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Review

Oriented immobilization of biologically active proteins as a tool for revealing protein interactions and function

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Abstract

The advantages of oriented immobilization of biologically active proteins are good steric accessibilities of active binding sites and increased stability. This not only may help to increase the production of preparative procedures but is likely to promote current knowledge about how the living cells or tissues operate. Protein inactivation starts with the unfolding of the protein molecule by the contact of water with hydrophobic clusters located on the surface of protein molecules, which results in ice-like water structure. Reduction of the nonpolar surface area by the formation of a suitable biospecifc complex or by use of carbohydrate moieties thus may stabilize proteins. This review discusses oriented immobilization of antibodies by use of immobilized protein A or G. The section about oriented immobilization of proteins by use of their suitable antibodies covers immobilization of enzymes utilizing their adsorption on suitable immunosorbents prepared using monoclonal or polyclonal antibodies, preparation of bioaffinity adsorbent for the isolation of concanavalin A and immobilization of antibodies by use of antimouse immunoglobulin G, Fc-specific (i.e. specific towards the constant region of the molecule). In the further section immobilization of antibodies and enzymes through their carbohydrate moieties is described. Oriented immobilization of proteins can be also based on the use of boronate affinity gel or immobilized metal ion affinity chromatography technique. Biotin–avidin or streptavidin techniques are mostly used methods for oriented immobilization. Site-specific attachment of proteins to the surface of solid supports can be also achieved by enzyme, e.g., subtilisin, after introduction a single cysteine residue by site-directed mutagenesis.

Keywords: Reviews; Oriented immobilization; Proteins

Contents

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1. Introduction

The basis of bioaffinity technique is the biospecific complex formation of biologically active compounds. Therefore this technique is not only an efficient method for the determination, isolation and application of antibodies, antigens and haptens, cells and cell organelles, cofactors and vitamins, enzymes, glycoproteins and saccharides, hormones, inhibitors, lectins, lipids, nucleic acid and nucleotides, toxins, transfer receptors and binding proteins or viruses [1], but also it is useful for the study of supramolecular structures in relation to their microenvironment. Oriented immobilization of various affinity ligands on porous or nonporous solid supports can not only provide us with efficient biospecific adsorbents suitable for a variety of applications but as well with models that may be used advantageously for studies of chemical processes in the living cell [2].

A biospecific complex formation for oriented immobilization of biologically active proteins was used first in 1978 with covalently attached Protein A for oriented immobilization of immunoglobulin G (IgG) [3,4,78]. Janis and Regnier [5] used the other bacterial Fc binding protein – Protein G – instead of Protein A which has the advantage of binding to a wider range of IgG species and subclasses. Information on many examples is in Section 2.

The high efficiency of most natural processes and their low energy demands depend on highly active and specific catalysts – enzymes. In nature, enzymes are produced by organisms for their own use: in regulated metabolic processes their low stability, narrow specificity and strictly defined requirements concerning the reaction medium are inherently connected with their function. However, it is important to take into account that, after completion of their function in living cells, i.e., catalytic function, their denaturation and splitting by proteinases occurs. For stabilization, conformation, as well as function of enzymes, hydrophobicity in vivo plays an important role. However, the contact of nonpolar amino acids with water is enthalpically disadvantageous and results in ice-like water structure. This contact of water with hydrophobic surface clusters of proteins in vitro is harmful for protein stability [6]. Hence, a reduction of the nonpolar surface area by use of carbohydrate moieties or by the formation of a suitable biospecific complex should stabilize proteins. Shami et al. [7] showed the dramatically increased activity of amylase in complexes with their antibodies. Some antibodies might interact at sites where protein unfolding is initiated, or where proteolytic digestion occurs, thereby stabilizing the protein. For the average bioaffinity antibody–antigen binding (10^8 M^{-1}), the interaction could reduce the free energy of the antigen by about 4.2 kJ mol^{-1}. This is sufficient to confer increased stability since the differences in free energy between the folded and unfolded states for active proteins are in the range of 2.1–6.3 kJ mol^{-1}.

Sheriff et al. [8] used X-ray crystallography to determine the three-dimensional structure of antibody–antigen complexes by use of antilysozyme Fab variable region of antibody and lysozyme. They demonstrated that more than 80 van der Waals bonds are formed during the interaction antibody–lysozyme. Formation of the complex resulted in exclusion of all molecules of water. Biospecific adsorption of biologically active proteins to immunosorbents prepared by use of suitable monoclonal (MAb) or polyclonal antibodies can also combine the isolation of molecules with their oriented immobilization. Oriented immobilization of enzymes and of other biologically active proteins by antigen–antibody interaction, which can result both in good steric accessibility of the active sites and in an increased stability is discussed in Section 3.

Glycoproteins, antibodies and enzymes contain their active site in the polypeptidic part of the

molecules. Therefore the immobilization through their carbohydrate moieties gives conjugates with very good steric accessibility of their active sites. A review about stabilization and oriented immobilization of glycoproteins through their carbohydrate moieties was published by Turková et al. [9]. Information about advantages of this way of immobilization is in Section 4. Section 5 contains studies on oriented and reversible immobilization of glycoprotein using a novel boronate affinity gel [10]. Reversible and oriented surface immobilization of functional proteins on oxide surfaces based on the immobilized metal ion affinity chromatography (IMAC) technique was described by Schmid et al. [11]. Oriented immobilization by use of metal complexes is described in Section 6.

The binding of water-soluble vitamin biotin to the egg white protein, avidin, or to its bacterial counterpart, streptavidin, is accompanied by a vast decrease in free energy compared to that observed for other noncovalent interactions. The extraordinarily high affinity constant of biotin–avidin complex (10^{15} M^{-1}) makes this system an efficient tool for oriented immobilization which has been used for study and application in various fields of biology, protein chemistry and biotechnology.

Information on biotin–avidin or streptavidin techniques is given in Ref. [12] (Avidin–Biotin Technology). Information on these techniques is in Section 7.

Orientation of immobilized proteins was elaborated also by Huang et al. [13]. To achieve this goal, a single cysteine residue was introduced into the cysteine-free enzyme by site-directed mutagenesis. More information on this method is in Section 8. The advantages of described oriented immobilizations are summarized in Section 9.

2. Immobilization of antibodies by use of Protein A or Protein G

Protein A, which is a coat protein extracted from the bacterium *Staphylococcus aureus,* has the unique capacity to bind mammalian immunoglobulins, especially IgG. This protein covalently attached to Sepharose was the first reagent employed for oriented immobilization by use of biospecific complex formation. Werner and Machleidt [3] used *N*-ethyl-*N′*-(3-dimethylamino-propyl)-carbodiimide hydrochloride to bind IgG to immobilized Protein A, while Gersten and Marchalonis [4] employed dimethylsuberamide as a crosslinking agent for covalent coupling of the same proteins. The binding of the IgG molecules through their Fc constant region was thus effected. This mode of immobilization makes the binding site of antibody, always located on the top of its Fab variable regions, well accessible for interaction with the antigen. The affinity constant of the complex created was high but the multiple use of immunoadsorbents under the conditions necessary for the elution of most antigens requires the crosslinkage of IgG with Protein A through a covalent bond. Applications of immobilized Protein A in immunochemical techniques were reviewed by Langone [14].

Immunoaffinity HPLC (HPIAC or HPIC) with immobilized antibodies has proved to be very effective for one-step isolation of antigens, even from very complex samples such as plasma membrane extracts. The problem with HPIAC is the quick deterioration of the columns, caused by increasing denaturing of the immobilized antibodies during elution. In order to solve this problem, Josic et al. [15] recommended an indirect method for analytical HPIAC. For this purpose, the antibodies were adsorbed to a staphylococcal Protein A HPIAC. The solution containing the antigens was then applied and after washing the antigen–antibody was eluted from the column.

