A Chemical Approach To Illustrate the Principle of Signal Transduction Cascades Using the Avidin−Biotin System

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Received August 7, 1998

Abstract: A new approach to illustrate the principle of signal transduction and to assemble protein multilayers is described. It is based on competing affinities of two different ligands for the same binding site of a protein. A low-affinity ligand can be attached *covalently* to the protein, where it will be buried in the binding site and thus be prevented to interact with other proteins that recognize it. However, if a high-affinity ligand (or a molecule containing this ligand) is added, it will displace the low-affinity ligand (which still remains covalently bound) from the binding site to the periphery. The low-affinity ligand is now available for interaction with other molecules, thus providing the means through which to assemble multilayers of proteins by a "recognition cascade". This principle was demonstrated using the protein avidin which binds two ligands, biotin and 4-hydroxyazobenzene-2-carboxylic acid (HABA), with markedly different affinities. Avidin was affinity labeled with HABA, the low-affinity ligand, to produce a red, covalently conjugated avidin−HABA derivative (red avidin). Anti-HABA antibodies failed to recognize HABA buried in the binding site of avidin. However, upon addition of the high-affinity ligand biotin, HABA was expelled from the binding site and immediately bound by the antibodies. Multilayer assemblies of HABAylated avidin and biotinylated anti-HABA antibodies could thus be constructed. This concept may find application in numerous fields, such as medicine, diagnostics, nanotechnology, and artificial intelligence.

Introduction

In nature, cellular functions are propagated by cascades of molecules, which interact with one another. Generally, one reaction or binding process is dependent on a previous step. In this way, a signal, for example, can be transferred from the outside of the cell en route to the nucleus—a process mediated by ligands, e.g., hormones, cytokines, and growth factors. The cascade starts with the interaction of the ligand with a receptor, which, upon binding, is either phosphorylated or undergoes a conformational change.[1,2] Such modifications serve to increase the affinity of the receptor for the subsequent ligand in the cascade.

We wanted to determine whether such a cascade can be formed artificially, whereby the binding of one molecule would depend on the "signaling" of another. Such a system could be achieved using two molecules, which display differing affinities for the same binding site of a protein. If the low-affinity ligand is coupled covalently to the binding site, the high-affinity ligand can be used to displace it from the binding pocket. The expelled moiety—still covalently attached to the protein—is now available for further interaction with other molecules that can bind to this low-affinity ligand. This process serves as a signal, which can promote a consecutive series of subsequent reactions. Consequently, one step will be dependent on a previous one, thus enabling us to trigger a cascade of binding, e.g., to construct an organized system of protein multilayers.

To demonstrate this principle, we used the egg-white protein avidin, deglycosylated avidin,[3] and streptavidin which bind to two different ligands, biotin and 4-hydroxyazobenzene-2-carboxylic acid (HABA), with different affinities, 10^{-15} and 10^{-6} M, respectively.[4] In the present study, HABA was coupled covalently to the binding site of avidin using an appropriate spacer arm. The introduction of biotin or biotin-containing molecules served to expel the HABA moiety from the binding site, thereby rendering it available for further interaction with other binding molecules, e.g., unmodified avidin, anti-HABA antibodies, or biotinylated anti-HABA antibodies (Scheme 1). This self-contained system can be used to generate growing cascades of interactions and layers.

Results and Discussion

As revealed by the 3D structure of the complex between avidin and the ligand HABA,[5] the azo dye binds to the protein as the hydrazoquinone tautomer. Binding of HABA to avidin is accompanied by an instantaneous shift in the absorption spectrum from λ_{max} 348 nm of the free HABA to λ_{max} 500 nm of the complexed tautomer. The X-ray structure also shows that the anthranilic acid portion of the HABA molecule is crucial for binding to the biotin-binding pocket. It has also been demonstrated that the phenolic part of HABA can be manipulated extensively, by attaching a variety of functional groups, without significantly affecting the binding.[6] Furthermore, avidin can be forced to catalyze the hydrolysis of HABA derivatives, in which the hydroxyl function is blocked with a protecting

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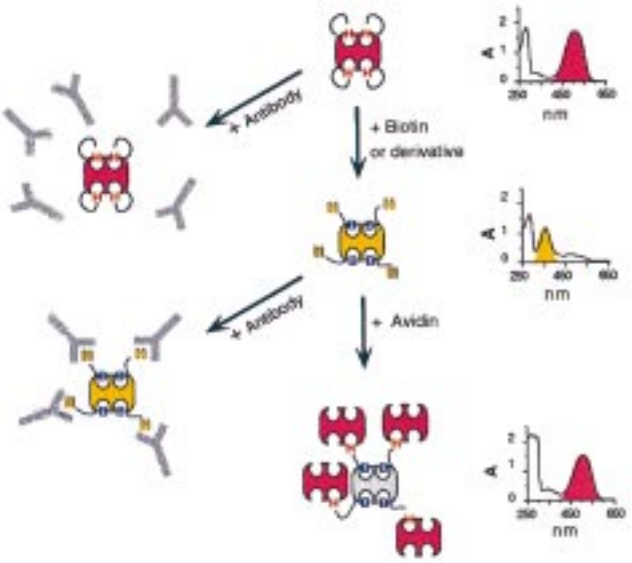
10.1021/ja982817m CCC: $15.00
Published on Web 11/25/1998

Scheme 1. Schematic Depiction of the Signaling System[a]



