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Easily reversible desthiobiotin binding to streptavidin, avidin, and other biotin-binding proteins: uses for protein labeling, detection, and isolation

James D. Hirsch,* Leila Eslamizar, Brian J. Filanoski, Nabi Malekzadeh, Rosaria P. Haugland, Joseph M. Beechem, and Richard P. Haugland

Molecular Probes, Inc., 4849 Pitchford Avenue, Eugene, OR 97402, USA

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Abstract

The high-affinity binding of biotin to avidin, streptavidin, and related proteins has been exploited for decades. However, a disadvantage of the biotin/biotin-binding protein interaction is that it is essentially irreversible under physiological conditions. Desthiobiotin is a biotin analogue that binds less tightly to biotin-binding proteins and is easily displaced by biotin. We synthesized an amine-reactive desthiobiotin derivative for labeling proteins and a desthiobiotin–agarose affinity matrix. Conjugates labeled with desthiobiotin are equivalent to their biotinylated counterparts in cell-staining and antigen-labeling applications. They also bind to streptavidin and other biotin-binding protein-based affinity columns and are recognized by anti-biotin antibodies. Fluorescent streptavidin conjugates saturated with desthiobiotin, but not biotin, bind to a cell-bound biotinylated target without further processing. Streptavidin-based ligands can be gently stripped from desthiobiotin-labeled targets with buffered biotin solutions. Thus, repeated probing with fluorescent streptavidin conjugates followed by enzyme-based detection is possible. In all applications, the desthiobiotin/biotin-binding protein complex is easily dissociated under physiological conditions by either biotin or desthiobiotin. Thus, our desthiobiotin-based reagents and techniques provide some distinct advantages over traditional 2-iminobiotin, monomeric avidin, or other affinity-based techniques.

Keywords: Desthiobiotin; Amine-reactive; Succinimidyl ester; Bioconjugates; Affinity chromatography; Reversible binding; Biotin; Streptavidin; Avidin

The ability of streptavidin, avidin, and other biotin-binding proteins (BbP)[1] to bind this vitamin has been exploited for more than 30 years [1,2]. Researchers have focused on this interaction because of its extremely high affinity, specificity, and almost universal applicability. Many reagents for biotinylating biomolecules using a variety of reaction chemistries are available [3,4]. Several solid-phase matrices incorporating biotin, 2-iminobiotin, diaminobiotin, and several BbP can be obtained for affinity-based isolation of biotinylated and biotin-binding target molecules. Different anti-biotin antibodies, numerous fluorescent dye- and enzyme-conjugated biotin derivatives, and variously labeled BbP are available for detecting desired targets using diverse strategies [3,4].

A well-recognized limitation of the biotin/BbP interaction is that it is essentially irreversible under physiological conditions. Thus, extremely low pH and high concentrations of chaotropic agents are required to dissociate the complex [3,5]. This can lead to inactivation of targets such as antibodies, enzymes, bioconjugates, or other labile molecules. Irreversibility also

* Corresponding author. Fax: 1-541-984-5698.
E-mail address: jim.hirsch@probes.com (J.D. Hirsch).

[1] *Abbreviations used:* BbP, biotin-binding protein(s); APC, allophycocyanin; BSA, bovine serum albumin; DAPI, 4′,6-diamidino-2-phenylindole; DDAO, 9H-(1,3-dichloro-9,9-dimethylacridin-2-one-7-yl) phosphate; DOS, degree of substitution; D-MEM, Dulbecco's modified Eagle's medium; FITC, fluorescein isothiocyanate; GAM, goat anti-mouse; GAR, goat anti-rabbit; HABA, 2-(4′-hydroxyazobenzene) benzoic acid; IgG, immunoglobulin G; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; MP, Molecular Probes, Inc.; MR, molar ratio(s); PBS, phosphate-buffered saline; PBST, phosphate-buffered saline containing 0.1% (v/v) Tween 20; R-PE, R-phycoerythrin; RT, room temperature (23 °C); SE, succinimidyl ester.

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prevents the use of direct biotin/BbP interactions for isolating viable cells from a complex mixture [6,7].

Five general approaches have evolved to address the irreversibility problem. A decade ago, based on earlier reports by Green [5], Fudem-Goldin and Orr [8] developed 2-iminobiotin-based reagents and affinity matrices exploiting the pH-sensitive binding of this biotin analogue to avidin and streptavidin. These authors reported that these proteins bound tightly to 2-iminobiotin–Sepharose at basic pH but eluted at pH 4 [8]. Others have shown that 2-iminobiotinylated ligands dissociated from BbP-based affinity matrices in a pH-sensitive manner [9–11]. Diaminobiotin–agarose has also been used in a similar fashion [12].

Biotin binding to nitrated avidin and streptavidin is also pH sensitive. In these derivatives, a significant percentage of the tyrosines in their biotin-binding sites are nitrated. Consequently, biotinylated ligands bind tightly to these proteins at acid pH, but dissociate at pH 10 [13–15]. Nitrated avidin–agarose (CaptAvidin agarose) has been commercially available from Molecular Probes (Eugene, OR) for several years. However, there are still situations where the desired targets cannot be exposed even briefly to the pH extremes needed to exploit the reversibility of 2-iminobiotin-, diaminobiotin-, and CaptAvidin agarose-based interactions.

Reversible biotin–avidin binding can be achieved by converting avidin tetramers to monomers. Monomerization reportedly lowers avidin's affinity for biotin by about seven orders of magnitude [3]. However, published reports indicate that, in practice, even matrix-bound avidin monomers reassociate to form oligomers with high affinity for biotin [3,16]. If these high-affinity sites are blocked with biotin before the sample is applied, a functionally monomeric matrix can result. Under these conditions, bound biotinylated ligands are reportedly eluted with millimolar concentrations of biotin or with low pH solutions [3]. Succinylated avidin–agarose has also been used for elution of biotinylated and 2-iminobiotinylated ligands by biotin [17]. More recently, a variety of mutant streptavidin proteins have been developed with reduced affinity for biotin or its analogues [18,19].

The fourth approach is based on a totally different strategy. Biomolecules can be biotinylated with derivatives that are subsequently cleavable by specific reagents. One example is biotin–HPDP (*N*-[6-(biotinamido) hexyl]-3′-(2′-pyridyldithio)propionamide), which reacts with sulfhydryl groups to create cleavable biotin–disulfide bonds [3]. Ligands labeled with biotin–HPDP can then be applied to a BbP-containing affinity matrix, where they bind. After unreacted species are washed out, bound ligands can be eluted with buffers containing dithiothreitol, β-mercaptoethanol, or sodium borohydride. These reagents reduce the biotin–disulfide bonds in the ligands, which are reportedly released from the matrix in their original form [3]. An analogous approach is based on attaching biotin incorporating a water-soluble, fluoride-sensitive $–CH_2OSi(Me)_2OC(Me)_2–$ linker to protein cysteines. The biotinylated protein is then reacted with streptavidin and immobilized on a biotin-containing matrix. Reportedly, the derivatized protein (papain) can be released by treating the complex with KF, which cleaves the silicon–oxygen bonds. Both biotin and part of the hydrolyzed linker remained attached to the matrix [20]. Although these techniques are typically performed under mild conditions, they may not be applicable in many situations. For example, the target protein(s) might not contain any exposed or nonessential cysteines. Reducing agents or fluoride may also have deleterious effects on protein integrity or activity.

The fifth approach is based on the lower affinity of BbP for selected biotin analogues such as D-desthiobiotin or dethiobiotin. This compound is a non-sulfur-containing biotin precursor and metabolite that binds less tightly to BbP than biotin ([5,21,22]; also see Discussion). Previous studies demonstrated that desthiobiotinylated proteins bound to BbP [21,22] and that biotin displaced BbP from immobilized desthiobiotin [23,24]. To build on these findings, we synthesized an amine-reactive desthiobiotin reagent for desthiobiotinylating proteins and prepared desthiobiotin–agarose for use in affinity chromatography. We show that desthiobiotinylated bioconjugates are biologically equivalent to their biotinylated counterparts and bind to BbP-based affinity matrices. Monoclonal and polyclonal anti-biotin antibodies recognize desthiobiotinylated proteins, but only the latter is partially displaced by free biotin. Agarose beads bearing amino-linked desthiobiotin bind BbP and bioconjugates containing them at protein-compatible pH values. However, in contrast to several of the techniques discussed above, the desthiobiotin/BbP interaction is easily reversed under physiological conditions by buffered solutions of either biotin or desthiobiotin. These versatile reagents (DSB-X biotin) may be advantageous for many bioconjugate-based labeling and detection strategies and for the facile affinity isolation of BbP-containing materials.