Protein A-coated glass beads were developed as a universal support medium for HPIAC by Phillips et al. [16]. The preparation of IgG antibody binding is demonstrated in Fig. 1. Protein A has the ability to bind to the Fc portion of IgG. Protein A is composed of five subunits, each with its own Fc receptor, although three of these receptors become inactive when the molecule is immobilized. If Protein A is applied as a coating to either solid or controlled pore glass beads, its Fc receptors become points of attachment on which the antibody can be immobilized. The bound antibodies are then covalently immobilized on the Protein A covered surface by treatment with carbodiimide. This attachment in turn helps to orient the antigen receptors of the antibody toward the mobile phase of the column. In addition,

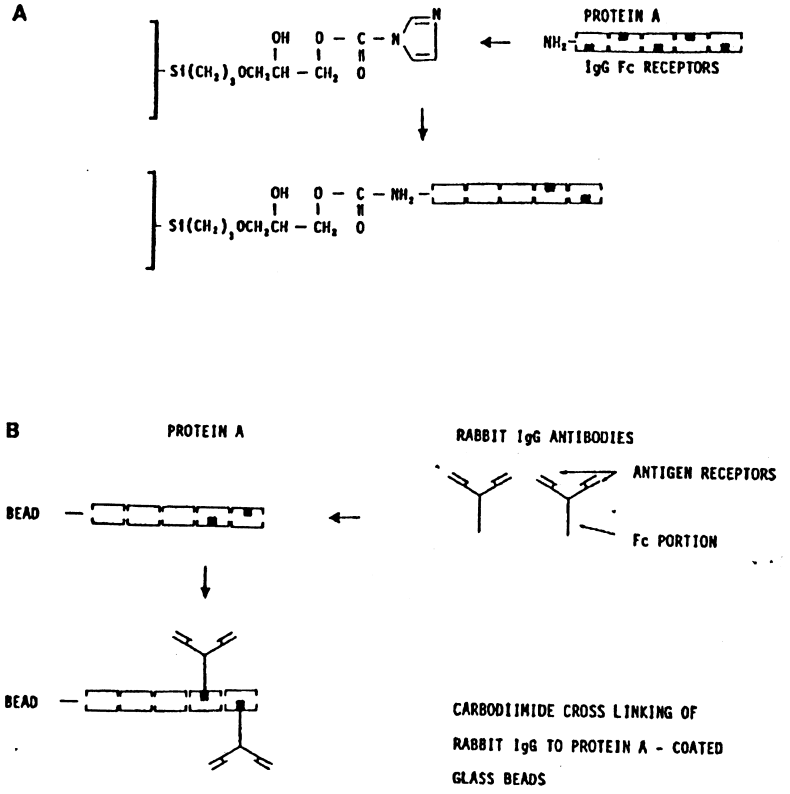

Fig. 1. Diagrammatic representation of the process for binding rabbit antibodies to Protein A-coated glass beads. (A) Illustration of the chemistry used to couple Protein A to the carbonyl diimidazole-derivatized glass beads. (B) Mechanism of IgG antibody binding to the Fc receptors on the Protein A-coated glass beads (from Phillips et al. [16] with permission).

the Protein A molecule becomes a spacer arm for the immobilized antibodies, thus preventing repulsion by the bead surface from inhibiting the formation of the antibody–antigen complex.

In order to increase the specific IgG-binding activity of immobilized Protein A, Solomon et al. [17] used tryptic digestion of Protein A for the preparation of its active fragments (FB). These FB fragments originate from almost identical five-repeated monoclonal Fc-binding units of 58 residues each. FB domain immobilized via glutaraldehyde to adipic dihydrazide-modified Eupergit CB6200 beads showed a higher antibody-binding capacity than intact Protein A, measured under the same experimental conditions.

However, the disadvantage is that only certain classes of immunoglobulin are able to bind to Protein A. Babashak and Phillips [18] overcame this problem by the development of avidin- or streptavidin-coated glass beads and the use of an avidin–biotin system after biotinylation of carbohydrate moieties of antibodies with biotin hydrazine. The avidin–biotin system will be more discussed in Section 7.

Compared to the receptor staphylococcal Protein A, streptococcal Protein G shows broader binding activity to immunoglobulins of different species as well as to immunoglobulin subclasses. Polzius et al. [19] determined kinetic rate constants and equilibrium constants by studying the interaction of human immunoglobulin with the immobilized Protein G. For the four human immunoglobulin subclasses a high affinity to protein G was determined with affinity constants ranging from 3.3 to $8.4 \cdot 10^{8}$ M^{-1}.

Riggin et al. [20] published that Protein G can be used to rapidly prepare an immunosorbent column that will interact specifically with antigens. Because

of its great flexibility and easy of use, a short high-performance Protein G column (30×2.1 mm I.D.) was used to immobilized both MAb and polyclonal antigrowth hormone antibodies. When a size-exclusion column was coupled witha high-performance affinity column, a comparison between the elution profile before and after the antibody immobilization was used to study antigen components present in the sample. Various human growth hormone structural variants and aggregates were studied using this approach which is simple, fast and does not involve the usage of radioactive material.

Immunoaffinity extraction using antibody–antigen interaction can provide high specificity for targeted analytes. Trace analysis of lysergic acid diethylamine (LSD) analogs and metabolites in human urine by use of online immunoaffinity extraction-coupled capillary high-performance liquid chromatography–tandem mass spectrometry was described by Cai and Henion [21]. Their system involved three columns: protein G immunoaffinity column with noncovalently immobilized antibody specific to the analytes of interest, a packed capillary trapping column, and a packed capillary analytical column. With use of a short packed capillary trapping column, the protein G column could be operated at flow-rates of 2.5–4 ml/min while the packed capillary analytical column was maintained at a flow-rate of 3.5 μl/min. Human urine diluted 1:1 with phosphate-buffered saline was pumped directly onto the immunoaffinity column without pretreatment and was analyzed by electrospray mass spectrometry following the column switching process. Sample handling and transfer procedures were eliminated. The system was optimized and evaluated for the determination of LSD, its analogs, and metabolites in spiked human urine at low part-per-trillion (ppt) levels using mass spectrometric detection. LSD-positive human urine specimens from LSD users were also analyzed. Concentrations as low as 2.5 ppt of LSD and several of its analogs were detected in spiked human urine using the described system. The technique was shown to be useful for the selective enrichment and characterization of drug metabolites with similar substructures as the parent drug.

Interaction of Protein G and IgG was used by Mauro et al. [22] in fiber-optic fluorometric sensing of polymerase chain reaction (PCR)-amplified DNA using an immobilized DNA capture protein. The gene for a chimeric protein composed of the IgG-binding β2 subdomain of streptococcal Protein G (54-residue) fused with the DNA binding domain of the yeast transcriptional regulator protein GCN4 was constructed, and this Protein G–GCN4 protein was overexpressed in *Escherichia coli*. The purified protein was noncovalently bound to IgG-modified fibers utilizing strong and specific interaction between the protein G β2 domain and goat IgG that had been covalently immobilized on the fiber surface. Nanomolar concentrations of amplified DNA labeled with the fluorophore tetramethylrhodamine and the AP-1 consensus nucleotide sequence recognized by GCN4 (5′-ATGACTCAT) were rapidly and selective bound within the evanescent zone of multimode laser-illuminated fibers. Signal from unincorporated fluorescent PCR primer was negligible. Individual fibers could be used for multiple sequential assays, since the fluorescent double-stranded DNA was rapidly and completely stripped from their surfaces with high salt solutions, leaving the IgG–Protein G–GCN4 DNA binding complex intact to accept another PCR sample. The described assay was carried out for purposes of pathogen screening, epidemiological studies, genetic evaluation, and forensic investigation.

Morais et al. [23] immobilized eight MAbs against the insecticide carbaryl on three sorbents, namely, controlled pore glass, hydrazide derivatized agarose beads and a hydrophilic polymer with immobilized Protein A/G. The interaction between immobilized antibodies and antigen was directly detected using a carbaryl hapten conjugated to horseradish peroxidase. Immunosorbent characterization was based on both sensitivity and reusability. Optimal immunosorbent regeneration was achieved using 0.1 *M* glycine–HCl, pH 2.0 as the desorbent solution. The best covalent immunosorbent was obtained by immobilization of MAb on hydrazide derivatized agarose beads. The best immunosorbent obtained by reversible immobilization was MAb on immobilized Protein A/G. Using this support the eventual irreversible denaturation of covalently immobilized Mabs was overcome.

Oriented immobilization of antibodies and its applications in immunoassays and immunosensors was reviewed by Lu et al. [24].

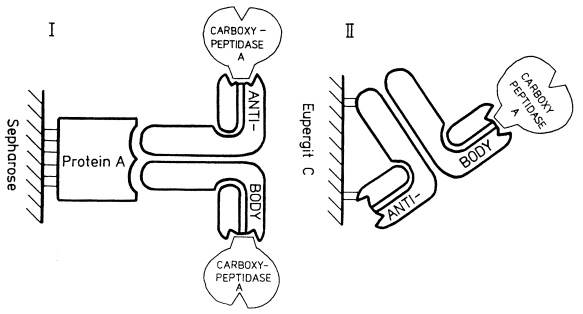

Fig. 2. Schematic drawing of the oriented immobilization of carboxypeptidase A by adsorption to MAb against carboxypeptidase A (with affinity constant=10^9 M^{-1}) which is (I) adsorbed to Protein A–Sepharose; (II) attached to acrylic polymer Eupergit C. Covalent bonds are shown as full lines (from Turková et al. [35] with permission).

3. Oriented immobilization of proteins by use of their suitable antibodies

3.1. Immobilization of enzymes by use of MAbs

Covalent crosslinkage of the complex employed for oriented immobilization is not necessary if the substance immobilized is to be used under milder conditions. The results reported by Solomon et al. [25] can be summarized by way of example. Fig. 2 demonstrates two modes for the immobilization of carboxypeptidase A (CPA) by these authors. In both cases they simply used adsorption of CPA to the antibody against the enzyme for its immobilization. The affinity constant of this complex was of the order of 10^9 M^{-1}. The attachment of the antibody to the solid support was performed by two different procedures. First it was again immobilized by mere adsorption, i.e., by the adsorption of the antibody to Protein A which had been covalently attached to Sepharose. Second the antibody was covalently attached to Eupergit C. Table 1 lists the catalytic constants for both peptide and ester hydrolysis by the two types of immobilized CPA and by native CPA. In all three cases the catalytic constants are nearly identical. The enzymatic activity of immobilized carboxypeptidase did not change after repeated incubation with the substrate in solution. The stability of the enzyme increased after immobilization.

