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Review

Application of avidin–biotin technology to affinity-based separations

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CONTENTS

1. INTRODUCTION AND PERSPECTIVES

In the past decade, the unprecedented interaction between the egg-white glycoprotein avidin and the vitamin biotin has provided the basis for establishing a new technology which has broad application in virtually all fields of biology and biotechnology[1–5]. The basis of this new technology resides in the exceptionally high affinity constant between avidin (or its bacterial counterpart streptavidin) and biotin, which is several orders of magnitude greater than other types of affinity interactions (Table 1). Originally designed to facilitate and improve purification (affinity chromatography) and localization (affinity cytochemistry) procedures for biologically active macromolecules, the application of avidin–biotin technology has also led to major advances in medical diagnostics (immunoassay, histopathology and gene probes). In addition, a variety of new applications have accumulated, including affinity targeting, cross-linking and immobilization studies, cell cytometry, blotting technology, drug delivery, bioaffinity sensors, fusogenic studies and hybridoma technology.

The general idea of the approach is that biotin, coupled to low- or high-molecular-weight molecules, can still be recognized by avidin, either as the native protein or in derivatized form containing any one of a number of reporter groups (*e.g.*, fluorescent groups, electron-dense markers, enzymes, immobilizing matrices). In this manner, mediation through the avidin–biotin complex often leads to a dramatic enhancement of signal and/or sensitivity levels.

The use of the avidin–biotin system as a "universal" tool in the biological

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TABLE 1

SOME AFFINITY PAIRS AND THEIR DISSOCIATION CONSTANTS

Binding protein	*Target molecule*	K_D *(M)*
Avidin	Biotin	*ca.* 10^{-15}
Streptavidin	Biotin	*ca.* 10^{-15}
Receptors	Hormones, toxins, etc.	10^{-9}–10^{-12}
Antibodies	Antigens	10^{-7}–10^{-11}
Transport proteins	Vitamins, sugars, etc.	10^{-6}–10^{-8}
Lectins	Carbohydrates	10^{-3}–10^{-6}
Enzymes	Substrates	10^{-3}–10^{-5}

sciences is the product of concepts which were defined in the mid-1970s. In rapid succession, a number of laboratories developed methodologies for the biotinylation of membranes[6], nucleic acids[7,8], antibodies and other proteins[9]. The biotinylated binders were then analysed in some way with an appropriate avidin-conjugated probe. Today, the same basic approach is commonly employed for a wealth of different applications (Fig. 1). Conceptually, little has changed from the original applications; currently reported contributions usually describe changes in the choice of target material, binders and probes, and new strategies for introducing biotin and avidin into the desired experimental system. There is no doubt, however, that the application of avidin–biotin technology is still on the increase[10].

2. EARLY ISOLATION STUDIES

In historical terms, the first example of the use of avidin–biotin technology was the isolation of avidin on a biocytin–Sepharose column[11]. Already in this instance was it clear that there would be problems in applying avidin–biotin technology for isolation purposes; although avidin bound readily enough to the biotin-containing column, the conditions required for its removal were extremely drastic (6 *M* guanidinium

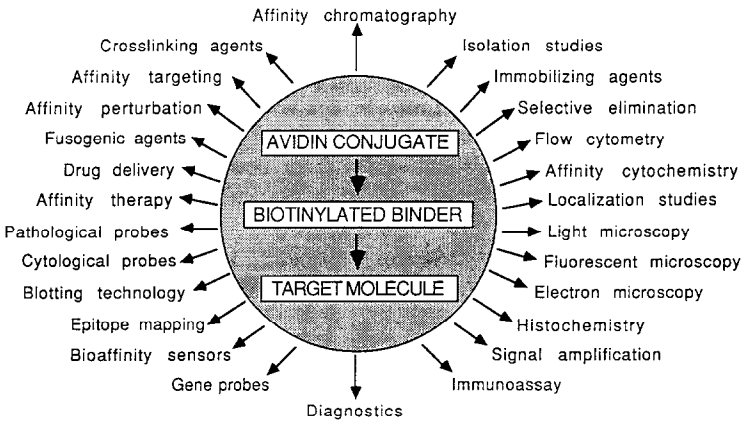

Fig. 1. General approach and major applications of avidin–biotin technology.

