilized on the matrix [6]. In addition, the interactions are strongly influenced by the ionic strength [3] and the type of ion employed in the buffer [7].

The preparation and novel protein binding properties of spacer-free thioalkyl agaroses was initially reported in 1984 [8]. These findings were substantiated in later work [9–11]. When comparing these thioalkyl agaroses with alkyl agarose gels prepared by the cyanogen bromide [2,11,13] or carbonyl diimidazole method [12] it was found that these gels adsorb proteins much stronger than expected from just changing the coupling procedure. In this paper the hypothesis will be discussed that the difference between alkyl-N- and alkyl-S-agarose gels with respect to protein adsorption is mainly due to an effect of the atom or atoms at the base of the alkyl residue, a phenomenon which has been called "base-atom recognition" [14].

MATERIALS AND METHODS

Preparation of proteins

Phosphorylase *b* (third crystals, 70–80 U/mg) was prepared from frozen rabbit muscle [15] and freed of AMP [15]. The enzyme was stored in 50% glycerol at −30°C. Enzyme activity [16] and protein concentration (standard bovine serum albumin, BSA) [17] were determined on an AutoAnalyzer I (Technicon, Tarrytown, NY, USA). For further details of

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the preparation of this protein ligand for adsorption see refs. 5 and 18.

Bovine testis calmodulin was isolated according to the method of Autric *et al.* [19] in conjunction with affinity chromatography [20]. The biological acitivity of purified calmodulin was tested with phosphorylase kinase [21] in the AutoAnalyzer test [22,23]. The calmodulin concentration necessary for the half-maximum activation of phosphorylase kinase was 30–50 n*M*.

Fibrinogen was isolated from bovine blood [24, 25] and further purified [26]. The clottability was measured according to the method of Jacobsson [27] as modified by Mahn and Müller-Berghaus [25] and was 96–100% for the preparations used here.

Preparation of alkyl agaroses

The preparation [7,28], analysis [7] and storage [29] of the alkyl derivatives of beaded agarose (Sepharose 4B, Pharmacia, Uppsala, Sweden) prepared by the cyanogen bromide method (alkyl-N_I-Sepharoses) have been described previously. The concentration of immobilized butyl residue was determined by the addition of the tracer [^{14}C]ethylamine (New England Nuclear, Boston, MA, USA) to the 2 *M* butylamine solution [7,30,31]. Uncharged alkyl-N-Sepharoses were prepared according to the carbonyl diimidazole method (alkyl-N_{II}-Sepharoses) of Bethell *et al.* [32]. For butyl-N_{II}-agaroses [^{14}C]ethylamine was used and for hexyl-N_{II}-agaroses [^{14}C]hexylamine (Amersham, Braunschweig, Germany) was used [11]. Tresyl-Sepharose was prepared according to the tresyl chloride method [33] and coupled to butyl mercaptan in an alkaline medium (alkyl-S-Sepharoses] [11]. The degree of substitution was determined with [^{14}C]butyl mercaptan as described previously [11]. The gels were stored at 5°C. Alkyl-S-Sepharose gels can be stored for many years [11] so that storage periods of 1 year or more for the gels used in this study are the rule. Only fresh, unregenerated gel was used in the experiments. The structure of the agarose derivatives is shown in Fig. 1.

Measurements of protein binding

The methods used for the measurement of adsorption and desorption isotherms of phosphorylase *b* to butyl-Sepharose at 5°C have been described previously [5,18]. Before use phosphorylase *b* was extensively dialysed against buffer A containing 10 m*M* tris(hydroxymethyl)aminomethane–maleate, 5 m*M* dithioerythritol, 1.1 *M* ammonium sulphate, 20% sucrose, pH 7.0 [5], which was used for all experiments. In the adsorption isotherms the free concentration of phosphorylase *b* at apparent equilibrium [18] was between 0.05 and 0.5 mg/ml. The adsorbed amount of phosphorylase *b* was calculated from difference measurements using the steel grid-syringe method [5] and is expressed in milligrams of adsorbed protein per millilitre of packed Sepharose [5]. Unsubstituted Sepharose 4B was used as a control.