Parekh et al. [26] by use of X-ray crystallography characterized the carbohydrate residues located on the Fc fragment of the antibody molecule which form the biospecific complexes with the complement. Since the antigen-binding sites of immunoglobulins are in polypeptide Fab moieties, modification of amino acid side-chains may result in either partial or complete loss of Ig activity. Therefore the coupling of antibodies through their carbohydrate moieties has been found to increase the steric accessibility of the immunospecific sites [27]. Fleminger et al. [28] used carbohydrate moieties after periodate oxidation for oriented immobilization of MAbs against CPA and horseradish peroxidase (HRP) on amino and hydrazide derivatives of Eupergit C for the preparation of multienzyme reactor. They immobilized MAbs which did not inhibit the respective enzymatic activities. The oxidation and coupling reactions were optimized to achieve highly active matrix-conjugated antibodies. Thus, antibody–matrix conjugates were obtained that possessed antigen-binding activities close to the theoretical value of 2 mol antigen bound/mol immobilized antibody. A higher CPA binding activity was also

Table 1
Kinetic constants for native and immobilized carboxypeptidase A (CPA)*

Preparation	Peptide hydrolysis[a]			Ester hydrolysis[b]		
	K_M $(M)\cdot10^{-4}$	V_{max}[c] $(M/min)\cdot10^{-5}$	K_i $(M)\cdot10^{-5}$	K_M $(M)\cdot10^{-5}$	V_{max}[c] $(M/min)\cdot10^{-5}$	K_i $(M)\cdot10^{-5}$
CPA	2.6	5.5	7.0	8.8	2.0	2.0
S–(PA–Ab)–CPA	2.2	5.0	6.8	7.9	3.5	2.0
E–Ab–CPA	4.0	9.0	7.5	9.0	5.0	2.5

* Sepharose–(Protein A–Antibody against CPA)–CPA=S–(PA–Ab)–CPA; Eupergit C–(Antibody against CPA)–CPA=E–Ab–CPA.
[a] Using hippuryl-L-phenylalanine as substrate and phenylpropionic acid as inhibitor.
[b] Using hippuryl-DL-β-phenyl lactic acid as substrate and phenylpropionic as inhibitor.
[c] Calculated per mg free or bound enzyme.

achived by Hadas et al. [29] by the direct immobilization of MAb against CPA to epoxy-containing Eupergit C after the reversible protection of the free amino groups of MAbs by dimethylmaleic anhydride. The activity of immobilized antibody against CPA after this treatment increased 3–10.7 fold.

Conformation and sequence of two carbohydrate chains located on the Fc fragment of the antibody molecule were determined by Parekh et al. [26] with *N*-acetylneuraminic acid and galactose as terminal sugars. Therefore, Solomon et al. [30] could use instead of periodate oxidation of carbohydrate moieties of antibodies against CPA and HRP enzymatic oxidation of these antibodies. Concomitant treatment of carbohydrate moieties of the antibodies with neuraminidase and galactose oxidase generated aldehyde groups in the oligosaccharide moieties of immunoglobulins. These groups reacted selectively with hydrazide groups of Eupergit C with attached adipic dihydrazide. Neuraminidase and galactose oxidase are glycoproteins. Both enzymes, after periodate oxidation, were immobilized on Eupergit C-adipic dihydrazide. The result of this immobilization was an efficient and selective system for the enzymatic oxidation of the MAbs with no impairment of their immunological activity. Oriented immobilization of enzymatically oxidized antibodies on a hydrazide-containing carrier led to the formation of an antibody matrix.

Sepharose particles with immobilized higher plant NADH: nitrate reductase (NR) were prepared by Ruoff et al. [31] by the use of immobilized MAb. To elucidate the question of the molecular basis of light-induced circadian rhythms of NR activity they prepared a system by adsorption of NR to immobilized antiNR MAb. They found that not all antibody-bound NR was eluted by 1 *M* NaCl, but that a substantial amount of enzyme remained bound to the antibodies (approximately 50%) and remained catalytically active, even after NaCl concentrations as high as 2 *M* were applied. They reported the first example of NADH substrate inhibition of higher plant NR in solution and for an immobilized enzyme using a novel immobilization technique with a MAb. The immobilized enzyme had greater thermal stability than the enzyme in solution (Fig. 3). This enhanced thermal stability of antibody bound enzyme might be of general interest, because many NR

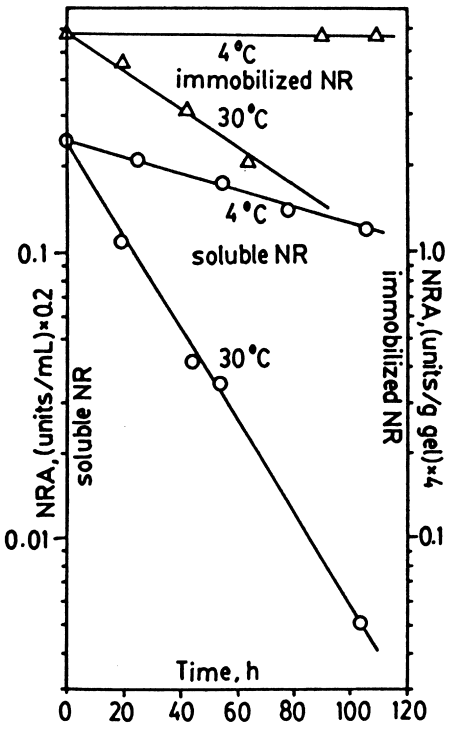

Fig. 3. Thermal stability of soluble and immobilized nitrate reductase (NR) preparations in 0.1 *M* Tris, pH 7.5. Calculated half-lifetimes for soluble NR: 4°C, 106 h; 30°C, 19 h; immobilized NR: 4°C, no significant decrease in activity observed during a 6 day period; 30°C, 46 h. (from Ruoff et al. [31] with permission).

extraction procedures, like the blue-Sepharose method, often result in quite unstable enzyme preparations.

MAb against HRP did not interfere with its enzyme activity. It also possessed a high affinity towards the enzyme and therefore it was used for the preparation of a highly active immobilized enzyme by Solomon et al. [32]. They prepared the complex of peroxidase and MAb in solution and attached this immunocomplex to immobilized antiFc antibodies.

The applicability of the MAbs for preparing immobilized multienzyme reactors containing acetylcholine esterase and choline oridase has been demonstrated by Gunaratna and Wilson [33] by using this reactor to detect acetylcholine and choline in brain tissue samples. Optimization of this multienzyme flow reactor used with HPLC and electrochemical detection has been developed for the determination of acetylcholine and choline at femtomole levels

[34]. Immobilized antibody goat antimouse IgG, Fc specific was used for the preparation of immunosorbent containing site directed MAbs against both enzymes. No inhibition of the enzyme activity was observed when an excess of each antibody was mixed in solution with enzyme. Before the introduction of the enzymes, the column of prepared immunosorbent was saturated with bovine serum albumin to prevent the nonspecific adsorption of the enzyme. The enzymes were immobilized by injecting an aliquot of enzyme solution in carrier buffer. Coimmobilization of both enzymes using immobilized MAbs provided higher sensitivity in the assay system due to the increased local intermediate concentration surrounding the first enzyme in the sequence which is then immediately available for reaction with the next enzyme.

3.2. Immobilization of proteinases by use of its suitable polyclonal antibodies

The use of cheaper pig polyclonal antibodies against chymotrypsin for oriented immobilization of enzyme was developed by Turková et al. [35] and Fusek et al. [36]. Fig. 4 shows the isolation of polyclonal antibodies suitable for oriented immobilization of chymotrypsin. It is schematic drawing (I) of oriented immobilization of chymotrypsin by use of covalent crosslinking of its active site to immobilized natural trypsin inhibitor antilysin with glutaraldehyde and (II) of use of this column for the isolation of IgG against antigenic sites outside the active site of chymotrypsin. Bílková et al. [37] used the same principle under better experimental conditions. Antilysin was covalently attached to bead cellulose Perloza MT 200 that had been activated with 2,4,6-trichloro-1,3,5-triazine. At pH 7.2 chymotrypsin was specifically and firmly bound to this biospecific adsorbent. The covalent coupling of chymotrypsin to immobilized antilysin was achieved by crosslinking with glutaraldehyde. The assay of zero chymotryptic activity with succinyl-L-phenylalanine-*p*-nitroanilide as substrate showed that the enzyme was immobilized via its active site only. Fig. 5 shows the chromatography of antibodies on a matrix composed of chymotrypsin immobilized via its active site to antilysin–cellulose. No decrease in proteolytic activity resulted from incubation of soluble chymotrypsin with isolated antichymotrypsin IgG in a 1:1 molar ratio. These isolated antibodies are thus suited for the preparation of a biospecific affinity matrix bearing immobilized chymotrypsin oriented such that substrate proteins are accessible to its active site. The antigenic affinity of the antichymotrypsin antibodies was essentially unchanged by periodate oxidation of their carbohydrate moieties. Therefore this procedure was used to covalently attach isolated antichymotrypsin antibodies to a hydrazide derivative of beaded cellulose. The molar ratio of biospecifically adsorbed chymotrypsin molecules to immobilized antibody was 2:1, which is the value predicted for enzyme occupancy of each of the two Fab binding sites in IgG. The immobilized chymotrypsin retained practically 100% of the native catalytic activity determined by use of the high molecular substrate–denatured hemoglobin. Its proteolytic activity did not change after more than 1 year.

