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A Spectrophotometric Assay for Biotin-Binding Sites of Immobilized Avidin

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ABSTRACT

A rapid and sensitive spectrophotometric assay was developed for the measurement of biotin-binding sites of immobilized avidin. The method is based on the reaction of avidin with excess biotin followed by assay of the unbound biotin using the HABA (2-[4'-hydroxyazobenzene]benzoic acid) method. Three solids possessing variable amounts of monomeric avidin were examined; viz., succinamidopropyl-controlled-pore glass (CPG-500), crosslinked 6% beaded agarose (Sepharose-CL-6B**), and crosslinked bis-acrylamide/azlactone (3M Emphaze Biosupport Medium AB_1). Results indicate that the total biotin-binding sites of monomeric avidin immobilized on CPG-500, Sepharose-CL-6B, and 3M Emphaze are 0.229, 0.093, and 0.218 μmol biotin per mL beads, respectively. Assays for exchangeable biotin-binding sites gave values greater than 90% of the total sites. The spectrophotometric HABA method described is an alternative to assays based on tracers, thus the handling of radioactive material is avoided.

Index Entries: Spectrophotometric assays; biotin-binding sites; avidin, immobilized.

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INTRODUCTION

Immobilized monomeric avidin is an ideal affinity support for reversibly binding biotinylated molecules (*1–5*). This property of immobilized monomeric avidin provides a very effective isolation/purification method for biotinylated proteins, peptides, and other molecules. Prior to any isolation/purification step, it is important to know the biotin-binding capacity of the affinity column in order to optimize the procedure.

A variety of methods have been developed for measurement of biotin-binding capacity. However, the available methods are often inconvenient or have other limitations. For example, the method based on the use of radioactive biotin (*6*) is very sensitive but very tedious and requires sophisticated instrumentation. The colorimetric assay based on 2-(4′-hydroxyazobenzene) benzoic acid (HABA) involves microtitration of the pink-colored HABA-avidin-(solid support) complex with biotin and observing the colored particles under a magnifying glass (*7,8*). This method is very time consuming, and accuracy of results is questionable. A direct spectrophotometric determination of the immobilized HABA-avidin complex is not possible and HABA cannot be released from the immobilized avidin without adding biotin, which would prevent assay of released HABA in solution.

However, the spectrophotometric HABA assay described here is precise, convenient, and can be used over a wide range of pH and salt concentration. HABA is a dye that binds to avidin and can serve as an indicator of unoccupied binding sites (*8,9*). The HABA-avidin complex exhibits an absorption at 500 nm with a molar absorptivity of $34{,}000 M^{-1}$ cm^{-1} (*8*), whereas HABA does not absorb at this wavelength. Biotin, owing to its higher affinity, stoichiometrically displaces HABA from the complex; thus, biotin can be assayed by the decrease in absorbance at 500 nm. Because of its potential rapidity and sensitivity, we have investigated HABA as a means for assaying the biotin-binding capacity of immobilized avidin. The method is based on the reaction of avidin with excess biotin, followed by assay of the unbound biotin using the HABA method. The reactions involved are represented in Fig. 1.

MATERIALS AND METHODS

The samples used in this study for the measurement of biotin-binding capacity of immobilized avidin, as indicated in Fig. 1, were avidin-succinamidopropyl-glass (CPG-500), avidin-bisacrylamide/azlactone (3M Emphaze Biosupport Medium AB_1), and avidin-agarose (Sepharose-CL-6B). The latter two samples were obtained from Pierce (Rockford, IL). Immuno-Pure Avidin, ImmunoPure HABA, and ImmunoPure D-Biotin were also from Pierce.