Materials and methods

Materials

Recombinant streptavidin is a Molecular Probes product (S-888). As determined by MALDI-TOF MS, the protein has an M_r of 52,800. Avidin (A-887), NeutrAvidin, streptavidin–agarose (S-951), CaptAvidin agarose (C-21386), streptavidin–R-PE (S-866), streptavidin–APC (S-868), DAPI (D-1306), (+)-biotin (B-1595), and the antibodies and bioconjugates used were also obtained from Molecular Probes. Catalogue

numbers are indicated in the text where appropriate. As described below, several bioconjugates were prepared in our laboratories and used for research only.

All other reagents were the highest grade commercially available and used as received. BSA for blocking (B-6003), D-desthiobiotin (5-methyl-2-oxo-4-imidazolinecaproic acid; D-1411), diaminobiotin (*cis*-3,4-diaminotetrahydrothiophenevaleric acid; D-1520), and goat anti-biotin antibody (B-3640) were from Sigma/Aldrich (St. Louis, MO). Mowiol 4-88 (475904) was from Calbiochem (La Jolla, CA). Aluminum TLC sheets (Silica gel 60 F_{254}) were from EM Sciences (Gibbstown, NJ). Nitrocellulose membranes (162-0145) were from Bio-Rad (Hercules, CA). A Milli-Q Synthesis dispenser (Millipore, Bedford, MA) provided the deionized water used in all experiments (18.2 mΩ/cm).

Stock solutions of biotin and desthiobiotin were prepared as follows. The reagent powders were first suspended in a small volume of anhydrous Me_2SO and stirred. Additional Me_2SO was added dropwise until all of the powder dissolved. The solution was then stirred vigorously and diluted with PBS (10 mM potassium phosphate, 150 mM NaCl, pH 7.2) to the final working volume. The final concentration of Me_2SO was $\leqslant 2\%$ (v/v), if possible. When Me_2SO could not be used (see Results), biotin and desthiobiotin were suspended in a small volume of water and stirred. Sodium hydroxide (1 M) or sodium carbonate powder was added in small aliquots until the compounds dissolved. The solutions were then rapidly diluted with PBS to the final working volume. The pH was adjusted to 7.2, if necessary. Aqueous solutions of biotin (MP, B-20656) and desthiobiotin (MP, B-20657) were also used.

Synthesis of desthiobiotin-X, SE

Desthiobiotin-X biotin, SE (6-((desthiobiotinoyl) amino)hexanoic acid, succinimidyl ester) was prepared from desthiobiotin by methods similar to those described previously [17]. The "X" represents a seven-atom spacer between desthiobiotin and the reactive ester moiety. TLC in chloroform/methanol (80:20), NMR in d_6-Me_2SO, and electrospray MS were used to establish the identity of the final product. The estimated purity (90%) was determined by reverse-phase HPLC. The structures of biotin, desthiobiotin, and desthiobiotin-X biotin, SE are shown in Fig. 1.

Preparation and purification of desthiobiotinylated proteins

In these experiments, protein concentrations were determined by absorbance at 280 nm using experimentally determined extinction coefficients. Lyophilized proteins were reconstituted in PBS or 100 mM bicarbonate buffer (pH 8.3) depending on the needs of the experiment. Antibodies in solution were usually in PBS containing 2–5 mM sodium azide.

To prepare bioconjugates, protein solutions in PBS were first brought to pH 8.0–8.3 by adding $\sim 1/10$ volume of 1 M sodium bicarbonate buffer (pH 8.3). The desthiobiotin-X biotin, SE was dissolved in anhydrous Me_2SO at 10 mg/ml and added gradually to the protein solution with gentle stirring at RT. After 60–90 min of incubation at RT, unreacted desthiobiotin-X biotin, SE was removed by dialysis or gel filtration, as described previously [25]. Unreacted desthiobiotin-X biotin, SE was removed from small volumes of conjugates by centrifugation through spin columns containing an appropriate gel filtration matrix. Approximate DOS values were determined with HABA–avidin using procedures published previously [3,26].

Residual desthiobiotin-X biotin in the conjugates was monitored by TLC on silica gel-coated plates in chloroform/methanol/acetic acid (70:25:5) or acetonitrile/H_2O (80:20). Like biotin, desthiobiotin-X biotin was visualized after wetting the chromatogram with an ethanol solution of *p*-dimethylaminocinnamaldehyde, followed by air-drying, as described previously [27].

Preparation of desthiobiotin-X biotin, SE–agarose

Sepharose beads (Sigma/Aldrich, CL4B-200) were converted to the amino derivative using minor modifications of a method described previously [28–30]. The resulting amino agarose (~ 0.45 μmol amines/ml of

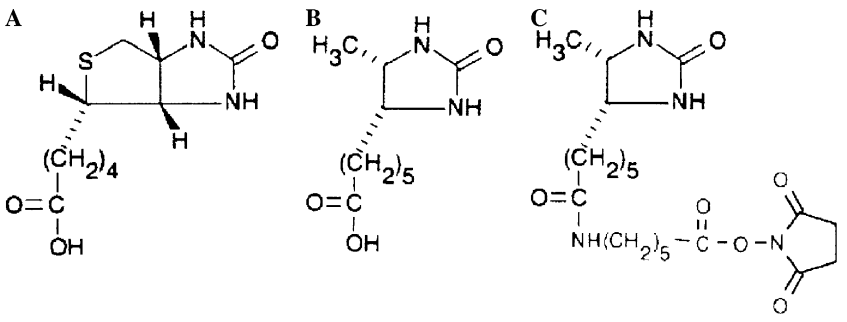

Fig. 1. The structures of: (A) biotin; (B) desthiobiotin; and (C) desthiobiotin-X-succinimidyl ester (desthiobiotin-X-SE).

beads) was mixed with desthiobiotin-X biotin, SE at a desthiobiotin-X biotin, SE to agarose amine MR of 0.4–0.5 and incubated at RT on a rocker platform for 90 min. Unreacted amines were capped with a 100-fold molar excess of acetic anhydride at pH 9.0. The desthiobiotin–agarose beads were rinsed extensively with PBS to neutrality and stored at 4 °C in PBS containing 2 mM sodium azide.

Characterization of reversible desthiobiotin/BbP interactions: multiwell plate assays

Reversibility of desthiobiotin/BbP binding was initially evaluated in assays performed in plastic round-bottom 96-well plates (Corning 25802). All incubations were at RT. In one assay variation, 100-μl aliquots of streptavidin, avidin, CaptAvidin, and NeutrAvidin BbP in 100 mM sodium carbonate buffer (pH 9.5) were added to quadruplicate wells at 50, 200, and 500 ng/well. After 2 h in a humidified chamber, the wells were washed with 3×100 μl of PBST. The wells were blocked for 1 h with 100-μl/well of PBST containing 1% (w/v) BSA and washed with PBST as before. Aliquots (100 μl) of desthiobiotin-X biotin–GAM IgG (desthiobiotinylated GAM, DOS = 2.3), prepared as described above, and diluted in PBST to 10 μg/ml, were added to each well. The plates were incubated for 2 h. After another round of washing with PBST, the wells were filled with 100 μl aliquots of the Alexa Fluor 488 donkey anti-goat IgG antibody (MP, A-11055) at 5 μg/ml and incubated for 2 h.

After washing with PBST, bound fluorescence (total binding) was determined in a CytoFluor II multi-well plate reader (PerSeptive Biosystems). Excitation and emission filters were set at 485 and 530 nm, respectively. The wells were filled with 100-μl aliquots of 5 mM biotin in PBST and the plates were incubated for 2 h at RT or overnight at 4 °C. After washing with PBST, residual bound fluorescence (nonspecific binding) was determined again in the plate reader. Following subtraction of blank values (buffer-only wells) from the experimental values, biotin-displaceable specific binding was computed by subtracting the nonspecific binding from the total binding (see Fig. 4).

Three variations of this multiwell plate experiment were performed to characterize reversible binding. In the first variation, streptavidin, avidin, NeutrAvidin, and CaptAvidin BbP (1 μg/well) were bound to the wells at pH 9.5. Following the protocol described above, desthiobiotinylated GAM (50, 200, and 500 ng/well) followed by Alexa Fluor 488 donkey anti-goat IgG antibody were then added. Bound fluorescence before and after incubation with 5 mM biotin was determined, as described above. In the second variation, desthiobiotinylated GAM was bound to the wells at 500 ng/well. Next, streptavidin–R-PE at 100, 200, and 500 ng/well was added. After incubating and washing, bound fluorescence was determined in the plate reader (excitation = 530 nm, emission = 580 nm). Bound fluorescence was determined again after incubation with 5 mM biotin, as described above. In the third variation, the concentration of the desthiobiotinylated GAM was varied (100, 200, and 500 ng/well) while the streptavidin–R-PE concentration was held constant at 500 ng/well. All other manipulations were as described above.