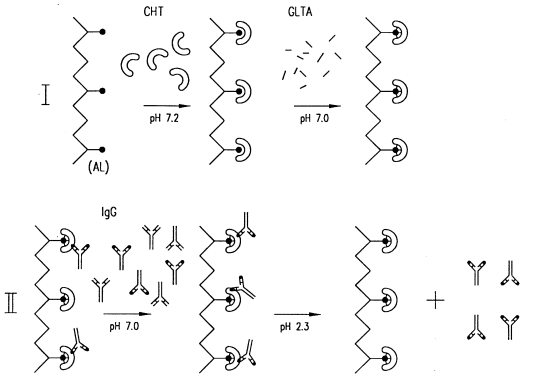

Fig. 4. Schematic drawing (I) of oriented immobilization of chymotrypsin (CHT) by use of covalent crosslinking of its active site to immobilized natural polyvalent trypsin inhibitor antilysin (AL) with glutaraldehyde (GLTA) and (II) of use of this column for isolation of immunoglobulin G (IgG) against chymotrypsin (with antigenic sites outside the active site of CHT). Covalent bonds are shown as full lines (from Turková et al. [35] with permission).

In order to eliminate the kinetic limitation of chymotryptic hydrolysis of protein, porous bead cellulose was replaced by 1.2 μm nonporous hydroxyethylmethacrylate beads [38]. Isolated antichymotrypsin antibodies, attached to nonporous beads containing 1.9 μmol of dihydrazide groups per 1 g of dry product, was used for the coupling of

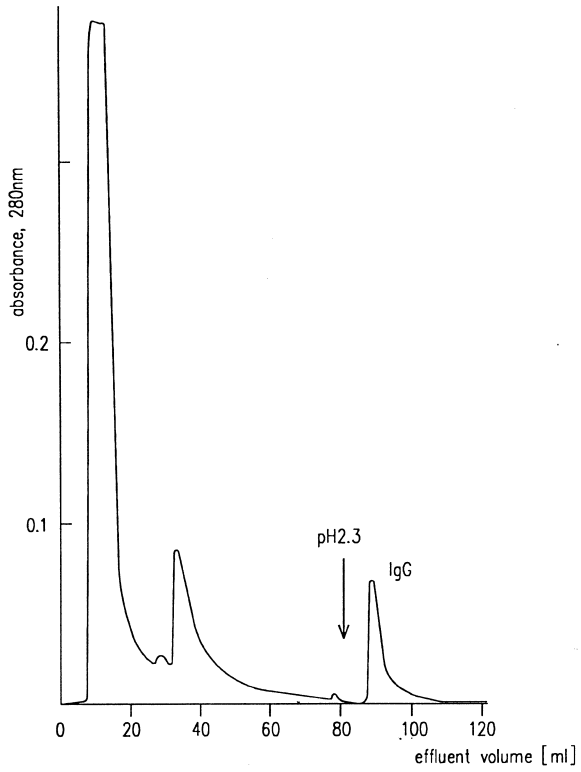

Fig. 5. Isolation of antichymotrypsin antibodies by chromatography on a matrix composed of chymotrypsin immobilized via its active site to antilysin–cellulose. The IgG fraction obtained by ion-exchange chromatography on DEAE-cellulose was applied to the column (10×0.7 cm I.D.) in 0.1 *M* phosphate buffer (pH 7), and the antichymotrypsin antibodies were eluted with 0.1 *M* glycine buffer (pH 2.3) containing 0.1 *M* NaCl. Flow-rate: 0.6 ml/min (from Bílková et al. [37] with permission).

antichymotrypsin antibodies. Chymotrypsin after the adsorption on the prepared immunosorbent (Fig. 6) retained practically 100% of the native proteolytic activity.

Štovíčková et al. [39] described a further method for the preparation of polyclonal antibodies suitable for biospecific adsorption of trypsin. Pig polyclonal antibodies were prepared against the biospecific complex of trypsin with its natural basic pancreatic inhibitor, antilysin, wherein the active site of trypsin is buried inside the complex. The active sites of enzyme and inhibitor therefore do not elicit an antibody response. Antibodies against trypsin were isolated from prepared antiserum by bioaffinity chromatography on trypsin-bound beaded cellulose. The catalytic activity of trypsin was not affected by interaction with these antibodies, even in the presence of excess of antibody. Antibodies covalently attached to Sepharose were used for the biospecific adsorption of trypsin. Comparison of enzymatic activities of trypsin randomly bound to CNBr-activated Sepharose 4B and biospecifically immobilized trypsin is described in Table 2. The results show that the method used represents a general technique which is suitable for the preparation of highly active immobilized enzyme preparations for the biochemical study of enzymes naturally bound in the organelle structures, because the kinetics of metabolic processes of an organism and heterogenous catalysis of enzyme reactors are governed by the same rules.

Only immobilized proteinases are suitable for the procedures for microscale analysis of proteins by peptide mapping to be obtained from picomole to femtomole quantities of proteins which were developed by Cobb and Novotny [40]. Peptide maps, obtained by enzymatic cleavage of proteins, can be employed for a range of purposes: (a) detection of pathological changes of proteins occurring in physiological fluids in submicrogram amounts, (b) detection of posttranslational protein modification, (c) identification and localization of genetic variants and (d) quality control and monitoring of genetically engineered protein products. The determination of the sequence of a protein is the first step in a comprehensive understanding of how it works. Already in 1988, a combination of immobilized proteinases, LC columns, and a thermospray LC–mass spectrometry was used for rapid protein sequencing by Stachowiak et al. [41].

3.3. Preparation of bioaffinity adsorbent for the isolation of concanavalin A (ConA)

To prepare an efficient biospecific adsorbent for Con A, ovalbumin (OA) was immobilized using an antiOA fraction of immunoglobulin that reacted with the antigenic determinants located in the protein part of OA only [27]. Fig. 7 shows the isolation of OA antibodies from rabbit antiserum by bioaffinity chromatography on a column of OA immobilized via only one of its carbohydrate moiety after its periodate oxidation to an hydrazide derivative of cellulose. The binding capacity of antibodies against the

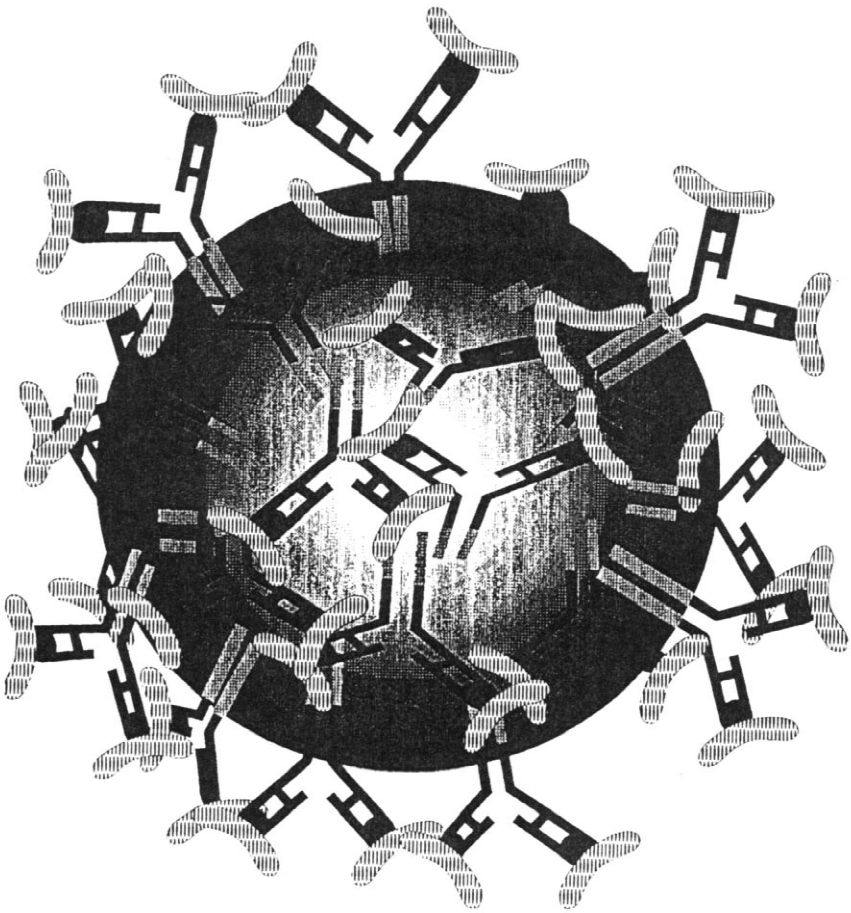

Fig. 6. Schematic drawing of chymotrypsin immobilized by use of suitable antibodies coupled to nonporous solid support (unpublished).