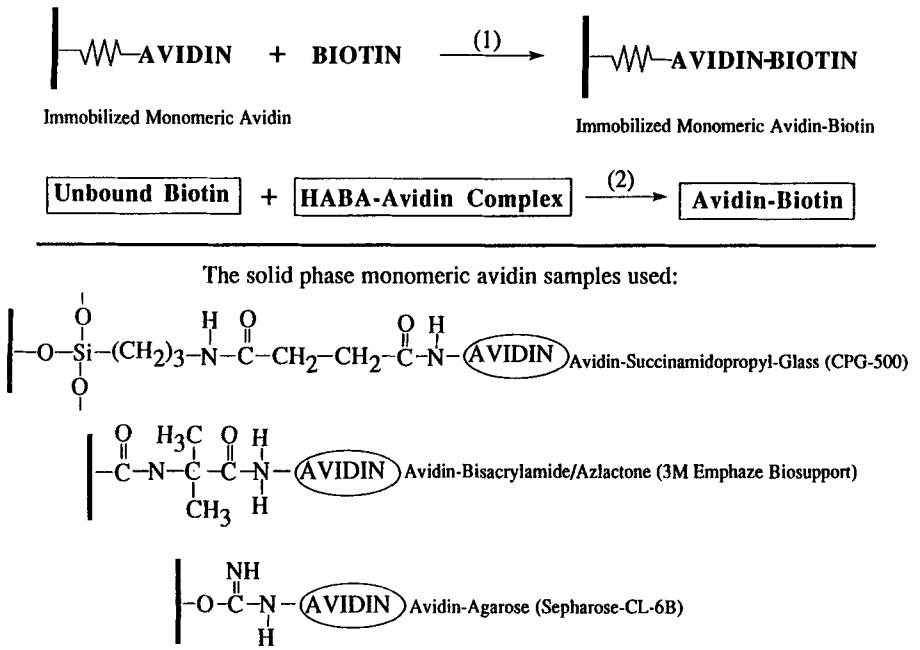

Fig. 1. Reaction of solid phase monomeric avidin with excess biotin (*1*), and subsequent reaction of unreacted biotin with the complex between 2-(4'-hydroxyazobenzene) benzoic acid and avidin (HABA-Avidin) to form avidin-biotin (*2*).

Preparation of Avidin-Succinamidopropyl-Glass

The glass beads (CPG-500) were derivatized by aqueous silanization (*10*) followed by nonaqueous succinylation (*11*). The cleaned porous beads were reacted with 10% aqueous solution of 3-aminopropyltriethoxysilane (Sigma, St. Louis, MO) at pH 4, for 3 h at 70°C. Nonaqueous succinylation was performed by placing the aminobeads in four volumes of a 1% (v/v) triethylamine solution in acetone made 10% (w/v) in succinic anhydride at room temperature for 5–20 min.

The basic procedure for immobilization of avidin (*12*) consists of:

1. Incubation of succinamidopropyl-glass beads with freshly dissolved 0.1*M* 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (Sigma) at 25°C in 0.2*M* NaH_2PO_4 adjusted to pH 4.75;
2. Rapid washing of the activated beads with pH 7.0, 0.047*M* phosphate buffer at 0°C; and
3. Recirculation of an avidin solution (~6 mg/mL) at pH 7.0 through the column of the washed activated beads for 16 h at 4°C.

Preparation of Immobilized Monomeric Avidin

Avidin immobilized on the three solid supports described were all in tetrameric form. Each matrix was packed in a 5-mL column. The solutions

of denaturants were freshly prepared and filtered before use (cellulose acetate filters, 0.45-μm pores). Three bed volumes of 6*M* guanidinium chloride (ICN Biochemicals, Ultra Pure) in 0.2*M* KCl (pH 1.5) were passed through the column, followed by washing with 0.047*M* phosphate buffer (pH 7) until the pH of the effluent was 6–7. Then two bed volumes of 3*M* guanidine thiocyanate (Boehringer Mannheim, Ultrapure Grade) in 0.2*M* KCl, pH 1.5, were passed through the column and the column was equilibrated with 0.047*M* sodium phosphate buffer (pH 7.0). A flowrate of 2–3 bed volumes per hour was used with the denaturants and the affinity matrix was never allowed to run dry. After the final equilibration with phosphate buffer, the monovalent avidin affinity matrix was stored at 4°C in pH 7.0 phosphate buffer with 0.02% sodium azide (*4*).

Quantification of Immobilized Avidin by the Modified Bradford Method

For the determination of avidin concentration on the three solid supports described, a modified Bradford dye-binding assay (*13*) was used. An aliquot of avidin bound to the solid phase was incubated with an excess of the dye reagent (Coomassie brilliant blue [Bio-Rad, Hercules, CA] stock solution diluted 1:5 in distilled water). The assay measures the decrease in absorbance of the solution at 465 nm owing to adsorption of the dye by the bound protein. The amount of protein immobilized was determined by using a standard curve prepared from data obtained with soluble avidin.

Reaction of the Solid Phase Monomeric Avidin with Biotin and Spectrophotometric Determination of the Excess of Biotin

Reagents

The reagents (R_1, R_2, and R_3) were prepared as described in the Pierce ImmunoPure HABA Reagent Manual (*14*). Descriptions of these reagents are as follows:

- R_1: 0.5 mg/mL avidin in 47 m*M* sodium phosphate, 0.9% NaCl, pH 6.0.
- R_2: 10 m*M* HABA in 10 m*M* NaOH.
- R_3: 0.5 m*M* Biotin in 47 m*M* sodium phosphate, 0.9% NaCl, pH 6.0 (Standard control).
- Sample (S): Supernatant from beads.