Effect of biotin and desthiobiotin on streptavidin tryptophan fluorescence

Several reports indicate that biotin quenches and blue shifts tryptophan fluorescence in streptavidin and avidin under steady-state conditions [31–34]. Since desthiobiotin binds less tightly, we were curious to learn how it would affect these fluorescence parameters. Only the results with streptavidin are reported here. These steady-state fluorescence experiments were performed in an Aminco-Bowman Series II Luminescence Spectrometer. To excite predominantly tryptophans, excitation at 295 nm was used [35]. Emission spectra were recorded at 5–10 nm/s from 310–390 nm. Excitation and emission band passes of 8 nm were used along with a step size of 1 nm.

Typically, samples of BbP were prepared in PBS at a concentration of 189 nM (756 nM biotin binding sites). For streptavidin, this represents 10 μg/ml. Aliquots of 2.997 ml of diluted proteins were dispensed into disposable 1-cm-path-length plastic cuvettes (Sarstedt, D-51588) containing 3 μl of either PBS or 1000-fold-concentrated biotin or desthiobiotin solutions prepared as described above. The final biotin/desthiobiotin concentrations used ranged from 5 nM to 10 μM, depending on the experimental design. The samples were mixed, incubated for 10 min at RT, and analyzed in the spectrofluorometer, as described. Fluorescence emission parameters were quantitated using the software provided with the instrument. The peak area and emission maximum for each spectrum were obtained.

In initial experiments, we found that biotin and desthiobiotin had opposite effects on streptavidin fluorescence. As reported, increasing concentrations of biotin quenched and blue-shifted fluorescence until all biotin-binding sites were filled [31–34]. In contrast, desthiobiotin increased and blue-shifted fluorescence over the same concentration range (see Results). These results provided a means to determine a biotin/desthiobiotin exchange rate with native streptavidin. Streptavidin at 19 nM (1 μg/ml/76 nM biotin-binding sites) in PBS was mixed with 100 nM desthiobiotin and the tryptophan fluorescence emission was determined. Biotin was added to a final concentration of 100 μM, the sample was rapidly mixed by hand, and emission from the same sample was measured periodically for 2 h. The peak areas and emission maxima at each time point were quantitated, as described above. These experiments were

also repeated with 19 nM streptavidin saturated with 100 nM biotin. In this case, desthiobiotin at a final concentration of 100 μM was added, and fluorescence emission was monitored as a function of time.

Characterization of reversible desthiobiotin/BbP binding by affinity chromatography

Desthiobiotin–agarose. Several modes of affinity chromatography were used to evaluate reversible desthiobiotin–BbP interactions. Desthiobiotin–agarose was used to isolate BbP and bioconjugates containing them. Typically, columns with a bed volume of 1 ml were used. Unless indicated otherwise, experiments were performed in PBS. Before applying protein samples, all desthiobiotin–agarose columns were conditioned with 10 column volumes of BSA at 1 mg/ml in PBS, followed by washing with PBS until $A_{280} = 0$.

In the first series of experiments, the binding capacity of desthiobiotin–agarose for BbP was determined by sequentially loading 100- to 500-μl aliquots of streptavidin, avidin, and NeutrAvidin (1 mg/ml) on the columns. Sample application continued until absorbance at 280 nm was detected in the outflow. To determine nonspecifically bound protein, separate 1-ml columns of unmodified Sepharose 4B were treated similarly. After washing until $A_{280} = 0$, retained proteins were stripped from these columns with 50 mM HCl. The binding capacity was taken as the total amount of protein loaded on the desthiobiotin–agarose prior to the first appearance of A_{280} in the outflow minus the amount of protein eluted from Sepharose 4B with acid.

BbP were then eluted from desthiobiotin–agarose to determine recovery. Typically, 10–20 column volumes of biotin or desthiobiotin (10–50 mM) prepared in PBS were used. Since these solutions had concentration-dependent A_{280}, this was subtracted from the total eluted absorbance before calculating recovery. Washing with five column volumes of 50 mM HCl stripped any remaining protein from the beads. A_{280} recovered in these fractions was also quantitated. Following reequilibration with PBS, these columns could be reused multiple times. Similar procedures were followed with protein samples containing streptavidin–R-PE, streptavidin–APC, streptavidin–antibody, and streptavidin–dye conjugates. Details of these experiments are provided under Results.

BbP–agaroses. The chromatographic behavior of desthiobiotinylated GAM and desthiobiotinylated GAR IgG (MP, D-20694) on streptavidin–agarose and CaptAvidin–agarose columns was determined. Conjugate samples (500 μg–1 mg) were applied in PBS to columns of streptavidin–agarose with 1-ml bed volumes. The conjugates were eluted with buffered 10–50 mM biotin or desthiobiotin and any residual protein was stripped with 50 mM HCl. When CaptAvidin–agarose columns were used, the desthiobiotinylated antibodies were applied in 25 mM citrate–phosphate buffer at pH 5.5 and eluted with 25 mM Tris buffer at pH 10.

Reversible cell staining with desthiobiotinylated GAM

Cultured bovine pulmonary artery endothelial (BPAE) cells were grown to 30–50% confluence on glass coverslips (18 mm^2) in D-MEM containing 20% (v/v) fetal bovine serum, 10 mM Hepes, and 2 mM glutamine at 37 °C in 5% CO_2/air. The cells were fixed for 15 min at RT with 4% (v/v) formaldehyde (Fluka) and washed three times with PBS. The cells were then permeabilized with 0.2% (v/v) Triton X-100 in PBS, washed three times with PBS, and blocked for 30 min with 6% (w/v) BSA in PBS. After more washing, the cells were incubated for 1 h with monoclonal mouse anti-α-tubulin (MP, A-11126) at 1 μg/ml in PBS containing 1% (w/v) BSA. After washing again, the cells were incubated for 1 h at RT with either biotin-XX-GAM (MP, B-2763) or desthiobiotinylated GAM (MP, D-20690) at 2 μg/ml in PBS/1% (w/v) BSA.

The cells were washed three times with PBS and stained with Alexa Fluor 488 streptavidin conjugate (MP, S-11223) at 1 μg/ml in PBS/1% (w/v) BSA for 30 min at RT. After washing with PBS, the cells were counterstained with DAPI at 0.2 μg/ml in PBS for 1 min at RT and imaged with a fluorescence microscope (Nikon Eclipse E400). The microscope was equipped with DAPI and FITC filters. For tubulin and DAPI imaging, the exposure times were 800 and 200 ms, respectively.

The cells were then incubated for 1 h at RT with four changes of 10 mM biotin in PBS and imaged again. For these experiments, biotin was dissolved in base and diluted with PBS to a final concentration of 10 mM, as described above. Me_2SO could not be used here because, at a final concentration of 2% (v/v), the solvent disrupted staining of cell nuclei by DAPI.

Reversible antigen detection on dot blots with desthiobiotinylated bioconjugates

The antigen used on model dot blots was a monoclonal mouse anti-c-myc antibody (MP, A-21280). Duplicate 1-μl spots containing 2.5, 5, and 10 ng of antibody were applied to nitrocellulose filter strips. All subsequent incubations were conducted at RT on a rocker platform. The filter strips were blocked for 1 h in 50 mM Tris–HCl buffer (pH 7.5) containing 150 mM NaCl, 0.02% (v/v) Tween-20, 0.5% (w/v) BSA, and 0.25% (w/v) Mowiol 4-88. After washing with PBST, the filters were incubated for 1 h with desthiobiotinylated GAM or biotin-XX-GAM at 5 μg/ml. After washing with PBS, the filters were incubated with Alexa Fluor 488 streptavidin at 2 μg/ml for 1 h. Fluorescence bound

to the antigen spots was detected with a Fuji FLA 3000 imaging scanner.

The blots were incubated for 2–16 h with 50 mM biotin, washed with PBS, blocked again, and imaged to determine the extent of Alexa Fluor 488 streptavidin conjugate displacement. The blots were then incubated with streptavidin–alkaline phosphatase (MP, S-921; 1:40,000 dilution) and DDAO phosphate (MP, D-6487) at 1.25 μg/ml for 10 min at RT in 10 mM Tris buffer (pH 9.5) containing 1 mM $MgCl_2$. The blots were washed extensively with PBS, air-dried overnight to minimize background, and imaged as described.