Quantitative hydrophobic affinity chromatography of calmodulin was performed on a column (12 × 0.9 cm) containing 2 ml of packed gel. The gel was washed and equilibrated with 20 volumes of buffer B (20 m*M* Tris–HCl, 1 m*M* $CaCl_2$, pH 7.0). As a sample, 1 mg of purified calmodulin was applied in a sample volume of 1 ml (in buffer B) and fractions of 1 ml were collected. The column was then washed with 9 ml of buffer B and then with 9 ml of buffer C (buffer B plus 0.3 *M* NaCl). The adsorbed calmodulin was eluted with buffer D [20 m*M* Tris–HCl, 0.3 *M* NaCl, 10 m*M* ethylene glycol-bis-(β-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA), pH 7.0].

Quantitative hydrophobic chromatography of fibrinogen was performed on a column (12 × 0.9 cm) containing 2 ml of packed gel. The gel was washed and equilibrated with 20 volumes of buffer E (50 m*M* Tris–HCl, 150 m*M* NaCl, 1 m*M* EGTA, pH 7.4). As a sample 1 mg of purified fibrinogen was applied in a sample volume of 1 ml (in buffer E) and fractions of 1.5 ml were collected. The column was then washed with 15 ml of buffer E and eluted with 7.5 *M* urea, and at high gel hydrophobicities with 1% sodium dodecyl sulphate (SDS).

Quantitative hydrophobic chromatography of the tripeptide Trp–Trp–Trp (Paesel & Lorei, Frankfurt, Germany; 98.5% purity) was performed on a column (12 × 0.9 cm) containing 2 ml of packed gel. The gel was washed and equilibrated with 20 volumes of buffer F (1 m*M* sodium β-glycerophosphate, pH 7.0]. As a sample, 1 mg of Trp tripeptide was applied in a sample volume of 1 ml (in buffer F) and fractions of 1.5 ml were collected. The column was then washed with 15 ml of buffer F and eluted with 1% SDS. The tripeptide in the fractions ob-

tained was measured directly according to the method of Lowry *et al.* [17] on a AutoAnalyzer using the Trp tripeptide as a standard instead of BSA.

All the quantitative analytical chromatography experiments were performed at room temperature, the flow was achieved by gravity and only fresh, unregenerated gel was used.

RESULTS AND DISCUSSION

The dramatically different binding properties of alkyl-S-Sepharoses were first noted when binding isotherms of phosphorylase *b* [relative molecular mass (M_r) 197 · 10^3] were determined on these gels [8–10]. These differences to alkyl-N-Sepharoses (for structures, see Fig. 1) are shown in Table I. Butyl-S-Sepharose adsorbs 8 mg of phosphorylase *b* per millilitre of packed gel at a degree of substitution of 1–2 μmol/ml packed gel, whereas alkyl-N-agaroses prepared either by the cyanogen bromide (alkyl-N_I-Sepharose) or by the carbonyl diimidazole method (alkyl-N_{II}-Sepharose) show no adsorption at all. Even at 4–5 μmol/ml packed gel the butyl-S-Sepharose binds about eight to ten times more enzyme at an apparent equilibrium concentration of 0.5 mg/ml for the free enzyme (for the definition of apparent equilibrium, see Jennissen and Botzet [18]). The butyl-N-agaroses thus show the typical sigmoidal protein adsorption isotherm (threshold phenomenon, critical hydrophobicity [7]) of the lattice-site

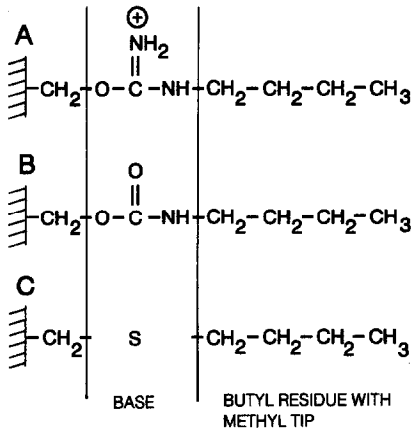

Fig. 1. Differentiation of base and tip elements on butyl-N_{II}- and butyl-S-Sepharose gels. The proximal "base element", which can consist of a single atom or a group of atoms, is shown schematically. The atom of the base element binding directly to the alkyl residue is the "base atom". The distally localized residue with the methyl tip is called the "tip element". The butyl residue is linked to the agarose gel as an isourea derivative (A), a carbamate derivative (B) and a thioether derivative (C). Alkyl-N_I-Sepharose contains a mixture of derivatives A and B [7,44], alkyl-N_{II}-Sepharose consists of derivative B and alkyl-S-Sepharose consists of derivative C (based on the structure proposed by Mosbach [33]).