Table 2
Comparison of enzymatic activities of randomly and biospecifically bound trypsin

Preparation	Free trypsin	Randomly bound trypsin	Biospecifically bound trypsin
Amount[a] of bound antibody	–	–	1.40 mg
Amount of bound trypsin	–	2.6 mg	66 μg
Specific proteolytic[b] activity of trypsin	6.3 U	1.0 U	6.21 U
Specific amidolytic[c] activity of trypsin	840 nmol	585 nmol	809 nmol
Relative specific proteolytic activity	100%	15%	98.6%
Relative specific amidolytic activity	100%	70%	96.3%

[a] Amount is related to 1 g of wet carrier.
[b] 1 U means increase of optical density at 280 nm for 1.0 per 1 min.
[c] Activity is related to 1 min and 1 mg of trypsin.

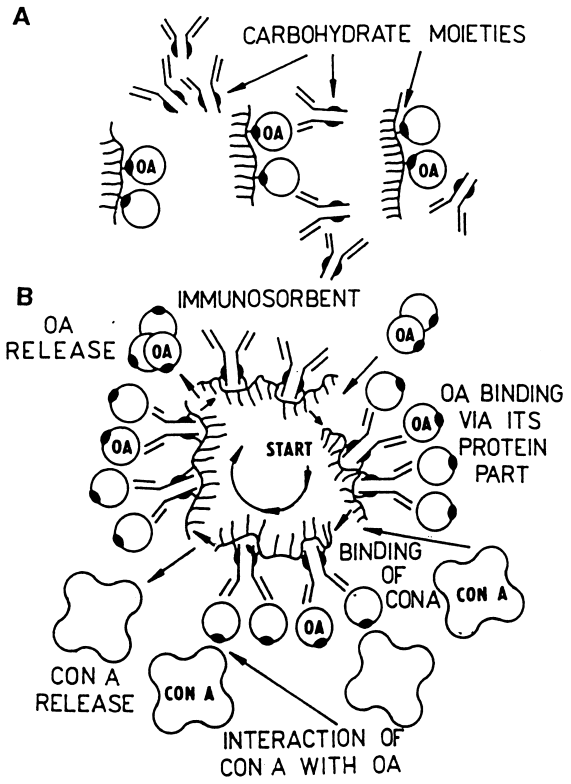

Fig. 7. Schematic drawing (A) of preparation of immunoglobulins (IgG) against ovalbumin (OA) by use of immunoaffinity chromatography on cellulose with OA attached through its carbohydrate moiety, and (B) of use of this oriented immobilized IgG (via carbohydrate moiety on hydrazide derivative of cellulose) after adsorption of OA for isolation of concanavalin A (Con A) (from Turková et al. [27] with permission).

protein moiety of OA did not decrease after periodate oxidation. Therefore this oxidized immunoglobulin fraction was coupled via its carbohydrate moieties to cellulose hydrazide. OA adsorbed onto this immunosorbent was an efficient sorbent for the isolation of Con A. The specificity of the Con A–OA interaction was examined and found unaltered. It was further demonstrated that Con A did not adsorb onto the starting support with immobilized immunoglobulin only. Because the free IgG reacted with Con A in solution, this finding may be considered as evidence that IgG was immobilized via its Fc part and that its carbohydrate moieties were not accessible for the interaction with Con A. Con A and OA were repeatedly bound and eluted and thus the chosen IgG–OA–Con A model strongly suggests the applicability of the principle of oriented immobilization to the preparation of various sorbents suitable for the purification of different compounds in satisfactory yields and with good reproducibility. It may be expected that oriented immobilization will find a use not only in separation processes, but also in analytical and research applications [2].

3.4. Immobilization by use of antimouse IgG, Fc specific

Oriented immobilization of murine MAbs specific to acetylcholine esterase and choline oxidase by use of immobilized goat antimouse IgG, Fc specific was described by Gunaratna and Wilson [33] and was already discussed in Section 3.1.

4. Oriented immobilization of glycoproteins by use of their carbohydrate moieties

The importance of the presence of carbohydrates in a molecule of acid phosphatase for its thermal stability and its protection against proteolytic digestion was shown by Barbarič et al. [42]. Chu and Maley [43] showed a decrease in the stability of carboxypeptidase Y with decreasing carbohydrate content. Galactosylation as a tool for the stabilization and immobilization of proteins was described by Turková et al. [44] and stabilization of trypsin by glycosylation by Vaňková et al. [45]. Immobilization of glycoproteins through their carbohydrate moieties protects against decrease of their biological activity caused by their deglycosylation.

4.1. Immobilization of antibodies through carbohydrate moieties

A schematic drawing of the immobilization of antibodies through their carbohydrate moieties is in Fig. 7B. The advantage of the immobilization of antibodies through their carbohydrate moieties to the hydrazide derivatives of solid supports has been already discussed in Section 3. Dakour et al. [46] coupled anti blood group A MAb to Con A-Sepharose for the separation and partial sequence analysis of blood group A-active oligosaccharides. Wolfe and Hage [47] published in their paper about automated

determination of antibody oxidation using flow injection analysis that the oxidation of antibody carbohydrate residues is a common approach used for site-specific antibody immobilization or modification.

4.2. Immobilization of enzymes through their carbohydrate moiety

The combination of several complementary binding sites on the surface of the enzyme molecule permits the formation of a relatively large number of biospecific complexes to be achieved. These complexes can be utilized for efficient isolation of enzymes as well as for their oriented immobilization. Fig. 8 shows the surface of an enzyme molecule covered with several complementary binding sites [48]. Such an enzyme could be, for instance, carboxypeptidase Y containing the complementary binding site for glycyl-glycyl-*p*-aminobenzylsuccinic acid, a specific inhibitor. The active site of this enzyme contains a free SH-group. Carboxypeptidase Y is a glycoprotein whose carbohydrate moiety specifically interacts with Con A, a lectin. The antigenic sites of the enzyme can form the biospecific complexes with its antibodies. Enzymes form also complexes with substrates and their analogues, cofactor, allosteric effectors, metal ions, etc.

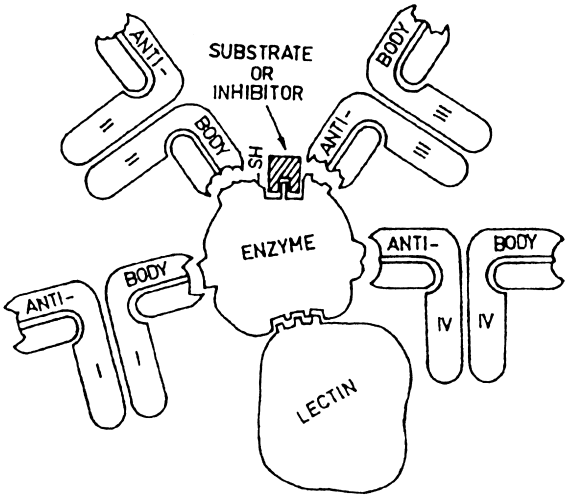

Fig. 8. Possible ways in which an enzyme may form biospecific complexes (from Turková et al. [48] with permission).

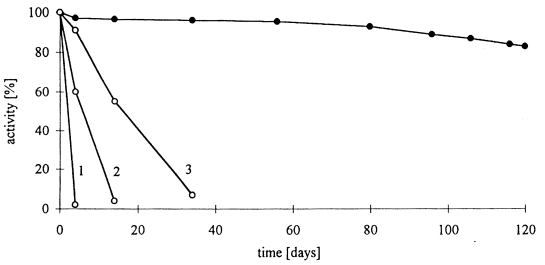

Fig. 9. Storage stabilities of glucose oxidase (GO) at 25°C. Activity of immobilized GO with concentration 0.36 mg/ml support (– • –), activity of free GO (– ∘ –) with concentration: 1–1 mg/ml, 2–0.1 mg/ml and 3–0.01 mg/ml. Specific activity determined by standard assay taken as 100% to calculate retained activity (from Ivanov et al. [49] with permission).

The type of the complex formed determines the mode of their action in chemical processes which take place in the living cell. Turková et al. [48] described affinity chromatography of carboxypeptidase Y by use of three biospecific adsorbents: Gly-Gly-*p*-aminobenzylsuccinic acid–Spheron, Mercury–Spheron and Spheron with attached Con A. Carboxypeptidase Y after adsorption onto Con A–Spheron was crosslinked with glutaraldehyde. The bound enzyme retained 96% of the native catalytic activity and showed very good operational stability. However, high stability of enzyme can be achieved also by the coupling via glycosidic residues to hydrazide derivative of solid support. Fig. 9 shows the storage stability of glucose oxidase from *Penicillium vitale* coupled after periodate oxidation of its carbohydrate moiety to the hydrazide derivative of polyacrylate-coated glass [49]. Activation of the galactose-containing carbohydrate moiety of glucose oxidase from *Aspergillus niger* by use of galactose oxidase was described for the same way of immobilization by Petkov et al. [50].