Recognition of desthiobiotin by anti-biotin antibodies and its reversal by biotin and desthiobiotin

We determined whether two anti-biotin antibodies recognized protein-bound desthiobiotin and biotin in multiwell plate assays. A monoclonal anti-biotin antibody was tested first. Aliquots (100 μl) of desthiobiotinylated GAM (MP, D-20690, DOS = 5.6) and two samples of biotin-X-GAM (DOS = 4.1 and 3.2) employed as positive controls were dispensed in quadruplicate wells of Corning 25802 96-well plates at 1 ng–1 μg/well in sodium carbonate buffer (pH 9.5). After 2 h at RT followed by washing with PBST, the wells were blocked for 1 h with PBST–BSA. After washing, the wells were filled with 100-μl aliquots (200 ng/well) of a mouse anti-biotin monoclonal antibody (MP, A-11242) diluted in PBST. The plates were incubated for 1 h at RT. After washing, 100-μl aliquots (1 μg/well) of Alexa Fluor 488-GAM were added to the wells. After 1 h, the wells were washed and refilled with 100 μl of PBST prior to analysis in the plate reader, as described above. Bound fluorescence was quantitated after subtraction of blanks (buffer-only wells), as described above.

To test the polyclonal anti-biotin antibody, desthiobiotinylated (see above) and biotinylated GAM (MP, B-2763, DOS = 5.3) were immobilized at pH 9.5 in 96-well plates at 5–100 ng/well, as described above. After washing and blocking, the wells were filled with 100-μl aliquots of a polyclonal goat anti-biotin antibody (200 ng/well). After 1 h and following a washing step, 100 μl aliquots (1 μg/well) of Alexa Fluor 488 donkey anti-goat IgG antibody were added. After incubating for 1 h, the plates were washed, refilled with PBST, and analyzed in the plate reader, as described above.

We also tested whether a large excess of biotin would displace the anti-biotin antibodies from desthiobiotinylated and biotinylated GAM. In these experiments, the assay protocol described above for the polyclonal anti-biotin antibody was employed for both antibodies and was performed exactly as described. After the final fluorescence determination in the plate reader, 100-μl aliquots of 50 mM biotin or desthiobiotin prepared without Me_2SO (see Materials and methods) were added to each well. The plates were incubated overnight at RT, washed with PBST, and analyzed again in the plate reader.

Results

Preparation of desthiobiotinylated proteins

Like other succinimidyl esters, desthiobiotin-X biotin, SE reacted optimally with protein amines at pH values > 8.0 [25]. Although the ideal desthiobiotin-X biotin, SE to protein MR for labeling varied somewhat depending on the protein, antibodies at 4–10 mg/ml were labeled at MR of 5–7 resulting in desthiobiotin-X conjugates with approximate DOS values of 4–6. The HABA–avidin method for quantitating protein biotinylation [3,26] was found to be equally suitable for measuring the approximate DOS of protein conjugates labeled with desthiobiotin-X biotin, SE.

We used desthiobiotin-X biotin, SE primarily to label IgG antibodies. These included GAM and GAR, GAM and GAR $F(ab')_2$ fragments, goat anti-human and chicken, donkey-anti-goat and sheep, and goat anti-rat. Desthiobiotinylated GAM μ chains were also produced. In addition, test batches of desthiobiotin-X biotin–R-PE, horseradish peroxidase, and alkaline phosphatase were prepared during the course of this research. These results suggest that any protein, peptide, or other macromolecule containing amines can be labeled with desthiobiotin-X biotin, SE.

Characterization of desthiobiotin–agarose

The binding capacity of desthiobiotin–agarose for streptavidin, avidin, and NeutrAvidin biotin-binding protein was 9, 3, and 2.5 mg per ml of packed matrix, respectively. Since this parameter was determined only at pH 7.2, and the isoelectric points of these proteins vary widely [3,4], binding capacities may be different at other pH values. Recoveries of input proteins ranged from 70 to 90%, depending on the protein and the biotin or desthiobiotin concentration used for elution (not shown). It was advantageous to use high biotin/desthiobiotin concentrations (> 25 mM) since this allowed recovery of the proteins in more concentrated form. After application of the biotin/desthiobiotin solutions, incubating the columns for 30–60 min before elution further reduced the final volume of recovered sample. A typical elution of streptavidin with 50 mM biotin is shown in Fig. 2A.

Although underivatized amines on the desthiobiotin–agarose beads were capped, and each column was conditioned with BSA prior to use to block nonspecific binding sites (see Materials), about 10–20% of input streptavidin would not elute in a single aliquot of 50 mM biotin or desthiobiotin. Incubating the column again in

50 mM biotin/desthiobiotin was often helpful for increasing recovery. Concentrations of biotin or desthiobiotin >50 mM were not evaluated. The tightly bound protein was removed from columns by washing the beads with 50 mM HCl. Desthiobiotin–agarose columns were reusable after stripping and reequilibrating with PBS. For example, one 1-ml column was used eight times in succession to isolate 500-μg samples of streptavidin–R-PE. Although we did not quantify recovery each time, no obvious decline in column behavior was observed.

The reversibility of desthiobiotin/BbP binding was exploited for isolating several BbP and bioconjugates. Avidin and NeutrAvidin binding protein were isolated from desthiobiotin–agarose by eluting with desthiobiotin (Fig. 2B). Streptavidin–R-PE and streptavidin–APC were also eluted from desthiobiotin–agarose with desthiobiotin (Figs. 2C and D). As noted, ligand recoveries were higher and final sample volumes were lower if the biotin or desthiobiotin eluents were left on the column for 30–60 min before collecting the sample. Figs. 2C and D also illustrate the use of multiple aliquots of biotin or

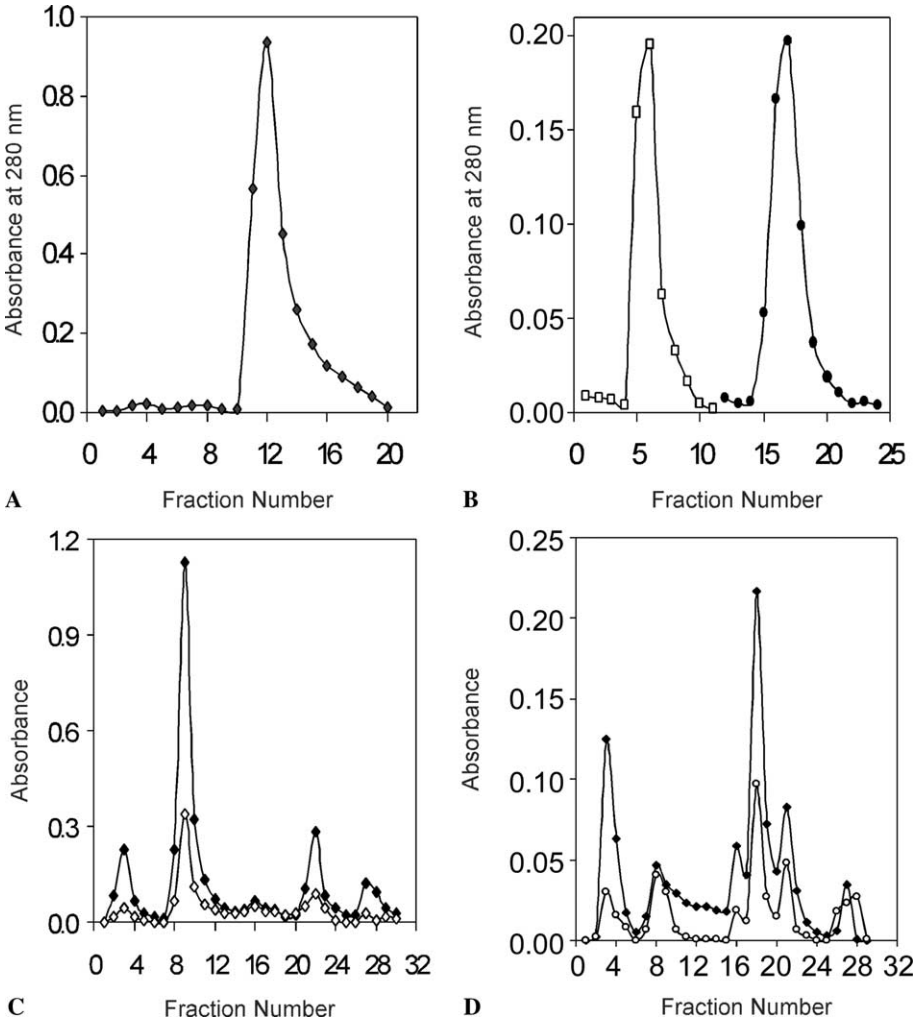

Fig. 2. Affinity chromatography with desthiobiotin–agarose. In each example, 1-mg samples of protein were applied to 1-ml bed volume columns of desthiobiotin–agarose equilibrated in PBS (pH 7.2). Fractions of 1 ml were collected. (A) Chromatography of streptavidin. The protein was eluted with a single 20-ml aliquot of 50 mM biotin applied after eight fractions had been collected. (B) Chromatography of avidin (open squares) and NeutrAvidin biotin binding protein (closed circles). After loading the avidin and washing with buffer, the protein was eluted with 50 mM desthiobiotin applied after three fractions had been collected. The column was washed with PBS, stripped with 50 mM HCl, and reequilibrated. The NeutrAvidin biotin-binding protein sample was then loaded and eluted with 50 mM desthiobiotin applied after two fractions had been collected. (C) Chromatography of streptavidin–R-PE. A sample of partially purified conjugate was applied to the column that was washed with PBS to elute unconjugated R-PE (fractions 1–5). The column was washed with 10 ml of 50 mM biotin (fractions 6–13) followed by 5 ml of 50 mM biotin (fractions 14–18). The column was then washed with 10 ml of 50 mM desthiobiotin (fractions 19–24) and finally with 50 mM HCl (fractions 25–30). Except for the HCl, each wash was run into the column and flow was stopped for 30 min before fractions were collected. Open diamonds, A_{280}; closed diamonds, A_{565}. (D) Chromatography of streptavidin–APC. The sample was a partially purified preparation and unconjugated APC was eluted with PBS (fractions 1–5). Fractions 6–23, containing the conjugate, were collected after incubating the column for 30 min in 50 mM desthiobiotin. The column was then stripped with 50 mM HCl (fractions 24–30). Open circles, A_{280}; closed diamonds, A_{650}.