binding function [34] described previously [4,5,22,35]. Table I also shows that this sigmoidal adsorption isotherm is not due to residual charges being incorporated into the alkyl gel as alkyl-N_{II}-

TABLE I

QUANTITATIVE ADSORPTION MEASUREMENTS OF PHOSPHORYLASE *b* ON BUTYL-N- AND BUTYL-S-SEPHAROSE GELS AS DERIVED FROM ADSORPTION ISOTHERMS

The data were obtained from corresponding adsorption isotherms measured in high ionic strength buffer A. The data for adsorption to butyl-N_I-Sepharose was taken from Demiroglou *et al.* [10] and Jennissen [34], the data for butyl-N_{II}-Sepharose is calculated from unpublished results and the data for butyl-S-Sepharose is taken from Demiroglou *et al.* [10]. c' corresponds to the apparent equilibrium concentration of free protein [18] at which the amount of bound protein was calculated from the isotherms. For definition of butyl derivatives, see legend to Fig. 1. For further details, see under Materials and methods.

Degree of substitution (μmol/ml packed gel)	Adsorbed protein (c' = 0.5 mg/ml) (mg/ml packed gel)		
	Butyl-N_I-Sepharose	Butyl-N_{II}-Sepharose	Butyl-S-Sepharose
1–2	0	0	8.1
2–3	0.55	0	ND
4–5	2.2	3.0	23.5
15–17	15.6	28.0	ND
30–40	17.2	39.2	24.0

Sepharose does not contain charges [12]. A sigmoidicity in the lattice-site binding isotherm of phosphorylase *b* on butyl-S-Sepharose was not found. This behaviour is in agreement with the conclusion that only one alkyl-S residue suffices for the adsorption of one molecule of phosphorylase *b* [34]).

Table II shows the Ca^{2+}-dependent binding of the Ca^{2+} modulator protein, calmodulin (M_r 17 · 10^3), to alkyl agaroses as measured by quantitative hyrophobic affinity chromatography. No binding of calmodulin to the butyl-N_{II}-Sepharose (28 μmol/ml packed gel) can be measured. In contrast, already propyl-S-Sepharose of 25 μmol/ml packed gel adsorbs over 60% of the 1 mg applied. Butyl-S-Sepharose adsorbs 80% of the applied amount at 5.2 μmol/ml packed gel. Only ethyl-S-agarose adsorbs poorly at the defined surface concentration and thus has similar properties as the butyl-N_{II}-Sepharose gel. At very high degrees of substitution about 40% of the adsorbed protein cannot be eluted with EGTA (EGTA irreversible binding). This fraction of calmodulin can, however, be eluted with detergents (*e.g.* SDS, not shown). The binding properties of the butyl-S-Sepharose gels can be simulated by hexyl-N_{II}-Sepharose, although the binding properties of these gels are not completely identical, especially at very low degrees of substitution. These findings of the enhanced binding of calmodulin to alkyl-S-Sepharoses are in agreement with the experiments shown above for phosphorylase *b* (Table I). In general, the total yield of protein decreases as a function of the degree of substitution indicating that the amount of "irreversibly" bound calmodulin increases (Table II). This singularity of butyl-S-Sepharose is also evident when comparing calmodulin binding with the findings of others [36,37], who reported that calmodulin is adsorbed on much more hydrophobic adsorbents, *e.g.* octyl- and aminooctyl-Sepharose. In addition, we observe a typical dependence of binding on the degree of substitution (Table II). A three-fold lowering of the de-