hydrochloride, pH 1.5). Clearly, if one wants to use an avidin column for the purification of target material via biotin-containing binder, in most instances it will be impossible both to regenerate the original column (avidin–Sepharose) and to recover active biotinyl binder. It was also unclear what if any short- or long-term advantages would be gained by preparing such binder–biotin:avidin–resin complexes. Thus, in the area of isolation studies, the unprecedented affinity constant which characterizes the avidin–biotin complex has remained its most important virtue as well as its most confounded vice. Nevertheless, such a system was used to isolate biotin-containing peptides from proteins which were modified with different biotinyl derivatives for sequencing purposes[12]. In addition, a similar approach was used to assist in the purification of peptides synthesized by the solid-phase method[12,13]. The approach which we used in these studies is shown schematically in Fig. 2.

Despite the difficulties encountered in applying avidin–biotin technology for separation purposes, many successful isolation procedures have been reported and the list continues to grow (Table 2). One approach has been to use avidin columns for the isolation or purification of target material by releasing the latter from the column. In many instances, researchers have depended on the stability of the complex which fastens the binder to the column, and even relatively harsh procedures (*e.g.*, high concentrations of detergents) can be employed to release the target molecule. In other strategies, avidin-containing columns have been used selectively to purge biotinylated molecules or cells from a complex mixture in solution or suspension. The first example of this approach was the removal of biotinylated lectins and antibodies from solution for analytical purposes[9]. An effective way of releasing the biotinylated molecule from an avidin column has also been developed[14]; columns consisting of immobilized avidin monomers take advantage of the lower affinity constant[15] which permits the subsequent release of the biotin-containing material under mild conditions. Another field in which avidin–biotin mediation is rapidly undergoing extensive development is the isolation or subtractive elimination of genes.

3. LARGE-SCALE ISOLATION OF ANTIGENS

In recent work, we have performed a pilot study in which avidin-derivatized matrices were proposed as universal columns for isolation and immobilization purposes[16]. In one of the examples, we chose to demonstrate the use of an avidin–Sepharose column for the large-scale isolation of an antigen using the appropriate biotinylated antibody. This relatively simple strategy is shown schematically in Fig. 3. In this study, we used transferrin as a model antigen and commercially obtained antibodies (BioMakor, Jerusalem, Israel) as the biotinylated binder. Using this approach, we were able to achieve remarkably high yields and purity using a single-step isolation procedure. A yield of about 0.75 mg of transferrin was obtained for every 1 ml of avidin–Sepharose column (which contained 2 mg/ml of biotinylated antibody and 1.5 mg/ml of avidin). Interestingly, the procedure proved more efficient than "conventional" affinity chromatography in which the antibody was directly attached to the resin. This was reflected in both the amount of antigen bound to the column and the percentage of antigen released. Essentially all of the target material could be released from the column using 0.1 *M* acetic acid.