desthiobiotin to increase recovery. Desthiobiotin–agarose could also be used batchwise to isolate streptavidin–R-PE. After mixing the conjugate with a matrix slurry and incubating at RT for 10 min, the beads were collected by brief centrifugation and washed twice with PBS. The bound conjugate was then eluted with two aliquots of 50 mM desthiobiotin (data not shown).

Desthiobiotinylated antibodies were isolated with streptavidin–agarose and CaptAvidin–agarose (Fig. 3). Streptavidin–agarose was used to isolate desthiobiotinylated GAM by eluting it with 10 mM biotin (Fig. 3A) while desthiobiotinylated GAM and GAR (not shown) were eluted from CaptAvidin-agarose by washing the column with pH 10 buffer (Fig. 3B). Since the protein-binding capacity of the streptavidin–agarose was only $\sim 750\,\mu g$ of desthiobiotinylated GAM/ml of packed beads, lower concentrations of biotin (5–10 mM) and desthiobiotin (10–20 mM) were adequate for eluting desthiobiotinylated proteins.

Other applications of reversible desthiobiotin/BbP interactions

Biotin-reversible binding of desthiobiotinylated GAM to BbP was also characterized in multiwell plate assays. When increasing amounts of streptavidin were immobilized in wells of 96-well plates, increasing amounts of desthiobiotinylated GAM were bound (total binding). The bound conjugate was almost totally released by 5 mM biotin (specific binding), leaving a small amount of residual fluorescence (nonspecific binding) (Fig. 4A). Similar results were obtained when increasing amounts of avidin, NeutrAvidin, and CaptAvidin biotin-binding proteins were immobilized on the plates (not shown).

Constant amounts of BbP were applied to the plates, followed by increasing concentrations of desthiobiotinylated GAM. There was a concentration-dependent increase in bound antibody conjugate that was almost totally displaced by 5 mM biotin. Fig. 4B shows the biotin-displaceable binding of desthiobiotinylated GAM to four immobilized BbP. Since the goal here was to evaluate reversibility, the apparent binding differences between the target proteins were not investigated. Presumably, the ability of CaptAvidin biotin-binding protein to bind desthiobiotinylated GAM was considerably lower than those of the other proteins because the assay pH was higher than CaptAvidin biotin-binding protein's optimum range for ligand binding.

The widely used phycobiliprotein conjugate, streptavidin–R-PE, also bound to increasing amounts of immobilized desthiobiotinylated GAM. The streptavidin–R-PE was almost totally displaced by 5 mM biotin. Similar results with streptavidin conjugates containing fluorescent Alexa Fluor dyes were also obtained (data not shown). This suggests that binding of any BbP-containing species to desthiobiotin can be reversed by biotin.

In the experiments just described, both the desthiobiotin and the BbP were conjugated to other molecules. Moreover, the bimolecular interactions took place with one ligand immobilized on a solid support. To evaluate ligand binding to native BbP in solution, we exploited the well-characterized effects of biotin on BbP fluorescence [27–30]. In these experiments, the proteins were excited at 295 nm to evoke emission from tryptophans primarily [35]. A typical emission spectrum for streptavidin is shown in Fig. 5 (curve 1).

Increasing concentrations of biotin (10 nM–10 μM depending on the streptavidin concentration) quenched and blue-shifted streptavidin tryptophan fluorescence emission, as reported previously [31–34]. The maximum quenching observed, at saturating biotin concentrations, was $23 \pm 1.5\%$ (mean $\pm$ SD, $n = 3$ separate experiments). Typical biotin quenching of streptavidin fluorescence is shown in Fig. 5 (curve 6). The MR of biotin to streptavidin yielding maximal quenching was approximately 4 in each experiment. Molar ratios >4 induced no additional quenching.

In contrast, over the same concentration range, desthiobiotin increased and blue-shifted streptavidin try-

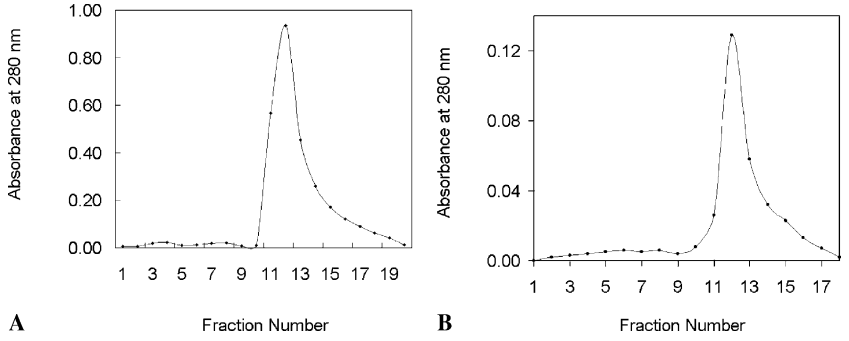

Fig. 3. Affinity chromatography with streptavidin–agarose (A) and CaptAvidin–agarose (B). In these examples, 1-mg samples of desthiobiotinylated goat anti-mouse IgG were applied to 1-ml bed volume columns in PBS (A) or 25 mM citrate–phosphate buffer (pH 5.5) (B). In (A), the conjugate was eluted with 10 mM biotin applied after seven fractions had been collected. In (B), the conjugate was eluted with 25 mM Tris buffer (pH 10) after nine fractions had been collected.

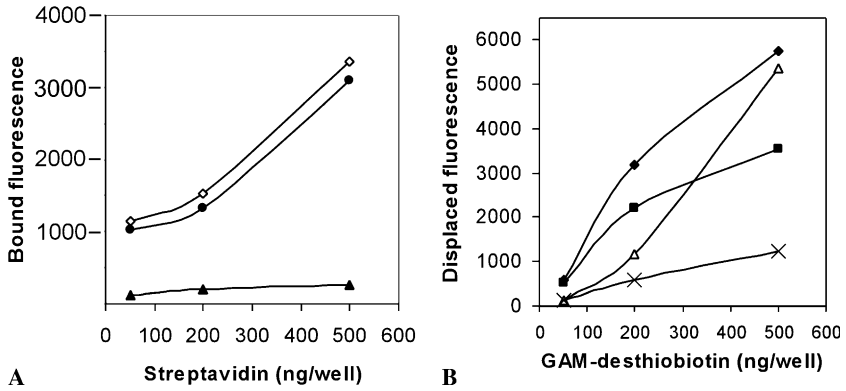

Fig. 4. Reversible desthiobiotin/BbP interactions. (A) Biotin-reversible binding of desthiobiotinylated goat anti-mouse IgG to streptavidin. Streptavidin was immobilized in wells of a plastic 96-well plate at the concentrations indicated. After blocking the wells with BSA, desthiobiotinylated goat-anti-mouse IgG followed by Alexa Fluor 488 donkey anti-goat IgG antibody were then applied, as described under Materials and methods. Bound fluorescence (open diamonds) was determined in a microplate reader and is referred to in the text as total binding. The wells were filled with 5 mM biotin solution, incubated for 2 h, and washed, and bound fluorescence was measured again (closed triangles). Fluorescence remaining bound to the plate after the biotin wash is referred to in the text as nonspecific binding. Biotin-displaceable specific binding (closed circles) was the difference between total and nonspecific binding at each streptavidin concentration. (B) Biotin-reversible binding of desthiobiotinylated goat anti-mouse IgG to immobilized BbP. As described under Materials and methods, fixed amounts of avidin (closed diamonds), streptavidin (closed squares), NeutrAvidin biotin-binding protein (open triangles), and CaptAvidin biotin-binding protein (Xs) were immobilized in wells of 96-well plates. Desthiobiotinylated goat anti-mouse IgG at the concentrations indicated, followed by Alexa Fluor 488 donkey anti-goat IgG antibody, were then added. Biotin-displaceable fluorescence was determined as described above. In this panel, only the specific, biotin-displaceable fluorescence is shown.

ptophan fluorescence (Fig. 5, curve 2). In this case, the maximum increase was $16 \pm 1.2\%$ (mean $\pm$ SD, $n = 3$ experiments). These results suggest that, at saturating concentrations (MR = 4), desthiobiotin was not as effective at enhancing fluorescence as biotin was at quenching it ($p = 0.0032$, unpaired t test). However, the blue shift induced by both compounds was from 334–335 to 329–330 nm. The biotin analogue diaminobiotin also quenched and blue-shifted streptavidin fluorescence, but was about 100-fold less potent than biotin (not shown). The effects of these compounds on fluorescence of avidin, NeutrAvidin, and CaptAvidin biotin-binding protein fluorescence were different from those presented here. Also, the magnitude and type of effect provoked by biotin and desthiobiotin with all four BbP varied with pH. Details of these experiments will be presented elsewhere.