TABLE II

QUANTITATIVE HYDROPHOBIC AFFINITY CHROMATOGRAPHY OF CALMODULIN ON ALKYL AGAROSES

The data (taken from Demiroglou and co-workers [10,11]) were derived from column experiments described in the text. Calmodulin was applied in buffer B (plus Ca^{2+}); the column was then washed with buffer C (plus 300 m*M* NaCl) and finally eluted with buffer D (plus EGTA). The excluded amount of calmodulin corresponds to the amount washed from the column in buffers B and C. The eluted amount corresponds to the amount eluted with buffer D. The total yield corresponds to the amount recovered in buffers B–D as a percentage of the amount applied. The difference between the amount applied and the amount recovered is the fraction which cannot be eluted with EGTA ("irreversibly" bound protein) which can, however, be eluted by detergents. For further details and the definition of gel types see under Materials and methods, the text and refs. 10 and 11.

Type of gel	Degree of substitution (μmol/ml packed gel)	Calmodulin (mg)				Total yield (%)
		Applied	Excluded	Bound	Eluted	
Alkyl-S-Sepharose:						
Homologous series:						
Ethyl-S-Sepharose	25.0	1.0	0.85	0.15	0.01	86
Propyl-S-Sepharose	25.0	1.0	0.34	0.66	0.42	76
Butyl-S-Sepharose	25.0	1.0	0	1.00	0.66	66
Degree of substitution series:						
Butyl-S-Sepharose	1.7	1.0	0.79	0.21	0.15	94
Butyl-S-Sepharose	5.2	1.0	0.19	0.81	0.70	89
Butyl-S-Sepharose	40.7	1.0	0	1.00	0.61	61
Alkyl-N_{II}-Sepharose						
Butyl-N_{II}-Sepharose	28.0	1.0	0.94	0.06	0	94
Hexyl-N_{II}-Sepharose	1.9	1.0	0.96	0.04	0	96
Hexyl-N_{II}-Sepharose	4.3	1.0	0.90	0.10	0.02	92
Hexyl-N_{II}-Sepharose	7.7	1.0	0.17	0.83	0.60	87
Hexyl-N_{II}-Sepharose	17.2	1.0	0.19	0.81	0.68	87

gree of substitution abolishes binding on hexyl-N_{II}-Sepharose altoghether and reduces it by a factor of five on butyl-S-Sepharose.

The third protein adsorbed onto the novel alkyl-S-agarose gels is fibrinogen (M_r 340 · 10^3) (see Fig. 2 and Table III [38,39]). The principle of the adsorption experiments is shown in Fig. 2. Fibrinogen is not adsorbed on the control gel of unsubstituted Sepharose 4B. As some protein is always lost during chromatography, the yield of this run is only 96%. At a low hydrophobicity (*i.e.* butyl-S-Sepharose 7 μmol/ml packed gel) the amount of protein absent in the run-through experiment can be eluted with 7.5 *M* urea. This is not possible on butyl-S-gels of high hydrophobicity (*i.e.* butyl-S-Sepharose 46 μmol/ml packed gel), where part of the strongly adsorbed protein can only be eluted with 1% SDS (not shown). Similar to calmodulin, no fibrinogen is adsorbed on the butyl-N_{II}-Sepharose of 21.7 μmol/ml packed gel. However, in contrast to the calmodulin experiments, ethyl-S-Sepharose of 25 μmol/ml packed gel strongly adsorbs up to 80% of the applied amount of fibrinogen. On butyl-S-Sepharose of 46 μmol/ml packed gel of only about 65% of the adsorbed protein can be eluted, even with 1% SDS (see above). Significant binding (70%) to hexyl-Sepharoses is found at about 8 μmol/ml packed gel with total binding of the applied amount being obtained at 17 μmol/ml packed gel. Here also the binding of fibrinogen reflects the different binding properties of the alkyl-S-Sepharoses, as described for phosphorylase *b* and calmodulin. Preliminary experiments with alkyl ether derivatives (butyl-O-Sepharose), also prepared by the tresyl chloride method, indicate a similar binding of fibrinogen as for butyl-N_{II}-Sepharose and not the enhanced binding of butyl-S-Sepharose (not shown). As can be concluded from the type of adsorption isotherm (Freundlich isotherm) [39], the adsorption mechanism for fibrinogen is probably similar to that of phosphorylase [34].