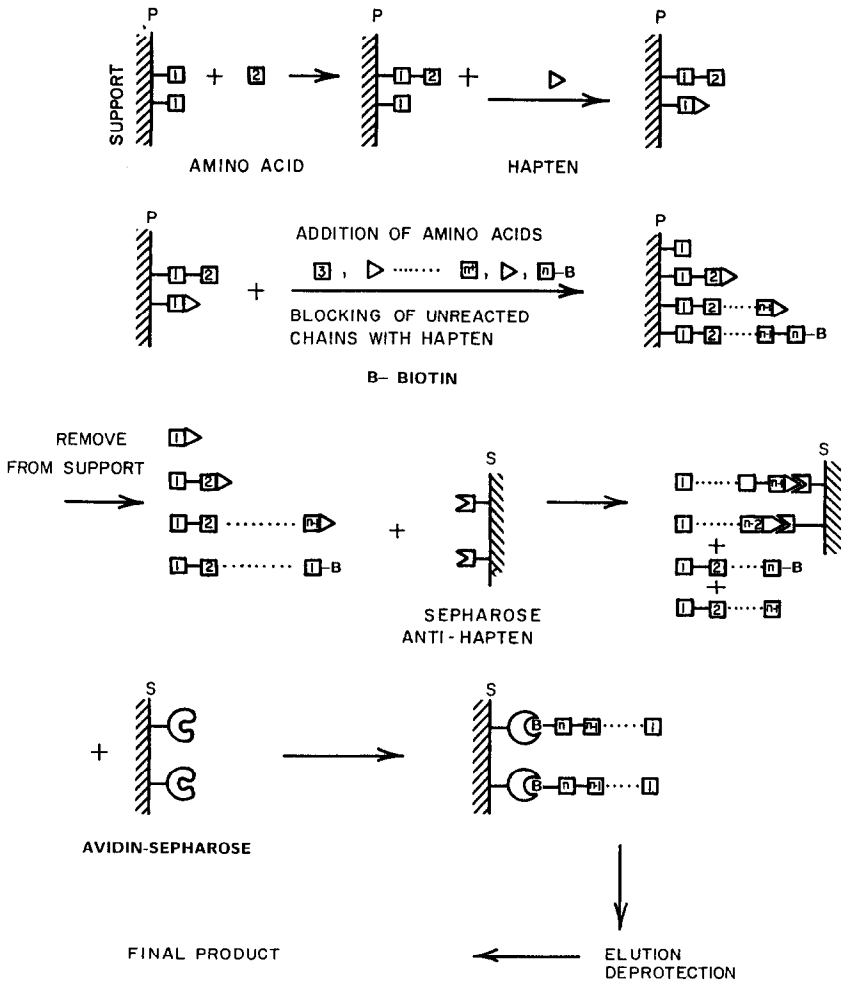

Fig. 2. Use of antibody and avidin columns for the isolation of solid-phase synthesized peptides. After each step in which tBoc-amino acid is added, the residual unreacted amino groups are blocked with a reactive hapten-containing reagent (*e.g.*, dinitrofluorobenzene). In the last step of the synthesis, either biotinyl methionine is added to the N-terminal residue or a terminal methionine is biotinylated using biotinyl N-hydroxysuccinimide ester. After removal of the synthetic peptide from the solid phase, the mixture is passed through an anti-hapten antibody column to remove all of the truncated peptides containing the hapten. The effluent which contains the complete peptide (containing the biotinyl methionine residue) is then passed through an avidin column. After removal from the column, the biotinylmethionine is split off the column with cyanogen bromide to yield the required peptide. This procedure, of course, is applicable to peptides that do not contain methionine. In such cases, an alternative procedure can be used by adding biotinyl homoserine or N^{α}-biotinyldiaminobutyric acid and the resultant derivative can be cleaved with acid. In yet another alternative, N^{ε}-tBoc-biocytin can be included in the last step and removed by Edman degradation. In many instances it may even be advantageous to leave the biotin on the synthetic peptide for identification purposes.

TABLE 2

EXAMPLES OF BIOLOGICALLY ACTIVE MATERIAL PURIFIED OR SEQUESTERED USING AVIDIN COLUMNS

Biotin-containing systems	*Systems mediated via biotinylated binder*
Native biotin-containing enzymes, subunits and peptides	Surface glycoproteins
Sodium transport enzyme (biotin-containing)	Soluble antigens
Biotinylated lectins	Membrane antigens
Biotinylated antibodies	Antibodies
Biotinylated enzymes	Enzymes
Biotinylated mitogens	IgE receptor
Biotinylated DNA	Hormone receptors
Biotinylated RNA	Opioid receptor
Biotinylated tRNA	Cell subpopulations
Biotinylated rRNA fragments	Antibody-producing cells
Biotinylated nucleosomes	DNA
Biotinylated membranes	Plasmid DNA
Iminobiotinylated membrane proteins and glycoproteins	Recombinant plasmids
Biotinylated synthetic peptides	Transcription factors
	Spliceosomes
	DNA-binding proteins

4. AVIDIN *VERSUS* STREPTAVIDIN

In many systems, the use of the egg-white glycoprotein avidin has led to high levels of non-specific binding. Consequently, we have witnessed a trend to replace avidin with another biotin-binding protein, streptavidin, the bacterial analogue from *Streptomyces avidinii*[17]. Despite the fact that streptavidin is currently about 100 times more expensive than avidin, the replacement is sometimes justified as non-specific binding can be averted. Some of the major characteristics of the two proteins are shown in Table 3.