The unexpected stimulatory effects of desthiobiotin on streptavidin fluorescence enabled us to determine a desthiobiotin/biotin exchange rate with native protein in solution. Streptavidin was mixed with a 4-fold molar excess of desthiobiotin to saturate its biotin-binding sites. This produced the maximum degree of emission enhancement and the typical blue shift of 4–5 nm (Fig. 5, curve 2). Biotin was then added at 1000-fold molar excess and tryptophan fluorescence was monitored periodically over the next 2 h. As biotin displaced desthiobiotin from the protein, fluorescence emission gradually decreased. Other curves in Fig. 5 depict fluorescence emission at 2 (curve 3) and 4 min (curve 4) after adding biotin, respectively. For clarity, data obtained at time points between 4 and 80 min are not shown in Fig. 5. After 120 min (Fig. 5, curve 5), emission had decreased and stabilized at the level of quenching typically obtained with streptavidin plus biotin (Fig. 5, curve 6). The usual 4 to 5-nm blue shift was observed.

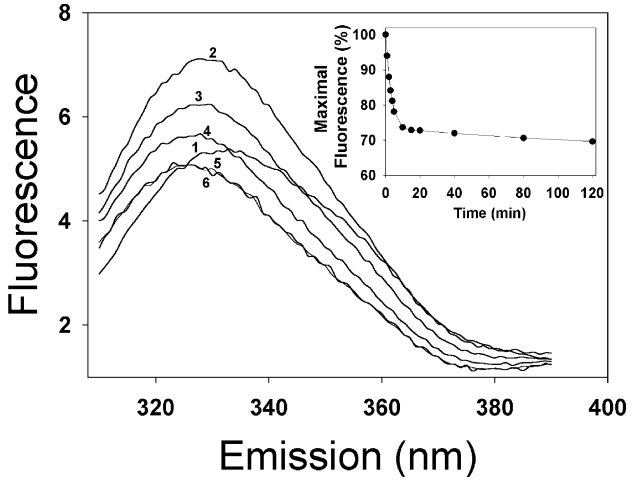

Fig. 5. Effects of biotin and desthiobiotin on steady state fluorescence emission from tryptophans in streptavidin and determination of the desthiobiotin/biotin exchange rate. Streptavidin at 1 μg/ml (76 nM) in PBS was excited at 295 nm and emission was recorded from 310 to 390 nm (curve 1). In the presence of 100 nM biotin, emission was quenched and blue shifted (curve 6), while desthiobiotin evoked an increase and blue-shift in fluorescence (curve 2). Biotin at a final concentration of 100 μM was then added and emission was monitored periodically for 2 h. Emission spectra at 2 (curve 3), 4 (curve 4), and 120 min (curve 5) after biotin addition are also shown. Spectra recorded between 4 and 80 min are omitted for clarity. *Inset*: Decrease in fluorescence as a function of time after adding biotin to streptavidin saturated with desthiobiotin (0 time). The data points represent the mean values from two separate experiments (0–20 min). Data for 40, 80, and 120 min are from a single experiment.

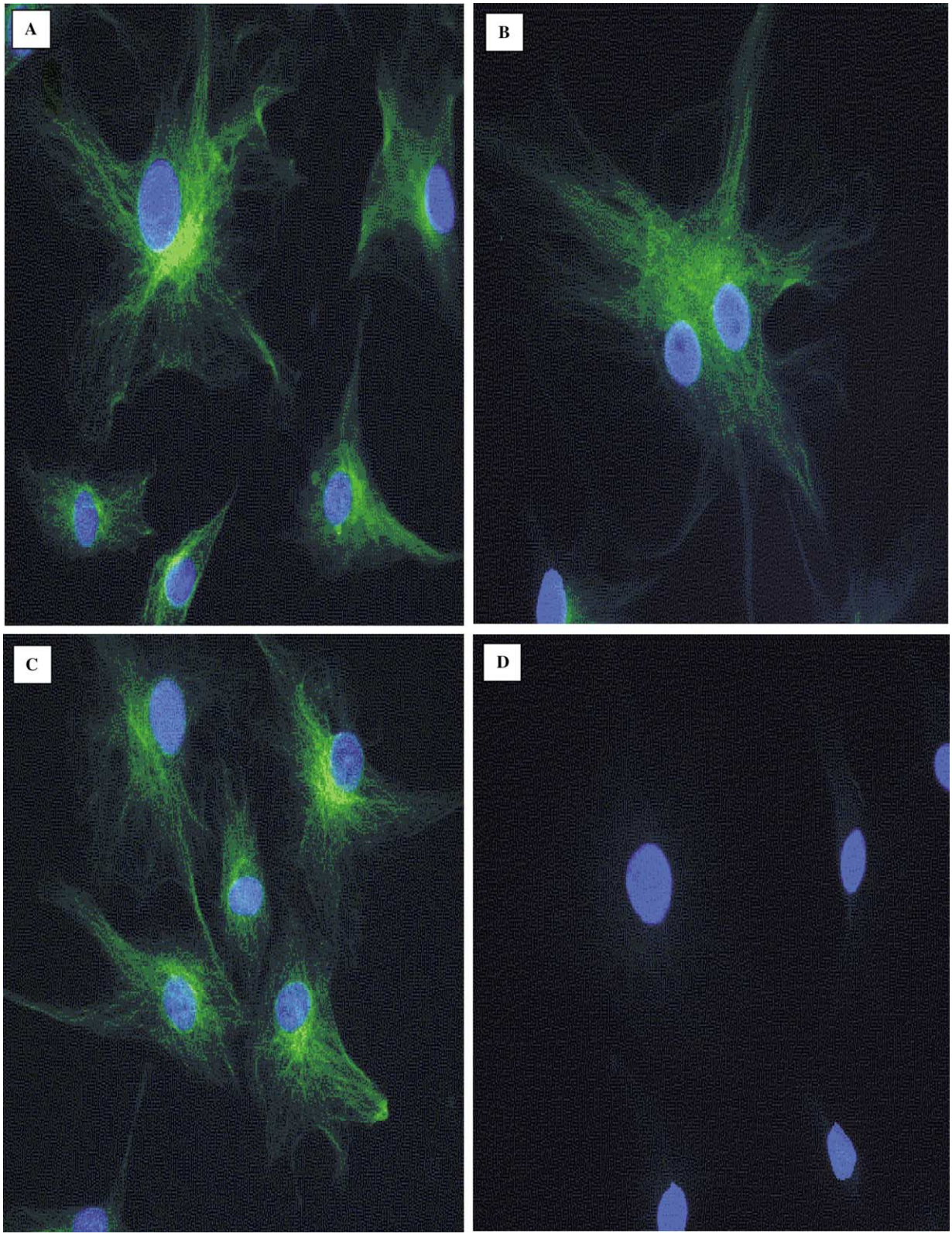

Fig. 6. Biotin-reversible cell staining using a desthiobiotinylated secondary antibody. Desthiobiotin-GAM was used as a secondary antibody to stain α-tubulin in fixed and permeabilized BPAE cells. Mouse anti-α-tubulin antibody was applied followed by either desthiobiotinylated or biotinylated GAM. α-Tubulin was then visualized after incubating with Alexa Fluor 488 streptavidin. The latter labeled both secondary antibodies (A) and (B). However, when the desthiobiotinylated GAM-treated cells were washed with 10 mM biotin, the fluorescent signal disappeared, leaving only the DAPI-stained nuclei (D). Washing with biotin had no effect on cells treated with biotinylated antibody (C).