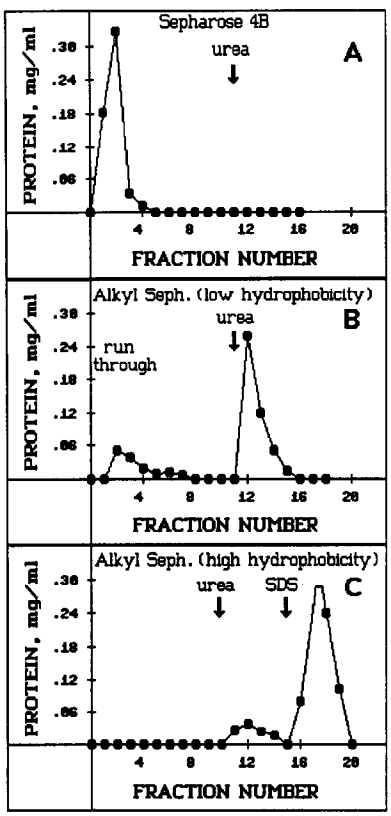

Fig. 2. Quantitative hydrophobic chromatography of fibrinogen on butyl-S-Sepharose gels. Fibrinogen (1–2 mg, see below) was applied in 1–2 ml aliquots to a column (12 × 0.9 cm) containing 2 ml of packed gel in buffer E (50 m*M* Tris–HCl, 150 m*M* NaCl, 1 m*M* EGTA, pH 7.4). Fractions of 1.5 ml were collected. The column was then washed with 15 ml of buffer E followed by elution either with 7.5 *M* urea or, at high hydrophobicities of the gel, with 1% SDS. (A) Sepharose 4B, unsubstituted agarose gel, 1 mg of fibrinogen; (B) Alkyl Sepharose (low hydrophobicity), butyl-S-Sepharose 7 μmol/ml packed gel, 1 mg of fibrinogen; and (C) Alkyl Sepharose (high hydrophobicity), butyl-S-Sepharose 46 μmol/ml packed gel, 2 mg of fibrinogen. For further details, see under Materials and Methods and Table III.

The binding of proteins to interfaces can be strongly influenced by conformational changes of the protein on the surface (see adsorption hysteresis [18,34]). Direct measurements of such changes in conformation by the total internal reflection fluorescence method have been reported for phosporylase *b* on substituted silica surfaces [40]. The enhancement of protein binding found for the alkyl-S-Sepharoses could thus also be due in part to induced conformational changes of the protein on the surface itself. In addition, the accessibility of the immobilized residues could be different for the three proteins depending on their molecular weights. That these arguments are probably irrelevant with the tripeptide Trp–Trp–Trp (Table IV). On butyl-N_{II}-Sepharose (41 μmol/ml packed gel) the Trp tripeptide is retarded (not shown), but runs fully through the column (no retention). In contrast, the total applied amount of the tripeptide is strongly

TABLE III

QUANTITATIVE HYDROPHOBIC CHROMATOGRAPHY OF FIBRINOGEN ON ALKYL AGAROSES

The data were derived from column experiments as described under Methods. Fibrinogen was applied in buffer E. The column was then washed with the same buffer and eluted with 7.5 *M* urea. The excluded amount of fibrinogen corresponds to the amount washed from the column in buffer E. The total yield corresponds to the amount recoverd in the buffer wash and after elution with urea. The difference between amount applied and the amount recovered is the fraction which cannot be eluted ("irreversibly" bound protein). For further details see Fig. 2, Methods and Demiroglou and Jennissen [38,39].