5. Oriented immobilization using boronate affinity gel

Studies on oriented and reversible immobilization of glycoprotein using novel boronate affinity gel were described by Liu and Scouten [10]. Borate chromatography was reviewed by Bergold and

Scouten [51]. Boronates react readily and under mild conditions with a wide range of polar functional groups that are oriented in the proper geometry. Although complexes of 1,2-diols, 1,3-diols, and the catechol type compounds are the most studied, a variety of other functional groups such as 1,2-hydroxy acids and 1,2-hydroxylamines will also react with boronates to form complexes. Many substances possessing these types of functional groups are biologically important. Examples of biological substances expected to form boronate complexes are carbohydrates, glycoproteins, catecholamines, nucleosides, α-hydroxy acids, dihydroxy compounds and aromatic α-hydroxy acids and amides. In a sense, boronates can be considered a ''general lectin''. Although the specificity for a given carbohydrate is less than for a true lectin, boronates have several advantages over lectins. First, the boronates are much more stable than the lectins. Second, as a consequence of the greater stability and the varying degree of interaction of boronates with different substances, the limits one must work in to obtain a separation are more flexible. Finally, the low cost of boronates makes boronate affinity chromatography an attractive tool for biochemical investigations.

Phenylboronic acid (PBA), like boric acid, is a Lewis acid and it ionizes not by direct deprotonation but by hydration and subsequent ionization to give the tetrahedral phenylboronate anion. PBA (pK_a 8.86) is three-times as strong an acid as boric acid and approximately ten-times as strong as *n*-butaneboronic acid. Liu and Scouten [10] used HRP for their studies on oriented and reversible immobilization of glycoproteins. At pH 7.0 they bound this enzyme to cellulose beads with attached catechol [2-(diethylamino)carbonyl - 4 - bromomethyl]phenylboronate. In comparison, commercial *m*-aminophenylboronic acid-agarose did not bind HRP below pH 8.0. HRP was immobilized in an oriented and reversible fashion using this gel. The immobilized enzyme retained 90% of its original activity, probably due to its attachment via the carbohydrate moiety of the enzyme. After repeated use, the activity remaining on the described gel was twice as high as that on conventional *m*-aminophenylboronic acid-agarose. The column was regenerated easily by washing with dilute acid because of reversibility of the boronate glycol bond.

6. Oriented immobilization by use of metal complexes

Reversible immobilization of histidine-tagged biologically active proteins on quartz in an oriented manner is based on the IMAC technique, which was described by Porath et al. [52] already in 1975. Schmid et al. [11] covalently bound the chelator nitrilotriacetic acid to the hydrophilic quartz surface and subsequently loaded it with the divalent metal cation (Ni^{2+}). The free coordination sites of the chelator–metal complex were subsequently filled by additional electron-donating groups such as histidine residues. Reversible binding of the green fluorescent protein from the jellyfish *Aequorea victoria*, modified with a hexahistidine extension, was monitored in situ using total internal reflection fluorescence. The immobilized protein is interesting in this context because it carries an intrinsic chromophoric group which emits efficiently in the green region only if the protein is structurally intact. This offers a way to probe the formation of functional protein layers on the quartz surface. The described reversible, directed immobilization of proteins on surfaces opens new ways for structural investigation of proteins and receptor–ligand interaction.

The IMAC technique was also used by Hale [53] for the preparation of immunoaffinity sorbent by irreversible, oriented immobilization of antibodies to cobalt–iminodiacetate resin. Antibodies that were reversibly bound to a cobalt–iminodiacetate resin were attached in an ''exchange inert'' fashion when the cobalt was oxidized from the 2+ to 3+ state. Antibodies bound in this fashion are not removed by metal chelating reagents, high salt, detergents, or chaotropic agents. Only reagents which reduce the metal are capable of removing the antibody. Since the metal binding site in antibodies is in the C-terminus of the heavy chain Fc, immobilized antibodies allow maximal antigen binding. The oxidation conditions used are mild and do not subject the antibody to harsh immobilization chemistry. This immobilization strategy may be useful not only for the described construction of affinity columns but it may also be utilized for other applications where oriented, immobilized antibodies are needed such as in surface plasmon resonance or the scintillation proximity assay format.

7. Biotin–avidin or streptavidin techniques

The binding of water-soluble vitamin biotin to the egg white protein, avidin, or to its bacterial counterpart, streptavidin, is accompanied by a vast decrease in free energy compared to that observed for other noncovalent interactions. The change of enthalphy, ΔH, for biotin bound to avidin and streptavidin was determined by Green [54] as −86 and −98.9 kJ/mol biotin. Each avidin or streptavidin can bind four molecules of biotin. The change in entropy for this reaction is essentially zero. The result for affinity constant for biotin–avidin of approximately 10^{15} M^{-1} is that this complex is suitable for study and application in various fields of biology, protein chemistry and biotechnology.

In the past decade the interaction between biotin and avidin or streptavidin has provided the basis for establishing a new avidin–biotin technology [12]. The basic concept is that biotin, coupled to either low- or high-molecular-mass molecules, is recognized by avidin. Methods for the biotinylation of membranes, nucleic acids, antibodies and other proteins has been developed in many laboratories. Fig. 10 shows biotin and various derivatives, suitable for various types of bonds. However, a spacer arm should be used when proteins are modified by biotin. Such a modification can be used for the preparation of complexes with immobilized avidin or streptavidin.

A literature survey of applications of avidin–biotin technology was published by Wilchek and Bayer [55] containing references of papers about the use of avidin columns in the isolation of biologically active materials, direct labeling, immunocytochemical and nonimmunocytochemical localization of biologically active materials, direct and indirect labeling of protein blots, immunoassays mediated by avidin–biotin technology and the use of biotinylated gene probes.

Information on biotin-binding proteins, the preparation of biotin, avidin and streptavidin derivatives, assays for avidin and biotin, and applications, classified in a similar way as a literature survey, is given in Ref. [12] (Avidin–Biotin Technology).

The use of an avidin–biotin complex in bioaffinity chromatography was the isolation of avidin from egg white on a biocytin-Sepharose column [56]. The

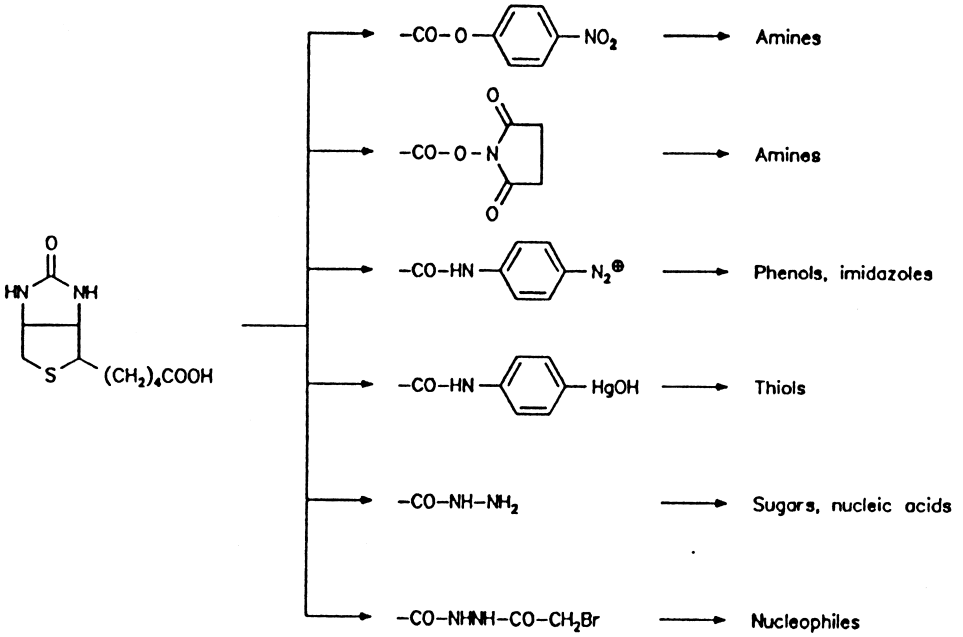

Fig. 10. Biotin and derivatives (from Wilchek and Bayer [12] with permission).

conditions required for its dissociation were extremely drastic requiring elution by 6 *M* guanidinium hydrochloride, pH 1.5. In order to decrease the strong affinity of streptavidin to biotin Bayer et al. [57] developed an improved method for the preparation of stable iminobiotin-containing Sepharose and used it for the single-step purification of streptavidin from culture broth of *Streptomyces avidinii*.