The major differences between avidin and streptavidin lie in the fact that streptavidin is a neutral non-glycosylated protein whereas avidin is highly alkaline and usually consists of a single oligosaccharide chain per subunit. In many systems,

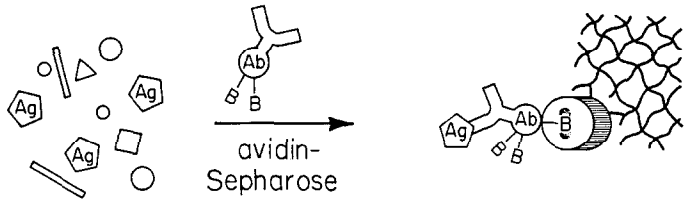

Fig. 3. Schematic diagram illustrating the isolation of an antigen on an avidin-containing resin via a biotinylated antibody. In this particular instance, avidin–Sepharose is loaded with biotinylated anti-transferrin antibody (B-Ab) and serum which contains the antigen (Ag) is applied to the column. The transferrin binds to its immobilized antibody and can be purified using acetic acid. Under these contitions, only the antigen is released from the column; the antibody remains owing to the stability of the avidin–biotin complex.

TABLE 3

SOME IMPORTANT CHARACTERISTICS OF AVIDIN AND STREPTAVIDIN

Values given for oligosaccharide, sugar and amino acid residues refer to the number of the respective groups per subunit.

Property	*Avidin*[a]	*Streptavidin*[b]	*Property*	*Avidin*[a]	*Streptavidin*[b]
Molecular weight:					
Tetramer	67 000	67 000	A_{282} (1 mg/ml)	1.54	2.7
	(60 000)	(60 000)		(1.70)	(3.4)
Subunit	16 500	16 500	Trp	4	4
	(15 000)	(15 000)	Tyr	1	6
Biotin bound[c]	1	1	p*I*	>10	<7
K_D (*M*)	*ca.* 10^{-15}	*ca.* 10^{-15}	Lys	9	8 (4)
			Arg	8	4
Oligosaccharide	1	0			
Man	4–5	0			
GlcNAc	3	0			

[a] Native avidin (non-glycosylated avidin in parentheses).
[b] Native streptavidin (truncated core streptavidin in parentheses).
[c] Per subunit.

egg-white undergoes extensive interaction with negatively charged macromolecules, *e.g.*, nucleic acids and acidic proteins. In other systems, lectins or other sugar-binding materials might interact "non-specifically" with avidin.

In view of the high cost of the bacterial protein, attempts have been made to rectify the two major undesirable characteristics of avidin. In terms of correcting the positive charge, the lysines of avidin can be easily derivatized by acetylation, succinylation, etc. Thus, a variety of avidin derivatives are commercially available with average p*I* values of 7 or lower. On the other hand, it is much more difficult to remove the carbohydrate residue from avidin. The native avidin tetramer is not generally susceptible to the action of commercially available glycosidases. However, we have recently demonstrated the isolation of a non-glycosylated form of the avidin tetramer from a commercial preparation of avidin (from Belovo, Bastogne, Belgium) which contained a heterogeneous combination of glycosylated and non-glycosylated subunit types[18]. Unfortunately, this procedure was applicable to only one batch of avidin dating from 1983 and more recent batches of avidin are heavily glycosylated. We are currently designing a procedure which may lead to the preparation of gram amounts of non-glycosylated avidin, and we are hoping that this product will eventually become commercially available.

5. STRUCTURAL STUDIES ON AVIDIN AND STREPTAVIDIN

The last few years have seen dramatic progress in the understanding of the molecular basis for the avidin–biotin interaction[10]. The primary sequences of both avidin and streptavidin are now known[19,20]. The genes for both proteins have been cloned and expressed in *E. coli*[20,21].

There is a remarkable conservation in the primary structure of the two proteins.

Avidin and the truncated form of streptavidin show an overall homology of about 40%. The conserved residues are usually confined to short homologous stretches which form relatively defined domains (Fig. 4). These homologous domains are more or less coincident with amino acid residues thought to be important to the construction of the binding site. Chemical modification studies have shown that the single tyrosine in avidin (Tyr-33) and its homologue in streptavidin (Tyr-43) play a role in biotin binding[22]. The majority of the tryptophans of both proteins, specifically those homologous to the two proteins, also appear to be involved in the binding site[23,24]. In addition, the homologies seem to be extended to the respective predicted secondary structures, which indicate a high preponderance of β-strands in both proteins connected by successive turn structures[20].