Type of gel	Degree of substitution (μmol/ml packed gel)	Fibrinogen (mg)				Total yield (%)
		Applied	Excluded	Bound	Eluted	
Sepharose 4B:						
Unsubstituted control		0.82	0.76	0.06	0	96
Alkyl-S-Sepharose:						
Homologous series:						
Ethyl-S-Sepharose	25.0	1.0	0.19	0.81	0.80	99
Propyl-S-Sepharose	25.0	1.0	0	1.00	0.73	73
Butyl-S-Sepharose	25.0	1.0	0	1.00	0.46	46
Degree of substitution series:						
Butyl-S-Sepharose	1.7	1.0	0.87	0.13	0	87
Butyl-S-Sepharose	4.0	1.0	0.63	0.37	0.22	86
Butyl-S-Sepharose	46.0	1.0	0	1.00	0.27[a]	27
Akyl-N_{II}Sepharose:						
Butyl-N_{II}-Sepharose	21.7	1.0	0.92	0.08	0	92
Hexyl-N_{II}-Sepharose	1.9	1.0	0.81	0.19	0	81
Hexyl-N_{II}-Sepharose	4.3	1.0	0.83	0.17	0	83
Hexyl-N_{II}-Sepharose	7.7	1.0	0.30	0.70	0.55	85
Hexyl-N_{II}-Sepharose	17.2	1.0	0.04	0.96	0.80	84

[a] Final elution with 1% SDS desorbed an additional 0.52 mg (total yield after urea and SDS elution 79%).

adsorbed onto butyl-S-Sepharose (46 μmol/ml packed gel) and can be eluted by 1% SDS, as shown for fibrinogen. Thus the tripeptide shows a very similar binding behaviour as the three proteins above. It may therefore be concluded that the differences in the binding properties of the gels are quite independent of possible conformational changes and the residue accessibility as indicated by

TABLE IV

QUANTITATIVE HYDROPHOBIC CHROMATOGRAPHY OF THE TRIPEPTIDE Trp–Trp–Trp ON BUTYL-AGAROSES

The data were derived from column experiments as described in the text. The Trp tripeptide was applied in buffer F. The column was then washed with the same buffer and eluted with 1% SDS. The excluded amount of tripeptide corresponds to the amount washed from the column in buffer F. The eluted amount corresponds to the amount eluted with SDS. The total yield corresponds to the amount recovered in the buffer wash and the elution with SDS. For further details see legend to Table III and the text.

Type of gel	Degree of substitution (μmol/ml) packed gel)	Trp–Trp–Trp (mg)				Total yield (%)
		Applied	Excluded	Bound	Eluted	
Butyl-N_{II}-Sepharose	41	1.0	1.05	0.03	0.03	108
Butyl-S-Sepharose	46	1.0	0	1.0	0.99	99

the independence of adsorption on the relative molecular masses of the ligands in a range between about 0.3 and 340 · 10^3.

Why do the alkyl-S-Sepharoses bind proteins and the Trp tripeptide more strongly than the alkyl-N-Sepharoses? Excluding the generation of sulphur–metal complexes as very improbable [11], three other possible explanations exist. Firstly, it could be reasoned that the sulphur atom shows properties similar to a carbon atom, as sulphur is also *non-polar* [41]. As sulphur increases the free energy of the system as it is transferred, *e.g.* from the interior of a protein to water [41], it may have similar water-structuring properties as carbon. Based on the free energies of transfer, neutral oxygen and nitrogen (N/O) belong to a different (polar) class of atoms than the sulphur atom [41] and this might explain the different properties of butyl-S- and butyl-N-agaroses. On the other hand, a major difference between the sulphur atom, a carbon atom and the N/O class atoms lies in the *d*-electrons of the sulphur atom. A plausible conclusion is that there might be an aromatic amino acid near the hydrophobic pocket or area of protein binding close to the base of the thioalkyl residue (see Fig. 3), leading to an enhancement of the binding-affinity by $d\pi$–π interactions [11]. Similar conclusions have been reached by Porath [42] for other sulphur-bearing agarose derivatives. However, it is not possible at this time to define differences or similarities between these two gel systems, *i.e.* those proposed by Demiroglou and Jennissen [11] and Porath [42] on a mechanistic basis. A third possibility for the significant difference between alkyl-N_{II}- and alkyl-S-Sepharose gels could lie in the different *binding angle* between the carbon and nitrogen atoms compared with the angle between the carbon and sulphur atoms possibly allowing a stronger interaction with the protein on alkyl-S-agaroses. Steric hindrance by a bulky base element should also be considered in this context.