The isolation of peptide or protein receptors posed a number of difficult problems owing to their scarcity, their location in a complex, lipid-rich environment, and the difficulty with which even a starting plasma membrane is prepared. Finn et al. [58] have devoted their efforts to developing biospecific sorbents based on avidin–biotin technology where the hormone is attached in a targetted fashion. Fig. 11 shows the application of the avidin–biotin systems to the isolation of hormone receptors [59]. Soluble receptor is percolated through a bioaffinity column to form the complex shown in the center of Fig. 11. The column is then exhaustively washed to remove contaminating materials. Alternatively, the biotinylated hormone ligand can be added to a solution of solubilized receptor to form the soluble complex biotinylated hormone–soluble receptor. Percolating a solution containing this complex through a column of immobilized succinylated avidin, as in Fig. 11B, will result in the formation of the same complex obtained by method A. Both these schemes have been used for the isolation of insulin receptors from human placenta. Removal of functional insulin receptor can be achieved by eluting with acetate buffer, pH 5 containing either 1 *M* NaCl or biotin.

Some important characteristics and structural studies of avidin and streptavidin have been published by Bayer and Wilchek [60]. Both proteins are tetramers with molecular mass 67 000. The genes for both proteins have been cloned and expressed in *E. coli*. The primary sequences of both proteins are known. Avidin and the truncated form of streptavidin show an overall homology of about 40%. Chemical modification studies have shown that the single tyrosine (Tyr-33) and its homologue in streptavidin (Tyr-43) play a role in biotin binding. This was confirmed by X-ray crystallographic studies of streptavidin. The elucidation of the three-dimensional structure of streptavidin showed that the streptavidin subunit consists essentially of an extremely stable β-barrel consisting of a series of eight juxtaposed β-structures connected by turns. The biotin site is inside the barrel and, in binding biotin, some of the turns fold over to stabilize the complex.

Despite the fact that streptavidin is currently about 100-times more expensive than avidin, its use is sometimes justified since immobilized streptavidin exhibits less nonspecific binding. Avidin is highly positively charged at neutral pH, with an isoelectric point above 10. Consequently, it binds negatively charged molecules such as nucleic acids, acid proteins or phospholipids in a nonspecific manner. This

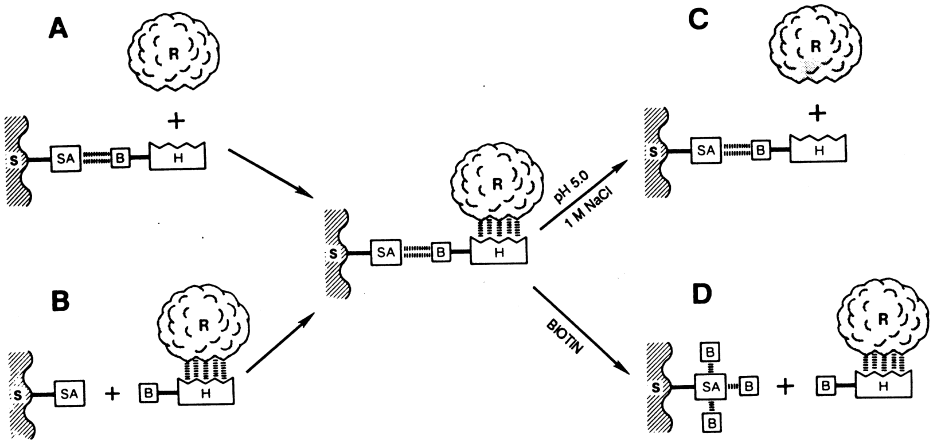

Fig. 11. Application of the avidin–biotin system to the isolation of hormone receptors. Dashed lines represent noncovalent bonds, SA succinylated avidin, H hormone, B biotin and R receptor (from Finn and Hofmann [59] with permission).

can result in nonspecific staining of, for example, the nucleus and cell membranes. Avidin is also a glycoprotein and therefore interacts with other biological molecules such as lectins or other sugar-binding materials via the carbohydrate moiety. The advantage of streptavidin lies in the fact that it is a neutral, nonglycosylated protein (p*I* lower than 7). To decrease its positive charge, the lysines of avidin can be easily derivatized by succinylation, acetylation, etc. A variety of avidin derivatives with average p*I*-values 7 or lower are now commercially available. The removal of the carbohydrate residue from avidin is much more difficult.

Haeuple et al. [61] have demonstrated that a far more effective approach in the purification of lactogenic and somatogenic receptors from rabbit liver or mammary membranes by use of biotinylated growth hormone is to replace the immobilized avidin with the immobilized streptavidin. They compared adsorptions of biotinylated and nonbiotinylated growth hormone–binder complexes to both bioaffinity columns. Nonspecific adsorption of nonbiotinylated hormone–binder complexes was found only on avidin-agarose. The purification of membrane proteins utilizing the biotin–streptavidin affinity has been reviewed by Kraehenbuhl and Bonnard [62].

The separation of complementary strands of plasmid DNA using the biotin–avidin system was described by Delius et al. [63]. Biotin-labeled strands were eluted from avidin-agarose with 50% guanidine isothiocyanate–formamide. Rapid and efficient recovery of the DNA–protein complexes was achieved by Herman et al. [64], when they used DNA labeled with the chemically cleavable biotinylated nucleotide Bio-19-SS-dUTP and streptavidin-Sepharose. The elution conditions in this isolation are SS-reduction of cleavable biotin analogue. The advantage of the use of streptavidin column was demonstrated by the fact that, in low ionic strength buffers, nonbiotinylated DNA bound to avidin but not to streptavidin. Therefore, the biotinylated DNA–protein complexes can be selectively bound to a streptavidin column under conditions that should stabilize labile DNA–protein complexes.

Biotin–avidin–biotin technology was used for the preparation of trypsin-modified fused-silica capillary for online digestion of picomole quantities of protein [65]. Biotin-labeled trypsin was adsorbed onto avidin molecules adsorbed to biotinylated inner surface of an aminoalkylsilane-treated fused-silica capillary. Subsequently the enzyme-modified capillary was used to digest β-casein simply by flowing a protein solution through it at a rate of 40 nl/min and collecting the effluent. This effluent contained the peptide fragments of the protein, which could be separated by capillary zone electrophoresis to yield the tryptic map for characterization and identification of the protein. Amankwa and Kuhr [66] demonstrated that is possible to interface two 50-μm-I.D. fused-silica capillaries through a solution gap to allow online transfer of the contents of one capillary into the second. Most of the typical problems with protein digestions, including sample loss and contamination are overcome by performing both steps online. The small size of the reactor makes it especially compatible for use in the analysis of small quantities (picomole) of protein as well as for analysis of small volumes of samples (typically, a few nanoliters). This technique is particularly advantageous in mapping proteins because of the high reproducibility requirement of the maps, and also because of the continuously decreasing size of protein samples that are presently of interest to the medical, bioanalytical and the biotechnological communities.

Fishel et al. [67] described the immobilization of DNA substrate by its 3′- or 5′-end to a chromatography matrix via streptavidin–biotin linkage. Biotin–streptavidin affinity selection as a valuable tool permitting the analysis of the RNA components of splicing complexes assembled on a wide variety of pre-mRNA substrates has been reviewed by Grabowski [68]. A review of the isolation of cell surface glycoproteins by the use of biotinylated lectin was published by Cook and Buckie [69]. They demonstrated the use of immobilized streptavidin to obviate dissociation of complexes formed between biotinylated Con A and membrane glycoproteins.

The advantage of the biotinylation of antibody carbohydrate moieties using biotin hydrazine for use in immunoaffinity chromatography has been described by Phillips [70]. HPIAC of specific phosphorylcholine receptors can be employed using biotinylated mouse monoclonal antiidiotypic antibody adsorbed to streptavidin-coated glass beads.

Using this technique HPIAC analysis was performed on isolated membranes from primed T cells obtained from mice immunized with phosphorylcholine. Rucklidge et al. [71] described a method combining the affinity purification and biotinylation of antibodies utilizing the high-capacity binding of blotting membranes (prepared from Immobilon P, Millipore or nitrocellulose, Hybond C, Amersham) and glutaraldehyde treatment to immobilize antigen. Following reaction of antisera to denatured collagen with membrane-bound antigen (collagen type II), the biotinylation of antibodies was performed in situ, before release of the IgG–biotin complex with low pH buffer. Biotinylation in situ may protect the variable region of the IgG compared to antibodies biotinylated in solution, thus increasing their antigen recognition.

Nucleic acid hybridization is an essential component in many of the standard molecular biology techniques. O'Meara et al. [72] employed real-time biospecific interaction analysis to monitor direct capture of a hepatitis C virus (HCV) derived polymerase chain reaction (PCR) product by nucleic acid hybridization. Different formats for hybridization were used to study the interaction between a single-stranded HCV PCR product and capture oligonucleotides immobilized on a sensor chip via streptavidin–biotin chemistry. The biotinylated PCR products were immobilized onto streptavidin-coated paramagnetic beads Dynabeads M-280.