Procedure

Packed immobilized avidin (100 μL) was incubated in a 1.5 mL polypropylene flat top microcentrifuge tube with 500 μL of R_3 for 5 min at ambient temperature. After centrifugation for 5 min, 50 μL of the supernatant were added to 2.05 mL of the HABA-avidin complex formed by

Table 1
Determination of Biotin-Binding Sites in Immobilized Monomeric Avidin

Solid Support	Biotin-binding sites (μmol/mL packed solid support[a])		
	Total	EXS[b]	NES[c]
Controlled-pore glass (CPG-500)	0.229 ± 0.009	0.211 ± 0.010	0.018
Crosslinked 6% beaded agarose (Seph-CL-6B)	0.093 ± 0.009	0.088 ± 0.010	0.005
3M Emphaze biosupport medium AB_1	0.218 ± 0.017	0.202 ± 0.009	0.016

[a]Average of triplicate determinations, ± standard deviations.
[b]Exchangeable biotin-binding sites were prepared for assay by blocking all the non-exchangeable biotin-binding sites with biotin.
[c]Nonexchangeable biotin-binding sites (total minus exchangeable).

combining 2 mL of R_1 with 0.05 mL of R_2. A standard control reaction mixture consisted of 50 μL of R_3 added to 2.05 mL of the HABA-avidin complex. Absorbance of the HABA-avidin complex at 500 nm was measured before and after addition of the sample supernatant or the standard control.

The concentration of the biotin-binding sites was calculated from the difference between the decrease in absorbance at A_{500} of the standard control $\Delta A(C)$ and the sample $\Delta A(S)$ using the molar absorptivity. The μmol of biotin-binding sites per mL of support was calculated using the relationship

$$\mu\text{mol/mL} = 6.18\,[\Delta A(C) - \Delta A(S)] \quad (1)$$

where $\Delta A(C)$ is the decrease in absorbance measured at 500 nm before and after addition of standard control and $\Delta A(S)$ is the decrease in absorbance measured at 500 nm before and after addition of sample supernatant.

RESULTS AND DISCUSSION

The method developed for analysis of biotin-binding sites of immobilized avidin requires quantification of unbound biotin after incubation of the affinity matrix with excess biotin. Because of its higher affinity, unbound biotin may be quantified by its stoichiometric displacement of HABA from the HABA-avidin complex resulting in a decrease in the absorptivity at 500 nm.

Results given in Table 1 indicate that, per unit volume of support, avidin-succinamidopropyl-CPG contains the highest concentration of total and exchangeable biotin-binding sites. This observation is consistent with our previous studies that indicated enzymes immobilized on succinamidopropyl glass exhibited properties similar to soluble forms, that most of the

Table 2
Quantification of Immobilized Monomeric Avidin

Solid Support	mg Avidin/mL packed solid support[a]	
	HABA method[b]	Modified Bradford method[c]
Controlled-pore glass (CPG-500)	3.71 ± 0.147	3.55 ± 0.346
Crosslinked 6% beaded agarose (Seph-CL-6B)	1.51 ± 0.145	1.15 ± 0.155
3M Emphaze biosupport medium AB_1	3.53 ± 0.285	3.09 ± 0.085

[a]Average of triplicate determinations, ± standard deviations.
[b]The quantity of immobilized avidin was calculated by multiplying the concentration of the biotin-binding sites (Table 1) with the molecular weight of monomeric avidin (16.2 kD).
[c]The assay measures the decrease in absorbance of the dilute Coomassie brilliant blue solution after incubation of the solid phase monomeric avidin with excess of the dye reagent. The quantity of immobilized avidin was determined by using a standard curve prepared using soluble avidin.

activity loss could be accounted for by mass transfer limitations, and that high volumetric biocatalyst specific activities were achievable (*10,15–17*). For all three affinity supports the fraction of exchangeable biotin-binding sites was greater than 90% of the total (Table 1). The concentration of exchangeable biotin-binding sites is, of course, the most important parameter relative to the use of immobilized monomeric avidin as an affinity matrix.

If all of the immobilized avidin monomers have competent biotin-binding sites, then the concentration of immobilized protein calculated from the biotin-binding assay should agree with that determined by the Bradford protein-dye binding assay. Exchangeability of biotin or quaternary structure will not affect the results because that calculation is based on the initial ability to bind biotin. Results given in Table 2 show that these data are in agreement for each of the affinity supports. Again, the highest concentration of competent biotin-binding avidin monomers was observed for avidin-succinamidopropyl-CPG. Hence, this affinity matrix should prove to be extremely useful for capture of biotinylated molecules. Its potential usefulness is further suggested by the lack of nonspecific binding to this derivatized surface as indicated by studies of its use as a support for analytical affinity chromatography (*18,19*).

The spectrophotometric HABA method developed for assaying solid phase biotin-binding sites is relatively simple, requiring only three reagent solutions and a 5-min incubation, and does not require radiolabeled reagents or a scintillation counter. Although results for biotin binding to immobilized monomeric avidin only are reported here, we have also used the method to quantify biotin-binding sites in immobilized tetrameric avidin and streptavidin. The method should prove to be very useful for characterizing avidin or streptavidin affinity matrices.

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