Although crystallizations of avidin have been reported since the early 1970s[25], the three-dimensional structure of streptavidin was the first to be elucidated, and only recently by two independent groups[26,27]. These studies confirmed the predicted model[20] in 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.

X-ray crystallographic studies confirmed that one of the important residues for biotin binding is indeed the above-described tyrosine (Tyr-43), which is hydrogen-bonded with the carbonyl oxygen of the ureido ring. On the other hand, one of the fascinating discoveries from the X-ray studies on streptavidin is that none of the binding-site tryptophans seems to form direct bonds with biotin. Instead, four tryptophans appear to stabilize via hydrogen bonding other binding site residues which in turn interact directly with the biotin. Another surprising feature is that one of the four tryptophans (Trp-120) is donated by a neighbouring subunit. This also appears to add another factor of stability to a molecule which is already extremely resistant to dissociation and denaturation[28].

Just how close is the conservation of the three-dimensional structures and formation of the respective binding sites of avidin and streptavidin? Does the theme of secondary tryptophan interaction hold true for egg-white avidin as well as for the bacterial protein? Crystals are now available which are suitable for X-ray studies[29], and it is hoped that within a short time the three-dimensional structure of avidin will also be solved, which would allow comparative analyses of the binding site residues. The data obtained so far indicate subtle differences in the fine structures of the two proteins.

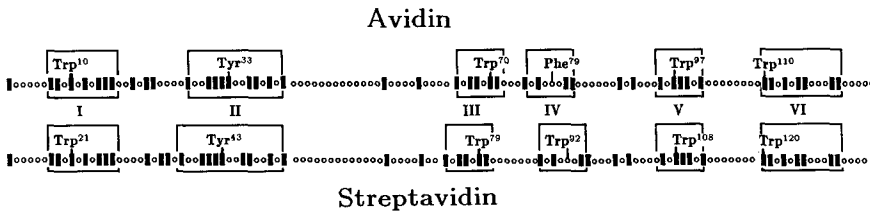

Fig. 4. Schematic description summarizing the sequence homology in egg-white avidin and a truncated form of bacterial streptavidin. Homologous amino acid resides in the two proteins are designated by ■ and unconserved residues by ○. Conserved regions are boxed in; each of the six regions contains a single aromatic amino acid residue of interest, and their respective positions are shown.

6. OTHER NATIVE BIOTIN-BINDING PROTEINS

There are other proteins in nature which bind biotin at different affinity levels (albeit much lower than those of avidin and streptavidin). These include the egg-yolk biotin-binding protein[30], the enzymes biotinidase[31] and biotin holocarboxylase synthetase[32], anti-biotin antibodies[33,34] and the biotin receptor in yeast[35] and other cells. Eventually, the primary and perhaps three-dimensional structures for many of the biotin-binding proteins will be known. In this context, the first 30 residues of the egg-yolk protein have been determined and the N-terminus bears striking resemblance to the N-terminal sequences of avidin and streptavidin[36]. The era of genetic engineering will undoubtedly contribute exciting information to this field. Indeed, a recent study of an EGF homologue from sea urchin has already revealed an unexpected and astonishing similarity in the sequence of the C-terminal domain with those of avidin and streptavidin[37]. On this basis, the authors suggested a biotin-binding function, despite the fact that the gene product has yet to be isolated.

It is hoped that such studies will eventually lead to a general definition of the contribution of given residues to the binding of biotin. This information should open the door to a flurry of site-directed mutagenesis studies, which should add a new dimension to this area. We hope that by deciphering the nature of the high–affinity avidin-biotin complex, we may be able both to improve its application further and to understand better other lower order affinity interactions.

7. ABSTRACT

During the last decade, avidin–biotin technology has become a commercially viable tool for research, medical and industrial applications. From the beginning, mediation via the avidin–biotin complex was proposed for affinity-based separations. This particular application, however, has been slow in gaining acceptance. One of the reasons is that the strength of binding between avidin and biotin is sometimes inappropriate for the desired affinity system. Another problem involves certain "undesirable" structural properties in the avidin molecule which may lead to high levels of "non-specific" binding. Recent progress in understanding the molecular requirements for binding biotin may eventually lead to the design of avidin-like proteins which will exhibit preferred recognition properties according to the desired application.

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