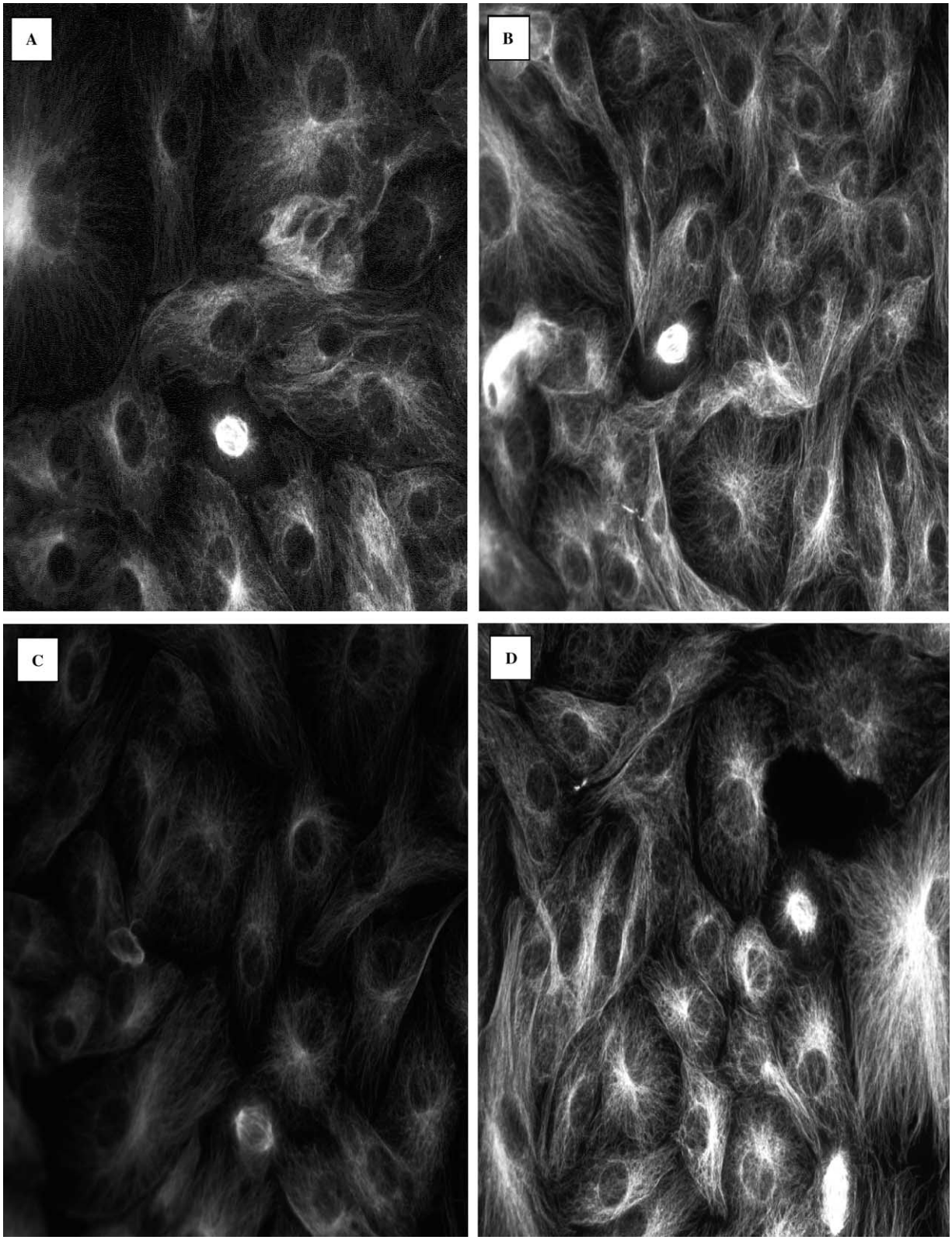

Fig. 7. Binding of Alexa Fluor 488 streptavidin conjugate saturated with desthiobiotin to α-tubulin in BPAE cells prelabeled with anti-α-tubulin and biotinylated antibodies. As described under Materials and methods, α-tubulin in fixed and permeabilized BPAE cells was stained with mouse anti-α-tubulin antibody, biotinylated GAM IgG, and either Alexa Fluor 488 streptavidin conjugate alone or this conjugate saturated with 10 μM desthiobiotin. All of the cells were then photographed periodically over the next hour at the same exposure settings (1 s). (A) Cells 5 min after application of Alexa Fluor 488 streptavidin conjugate; (B) cells 1 h after application of the fluorescent conjugate; (C) cells 5 min after application of the fluorescent conjugate in 10 μM desthiobiotin; (D) cells 1 h after application of the conjugate in desthiobiotin.

As shown in the inset of Fig. 5, the desthiobiotin/biotin exchange reaction was biphasic. Most of the exchange (~90%) took place over the first 10 min after biotin addition and this phase had a $t_{1/2}$ of ~ 2 min. The tightly bound desthiobiotin dissociated very slowly over the next 110 min. The biotin reversibility of the desthiobiotin/native streptavidin interaction was reinforced by an exchange experiment where the protein was first mixed with a 4-fold molar excess of biotin. This yielded the maximum degree of fluorescence quenching illustrated in Fig. 5 (curves 5 and 6). A 1000-fold molar excess of desthiobiotin was then added and fluorescence emission was monitored over 2 h, as described. Desthiobiotin did not displace biotin from streptavidin over the duration of the experiment (data not shown).

Biotin-reversible cell staining was also possible when desthiobiotinylated GAM was used as a secondary antibody to stain α-tubulin in fixed and permeabilized BPAE cells. In these experiments, the mouse anti-α-tubulin antibody was applied, followed by either desthiobiotinylated or biotinylated GAM. α-Tubulin was then visualized after incubating with Alexa Fluor 488 streptavidin. The fluorescent streptavidin (Figs. 6A and B) labeled both secondary antibodies. However, when the desthiobiotinylated GAM-treated cells were washed with 10 mM biotin, the fluorescent tubulin staining almost completely disappeared, leaving only the DAPI-stained nuclei (Fig. 6D). Washing with biotin had no effect on cells treated with biotinylated GAM (Fig. 6C).

After streptavidin-containing conjugates were eluted from desthiobiotin–agarose with biotin, the streptavidin component was fully and essentially permanently occupied by biotin. As such, the conjugates were unusable for cell staining. We determined, however, that a streptavidin conjugate saturated with desthiobiotin would still bind to a cell-bound biotinylated target (Fig. 7). α-Tubulin in BPAE cells was labeled with anti-α-tubulin antibody, followed by biotinylated GAM, as described under Materials and methods. Alexa Fluor 488 streptavidin conjugate alone or saturated with desthiobiotin (10 μM) was then applied to the cells to localize the secondary antibody. Within 5 min after application of the conjugate alone, intense α-tubulin staining was observed (Fig. 7A). Fifty-five minutes later, the staining intensity had increased only slightly (Fig. 7B). In contrast, 5 min after application of the desthiobiotin-saturated conjugate, α-tubulin staining was weak (Fig. 7C), but increased markedly over the next 55 min. At this point, the staining intensity appeared indistinguishable from cells labeled with Alexa Fluor 488 streptavidin alone (Fig. 7D). These results demonstrate that, in a cell-staining experiment, protein-bound biotin can displace desthiobiotin from streptavidin.

Biotin-reversible antigen detection was also performed on model dot blots (Fig. 8). Only the blots treated with desthiobiotinylated GAM are shown. In these experiments, dots of a mouse anti-c-myc antibody (2.5, 5, and 10 ng) were first labeled with either desthiobiotinylated or biotinylated GAM. The dots were then localized with Alexa Fluor 488 streptavidin and imaged (Fig. 8, top). The filters were then washed extensively with 10 mM biotin to strip off the fluorescent streptavidin and imaged again (Fig. 8, middle). Although stripping of the fluorescent streptavidin was incomplete at 10 ng/spot, it reached totality at 2.5 ng/spot. In contrast, the signal obtained with biotinylated GAM as the primary antibody was undiminished by the biotin washing step (data not shown). A second round of staining was then performed with streptavidin–alkaline phosphatase and DDAO phosphate to yield a permanent record of antigen localization (Fig. 8, bottom). In these experiments, it was not necessary to reapply the desthiobiotinylated GAM antibody since the biotin wash step removed only the fluorescent streptavidin conjugate.