The main conclusion from these experiments is the fact that the protein "sees" the difference in the base of the alkyl residue, which consists of a carbamate structure in the alkyl-N_{II}-agaroses and a thioether in the alkyl-S-agaroses (Fig. 1). The protein "recognizes" a nitrogen in one instance and a sulphur atom in the other, *i.e.* the "base atoms" (for definition, see legend to Fig. 1). This strong influen-

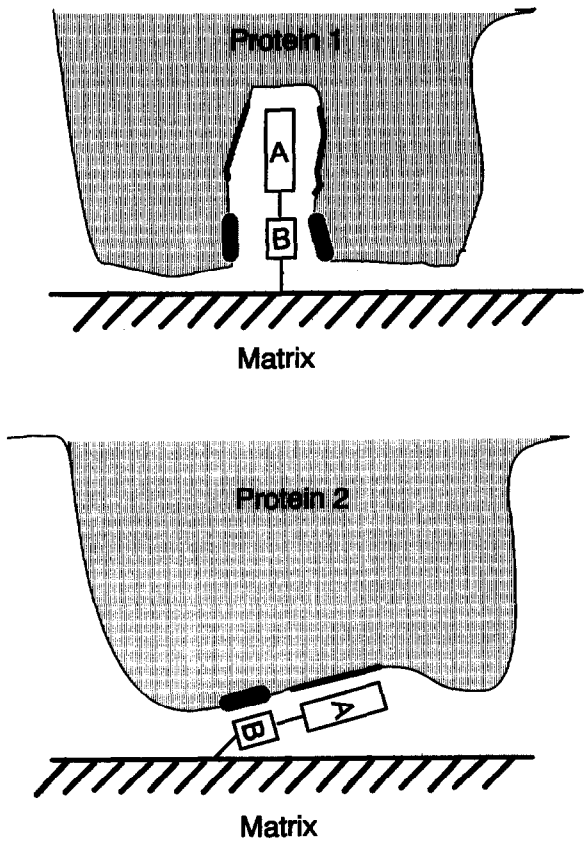

Fig. 3. Schematic models of "base-atom recognition". The models, not drawn to scale, schematically show a putative "binding site" [31] on a protein interacting with an immobilized alkyl residue as part of a multivalent interaction [4,5] in a "binding unit" [31] between the protein and the interface. Protein 1 shows a binding pocket whereas protein 2 shows a binding area. The immobilized residue is subdivided into the tip element (residue and tip) (A) and the base element (B). The hydrophobic tip element interacts with hydrophobic amino acids (thick solid line) lining the pocket in the upper panel or the area in the lower panel. (A hydrophobic lining on both sides of the pocket is probably the exception.) The base element of the alky-S-agaroses, *i.e.* the sulphur atom, might interact via charge transfer with aromatic amino acids (hatched area) lining the orifice of the pocket in the upper panel or an area to the left of the hydrophobic portion in the lower panel. If the charge transfer model is inadequate the sulphur atom would lead to a hydrophobic extension of the immobilized alkyl residue capable of interacting with the aromatic amino acids by hydrophobic interactions. These additional interactions would not be possible for the other base element, *i.e.* the carbamate group of the alkyl-N-agaroses, which would show a lower binding affinity. For further details see the text and legend to Fig. 1.

ce of the base atoms of otherwise identical uncharged and non-polar immobilized residues on protein binding is striking and has been called "base-atom recognition" [14]. The "base element" (Fig. 1) can consist of one atom or a group of atoms. The point is that this element can be of stronger influence than the "tip element" in itself and can largely determine the adsorption properties of the gel. How this interaction might occur is shown schematically in Fig. 3 for the two possibilities of a so-called (hydrophobic) pocket or (hydrophobic) area. In addition, different binding angles of the immobilized residue are also symbolized in the two instances. The base atom recognition capability of proteins sheds some doubt on the general validity of the spacer "arm hypothesis" [43] which suggests that immobilized molecules must be presented to a protein for "recognition" at a certain spacer distance from the matrix.

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