Higher eukaryotic organisms depend upon a dietary supply of vitamin A for a range of essential biological processes including vision, reproduction, growth, and development. Dietary vitamin A is first transported into the liver, where it forms a complex with a retinol-binding protein (RBP). The hypothesis that the cellular uptake of retinol involves the specific interaction of a plasma membrane receptor with serum RBP at the extracellular surface followed by ligand transfer to cytoplasmic cellular retinol-binding protein (CRBP) was investigated by Sundaram et al. [73]. The experimental system consisted of the [^{3}H]retinol–RBP complex, *E. coli*-expressed recombinant apo-CRBP containing the ten amino acid long streptavidin-binding peptide sequence at its C terminus (designated as CRBP-Strep) and permeabilized human placental membranes. [^{3}H]Retinol transfer from RBP to CRBP-Strep was monitored by measuring the radioactivity associated with CRBP-Strep retained by Streptavidin-agarose. Using this assay system, it was demonstrated that optimal retinol uptake is achieved with holo-RBP, the membrane receptor and apo-CRBP. The effects are specific: other binding proteins, including β-lactoglobulin and serum albumin, despite their ability to bind retinol, failed to substitute for either RBP or apo-CRBP. The process is facilitated by membranes containing the native receptor suggesting that this protein is an important component in the transfer mechanism. Taken together, the data suggest that RBP receptor, through specific interaction with the binding proteins, participates in the mechanism which mediates the transfer of retinol from extracellular RBP to intracellular CRBP.

8. Site-specific attachment of proteins to surfaces by use of site-directed mutagenesis

The key to controlling the orientation of immobilized protein molecules on a support is to selectively attach the protein from a predetermined site on the protein surface. Genetic engineering, a way to produce large amounts of desired proteins in simple organisms such as *E. coli* or yeast, makes it possible to modify the protein of interest on the DNA level in order to introduce desired properties to the protein by the introduction a unique amino acid residue with a specific side-chain functional group to the protein molecule by side-directed mutagenesis. The protein molecule can then be attached through this functional group, leading to a controlled orientation on the surface. Persson et al. [74] used genetic engineering for the preparation of the glucose dehydrogenase mutant containing a cysteine residue at position 44. Thiopropyl-Sepharose was the solid support for the oriented site-specific immobilization. In order to facilitate immobilization of the L-lactate dehydrogenase from *Bacillus stearothermophilus*, a single cysteine residue was introduced by site-directed mutagenesis. The result was a freely accessible thiol group which was located on the protein surface without interfering with enzyme catalysis [75]. The active lactate dehydrogenase mutant was coupled covalently to thiopropyl- or organomercurial-functionalized agarose beads with at least 56% recovery

of enzymatic activity. The immobilized catalyst showed saturation kinetics similar to the free enzyme, but had an increased thermal stability. However according to Huang et al. [13], glucose dehydrogenase and lactate dehydrogenase are multisubunit enzymes, and the support used in these studies are commercial porous agarose beads of different sizes. Under these conditions, it is difficult to study the effect of orientation of the immobilized protein molecules on their activity because the steric accessibility of active sites is not the same. Immobilization of dihydrofolate reductase by engineered cysteine residue attached to its C-terminal end was described by Iwakura and Kokubu [76]. McLean et al. [77] described engineering protein orientation at surfaces to control macromolecular recognition events. They genetically introduced single cysteine residues at different sites of the heme protein cytochrom b_5 and studied protein–protein interaction by use of site-specific immobilized protein.

Huang et al. [13] used the protease subtilisin BPN′ as a model protein to study how the orientation of immobilized enzyme molecules on surfaces affects their catalytic properties. To achieve this goal, a single cysteine residue was introduced into the cysteine-free enzyme by site-directed mutagenesis. This cysteine residue was designed to be away from the active site of the enzyme (Fig. 12). The enzyme molecules were immobilized through the side-chain on porous and nonporous solid supports. The conclusion of their results is that the porous agarose beads are not ideal for studying the effect of enzyme orientation on immobilization, and for comparing the site-specific and random immobilization. The reason is that the apparent activity of the immobilized enzyme does not necessarily reflect the activity of each enzyme molecule due to the internal diffusion limitation. Therefore, uniformly sized, nonporous silica particles were used as the support to study further the immobilization of the mutant subtilisin. The site-specific immobilization method led to ordered two-dimensional arrays of enzyme molecules on the support surface with active sites of the enzyme oriented toward the solution phase. Oriented immobilized subtilisin demonstrated a higher catalytic efficiency compared to subtilisin that was immobilized by a conventional method that lead to random immobilization.

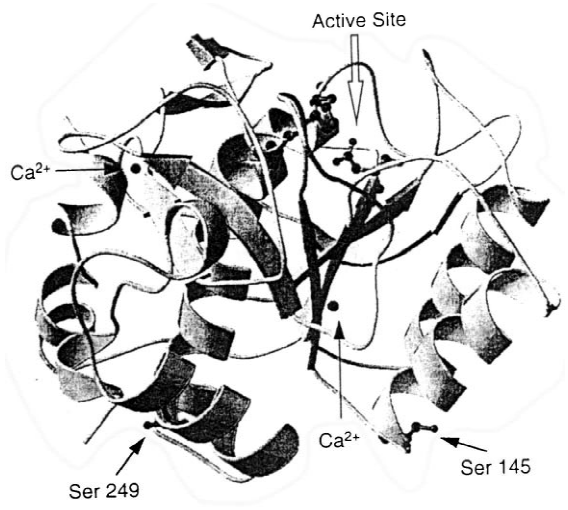

Fig. 12. Structure of subtilisin BPN′. The active site and Ca^{2+} cations are shown. Serine 249 and serine 145 are highlighted with ball and stick. Serines 249 and 145 were changed to cysteine residues in the mutants of subtilisin, subt-1 and subt-2, respectively. The coordinate data for this structure were obtained from the Brookhaven protein data bank (from Huang et al. [13] with permission).

The stability of the site-specifically immobilized subtilisin was evaluated over a 25-day period both when stored at room temperature and at 4°C. It was found that the stability of the immobilized subtilisin was superior when it was stored at 4°C, with the enzyme maintaining ~61% of its original activity after 25 days. Subtilisin stored in solution at 4°C showed similar behavior, with enzyme the retaining 62% of its activity after 25 days. The difference in long-term stability was significant when the enzyme was stored at room temperature. There, the oriented immobilized enzyme maintained 28% of its activity after 25 days, whereas the homogeneous enzyme in solution was only 12% active.

9. Conclusions

By common methods of immobilization, the activity of immobilized biologically active proteins is sometimes low, because the active site is hindered or even broken by the linkage to the solid support. It is also necessary to take into account, that hydrophobic surface clusters of proteins play an important role in

vivo, but in vitro their contacts with water result in ice-like water structure which starts the protein inactivation with unfolding of the protein molecule. The formation of suitable biospecific complexes or the protection of carbohydrate moieties cleavage can reduce the nonpolar surface area of proteins which results in increasing of their stability. Therefore the use of suitable biospecific complex formation for oriented immobilization of biologically active proteins or their coupling through their carbohydrate moieties can contribute to their stabilization.

The second advantage of oriented immobilization of biologically active molecules, first of all antibodies and enzymes, is the possibility of achieving good steric accessibility of active binding sites. Biospecific sorption of proteins to suitable immunosorbent gives also the possibility of combining their isolation with their oriented immobilization.

Many examples of oriented immobilization of antibodies by the use of biospecific complex formation with Protein A or G, oriented immobilization of proteins by the use of their suitable immunosorbents or immobilization of glycoproteins by the use of their carbohydrate moieties are described. Another type of oriented immobilization can use boronate affinity gel or IMAC techniques. The mostly used oriented immobilization of biologically active proteins is the biotin–avidin or streptavidin technique. The last example of oriented immobilization discussed in this review is the attachment of a unique amino acid residue with a specific side-chain functional group introduced to the immobilized protein by side-directed mutagenesis.

The described techniques may find application mainly in the following areas: (1) biochemistry and biotechnology; (2) analytical studies; (3) medical studies and (4) organic chemistry. The main point is, however, that application of the techniques described will enable more appropriate modelling of processes that make the cells or tissues function in the living body. Several examples were shown. Oriented immobilized proteinase trypsin was used for microscale analysis of picomole quantities of protein by peptide mapping [65,66]. This method can be employed for detection of pathological changes of proteins occurring in physiological fluids in submicrogram amounts, detection of posttranslational protein modification, identification and localization of genetic variants or quality control and monitoring of genetically engineered protein products. The technique using Protein G immunoaffinity columns with noncovalently immobilized antibody specific to the analytes of interest [21] was shown to be useful for the selective enrichment and characterization of drug metabolites with similar substructures as the parent drug. Interaction of Protein G and IgG was used in fiber-optic fluorometric sensing of PCR-amplified DNA using an immobilized DNA capture protein [22]. This assay was carried out for purposes of pathogen screening, epidemiological studies, genetic evaluation, and forensic investigation. However, oriented immobilization of proteins is suitable not only for their practical applications but also for the investigation of molecular interaction by surface-sensitive techniques. The formation of several biospecific complexes can be used also as a models for studies of chemical processes in the living cells [14]. Oriented immobilized biologically active proteins may, we hope, in the future serve mankind in many areas: for improving health and environment and last but not least for further progress in the basic biosciences.

Acknowledgements

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