We investigated whether anti-biotin antibodies recognized protein-linked desthiobiotin. To demonstrate this, increasing amounts of desthiobiotinylated and biotinylated protein (GAM) were bound to microplate wells and incubated with either a mouse monoclonal or a goat polyclonal anti-biotin antibody. The bound monoclonal and polyclonal antibodies were then detected with Alexa Fluor 488 GAM antibody and Alexa Fluor 488 donkey anti-goat IgG antibody, respectively. The results show that both antibodies recognized protein-linked biotin and desthiobiotin. Only the data

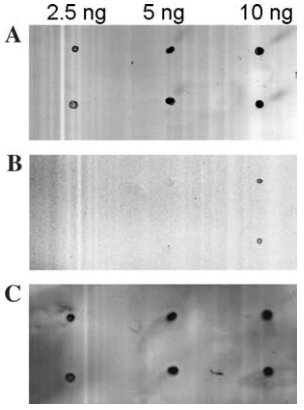

Fig. 8. Biotin-reversible antigen detection on dot blots with desthiobiotin-X biotin. Duplicate aliquots containing 2.5, 5, or 10 ng of a mouse anti-c-myc antibody were applied to nitrocellulose filter strips. After blocking, the antigen dots were detected by incubating the blots with desthiobiotinylated GAM IgG and Alexa Fluor 488 streptavidin conjugate (top). The strips were washed for 2 h with multiple changes of 50 mM biotin and imaged again (middle). After reblocking, the antigen dots were stained with streptavidin–alkaline phosphatase and DDAO phosphate, as described under Materials and methods, and imaged again (bottom). Equivalent dot blots stained with biotinylated GAM were carried through the entire procedure but are not shown here (see Results).

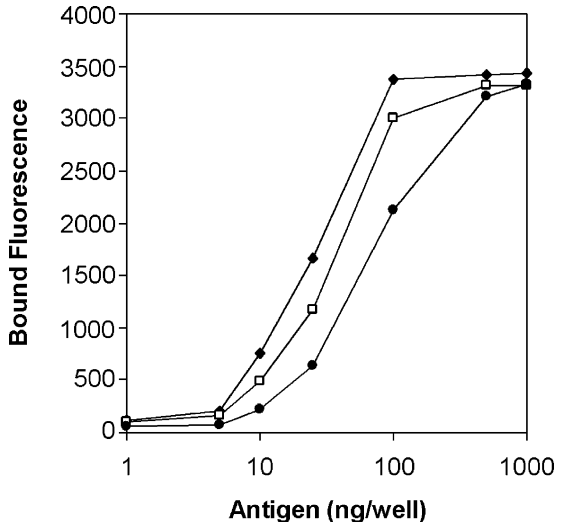

Fig. 9. Binding of an anti-biotin monoclonal antibody to biotinylated and desthiobiotinylated protein. Increasing amounts of biotinylated and desthiobiotinylated GAM IgG were immobilized in wells in 96-well plates and incubated with the anti-biotin antibody. The amount of anti-biotin antibody bound to the immobilized antigens was then detected with Alexa Fluor 488 GAM antibody (bound fluorescence). Antibody recognition of a biotinylated IgG with a DOS of 4 (closed diamonds). Antibody binding to a biotinylated IgG with a DOS of 3 (open squares). Antibody recognition of a desthiobiotinylated IgG with a DOS of 6 (closed circles).

obtained with the monoclonal antibody is shown in Fig. 9. Approximately 10 times more desthiobiotinylated than biotinylated target was required for equivalent degrees of recognition. A similar, but smaller difference in affinity for biotin and desthiobiotin was observed with the polyclonal antibody (data not shown). Fig. 9 also shows that this effect was unrelated to the relative biotin/desthiobiotin DOS values, since two biotinylated targets with lower DOS (4 and 3) were recognized better than the desthiobiotinylated one with a DOS of 6. Moreover, the apparent lower affinity of the anti-biotin antibodies for desthiobiotin was unrelated to the length of the spacer (7 or 14 atoms) between the desthiobiotin and the GAM antibody (data not shown).

Although biotin did not displace the monoclonal anti-biotin antibody from protein-linked desthiobiotin or biotin, free biotin and desthiobiotin partially displaced the polyclonal antibody from these targets. Fifty millimolar biotin decreased binding of the polyclonal antibody to desthiobiotinylated and biotinylated GAM (5 ng/well) by 42 and 24%, respectively. However, 50 mM desthiobiotin decreased binding to the two antigens by only 27 and 15%, respectively.

Discussion

Biotin binds so tightly to avidin and streptavidin that the interaction is essentially irreversible. In fact, Green [5] estimated a $t_{1/2}$ of $\sim$200 days for dissociation of biotin from avidin and determined binding constants in the fM range. Decades of basic research have yielded the details of biotin BbP interactions at atomic levels of resolution [12,36,37] and many practical applications of this knowledge have been developed [1–4]. However, irreversible binding is a handicap when the goal is to recover and use a biotinylated or BbP-containing target. As discussed in the introduction, multiple approaches to achieving reversible biotin/BbP interactions have been developed but most have drawbacks that limit their general applicability. The goal of our research was to develop an easily reversible biotin/BbP-based technology that would free researchers from the limitations imposed by the existing techniques. Our work suggests that desthiobiotin provides a means to achieve this goal. We have developed versatile desthiobiotin-X biotin-based reagents and techniques that may enable investigators to take fuller advantage of the biotin/BbP system.

Reversible desthiobiotin binding was demonstrated in a variety of assay formats. It was quite interesting to find that, in contrast to biotin, desthiobiotin binding increased the tryptophan fluorescence of streptavidin. This result enabled us to study biotin displacement of desthiobiotin from the protein. In this case, desthiobiotin dissociated biphasically. These results are similar to those published previously for biotin dissociation from avidin and streptavidin [38]. When the steady state was reached, the quenching of streptavidin fluorescence characteristic of biotin appeared (see Results and [31–34]). In contrast, when biotin was bound to streptavidin, desthiobiotin did not displace it. These findings are consistent with data showing that the affinity of streptavidin and avidin for desthiobiotin is lower than that for biotin. It appears that there is no agreement in the literature about how much lower this affinity is, although the consensus is at least several orders of magnitude [39–46]. In practical terms, however, the affinity of streptavidin for desthiobiotin is sufficiently low that biotin easily reverses desthiobiotin binding. This is the key feature of the approach described in this paper.

We examined reversible desthiobiotin binding in a variety of contexts. After proteins were desthiobiotinylated, they could be recovered using streptavidin–agarose and CaptAvidin–agarose affinity columns after elution with biotin or desthiobiotin and basic pH, respectively. Bioconjugates containing streptavidin could also be recovered after binding to desthiobiotin–agarose by elution with biotin or desthiobiotin. Since avidin and NeutrAvidin BbP also bound reversibly to desthiobiotin–agarose, it is probable that conjugates containing these proteins could be isolated with this matrix.

Hofmann et al. [17] reported that repeated applications of biotin led to higher, but not total, recovery of bound ligands from succinyl–avidin–agarose. We also observed this phenomenon with desthiobiotin–agarose.

In both studies, stripping the column at acid (present results) or basic pH recovered the very tightly bound protein fraction [17]. Since our streptavidin fluorescence results suggest that there are some desthiobiotin molecules bound very tightly to the protein, this may explain why total recovery of streptavidin-containing ligands from desthiobiotin–agarose with one or more aliquots of biotin or desthiobiotin is problematic. However, the fact that biotin has higher affinity for BbP than desthiobiotin may explain why eluting is more efficient with biotin than with desthiobiotin. In practice, since streptavidin conjugates saturated with desthiobiotin still bind to a biotinylated target, it is advantageous to elute conjugates from desthiobiotin–agarose with desthiobiotin rather than biotin.

The present results indicate that antigen detection in cells and on dot blots with desthiobiotinylated reagents can be reversed by washing with biotin under gentle conditions. This suggests that different detection strategies can be employed with the same target(s). For example, one can first detect an antigen with a fluorescent streptavidin probe, strip it off with biotin, and detect the antigen again with an enzyme-linked streptavidin probe that yields a permanent record of the results. Electron microscopic imaging is also possible in this case.

Although detection of the same antigen with two different probes is sometimes the goal, our results suggest that several different antigens can be localized in cells or on blots with desthiobiotinylated reagents. For example, it is often necessary to reprobe the same Western blot with different antibodies and detection systems. Unlike biotin-based reagents [47,48], using desthiobiotin-based reagents for one or more rounds of detection permits researchers to gently remove probes without damaging the target proteins on the blot.

Since two different anti-biotin antibodies bound to desthiobiotin, this suggests that researchers can detect desthiobiotinylated targets with these reagents in many experimental formats [49–51]. Affinity matrices containing anti-biotin antibodies might also be used to isolate desthiobiotinylated and biotinylated targets [3]. Although high concentrations of biotin (50 mM) did not reverse the binding of a monoclonal anti-biotin antibody to a desthiobiotinylated or biotinylated target, binding of a polyclonal antibody was partially reversed by biotin and desthiobiotin. This suggests that lower-affinity anti-biotin antibodies might be appropriate for affinity chromatography when displacement of targets by biotin or desthiobiotin is desired.

Taken together, our results suggest that desthiobiotin-based reagents provide some distinct advantages over other commercially available biotin/BbP-based techniques. Moreover, many other applications of these versatile reagents can be envisioned and are under study in our laboratories.

Acknowledgments

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