[a] Affinity-labeled red avidin (red symbols), $\lambda_{max} = 504$ nm (see spectrum), does not interact with the anti-HABA antibody (gray Y-shaped symbols). The cascade is triggered upon addition of biotin (blue B) or biotin-containing molecules, which expel the covalently attached HABA moiety (yellow H) from the binding site. The spectrum shows a shift to orange, $\lambda_{max} = 356$ nm. The HABA group is now available for subsequent interaction with anti-HABA antibody or avidin. Reaction with avidin restores the red color.

group (e.g., an acetyl moiety), to accommodate the azo dye in its tautomeric form at the binding site.[6]

For the present work, an HABA-containing affinity label was designed, such that the dye would remain covalently attached to the binding site of avidin. The ortho position of the HABA phenol was thus modified with a reactive functional group, which, due to steric constraints, forms an intramolecular cyclic carbamate (Figure 1). This cyclic HABA can be hydrolyzed by avidin, which enabled us to exploit the principle of forced catalytic hydrolysis[6] to attach the HABA moiety to an appropriate residue in or near the binding site of avidin. The cyclization of the reagent was necessary, since the activated linear *N*-hydroxysuccinimidocarbamate (B) would react with any amino group, whereas the cyclic compound (C) reacts in situ, i.e., selectively, after occupying the binding site of avidin.

Upon addition of the HABA reagent to avidin, an immediate shift in the spectrum to 504 nm was observed only with the linear, nonactivated HABA analogue.[7] A similar change was also observed with the cyclic HABA derivative, but in this case, the shift developed gradually. The latter reaction was allowed to proceed until no further increase at λ_{max} was observed. No shift was observed if the cyclic reagent was added together with biotin (Figure 2), indicating that the vitamin occupied the binding site and prevented the reaction with HABA. To verify this assumption, the reaction mixtures were passed through a Sephadex G-25 column or dialyzed. In mixtures that contained either the linear precursor or the cyclic reagent in the presence of biotin, it was possible to dissociate the orange-colored HABA from the colorless avidin. On the other hand, after reaction with avidin, the cyclic HABA reagent could no longer be separated from the protein; the red color remained associated with the protein, indicating covalent attachment to avidin.

Spectral analysis revealed that four HABA molecules were bound covalently to each molecule of avidin—one per subunit. The HABA moiety appeared to be coupled only to the biotin-binding site of avidin, as indicated by the red color. To verify this contention, biotin was added to the red avidin and an immediate shift to 356 nm was observed (see spectra in Scheme 1), indicating that biotin, due to its higher affinity for avidin, was capable of displacing the HABA moiety from the binding site. Upon dialysis or gel filtration on Sephadex G-25, the orange color continued to be associated with the protein. To further prove that HABA is bound covalently to avidin, deglycosylated avidin[3] was modified in the same way and analyzed by mass spectroscopy. MALDI analysis of the unlabeled protein showed a *m*/*z* value of 14 287−14 290, corresponding to that for a single subunit of deglycosylated avidin. The reaction product showed a single peak (*m*/*z* 14 727−14 734); the difference in mass (about 440 units) is consistent with the molecular weight of the HABA derivative (Figure 3). The HABA must be bound covalently since biotin-blocked avidin showed a *m*/*z* of 14 290 similar to that of the native avidin.

To determine which residue was modified, the protein was subjected to trypsin digestion. The hydrolysate was separated by HPLC (Figure 4), and the sequence of the orange peptide was analyzed. The analysis showed that Lys-111 was modified (Table 1). Interestingly, a neighboring residue, Trp-110, is an important part of the binding pocket,[8] which again indicates that the HABA moiety is located in the binding site (Figure 5).

To show that the HABA moiety is indeed buried in the binding site and not available for further interaction before its displacement by biotin, we used a second HABA-binding molecule for its detection. For this purpose, polyclonal antibodies against HABA were elicited in rabbits. After affinity purification, the antibodies were examined for their interaction with the HABAylated avidin. As seen in Figure 6, in the absence of biotin, the anti-HABA antibody fails to recognize the HABA, buried in the binding site. Upon addition of biotin, however, the HABA moiety was expelled and strong binding of the antibody was detected.

To demonstrate that this system meets the requirements of a signal transduction cascade and the assemblage of protein multilayers,[9] biotin-saturated HABAylated avidin was incubated with biotinylated anti-HABA antibodies, followed by additional cycles of HABAylated avidin and biotinylated antibodies. A stepwise increase in absorbance could be detected after the formation of each layer (Figure 7). The cascade could be initiated using HABAylated avidin and biotin or biotinylated macromolecules. In either case, the addition of biotin was crucial to expel the HABA from the binding site, thus enabling subsequent interaction with the anti-HABA antibodies.

In summary, we have demonstrated, by chemical means the interplay of molecular recognition and oriented protein assembly which serves as a prerequisite for the principle of signal transduction. Using the avidin−biotin system, biotin served as the effector or trigger of the cascade. Since avidin has four binding sites, if limited amounts of biotin are added to displace only one or two HABA molecules, the cascade can be triggered vectorially in different dimensions. Therefore, this system can be considered a chemical mimic of signal transduction, which,

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(7) The same effect was observed for deglycosylated avidin, Neutralite avidin, and streptavidin.

(8) Trp-110 is a part of the binding site but comes from the neighboring subunit: Livnah, O.; Bayer, E. A.; Wilchek, M.; Sussman, J. L. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 5076−5080.

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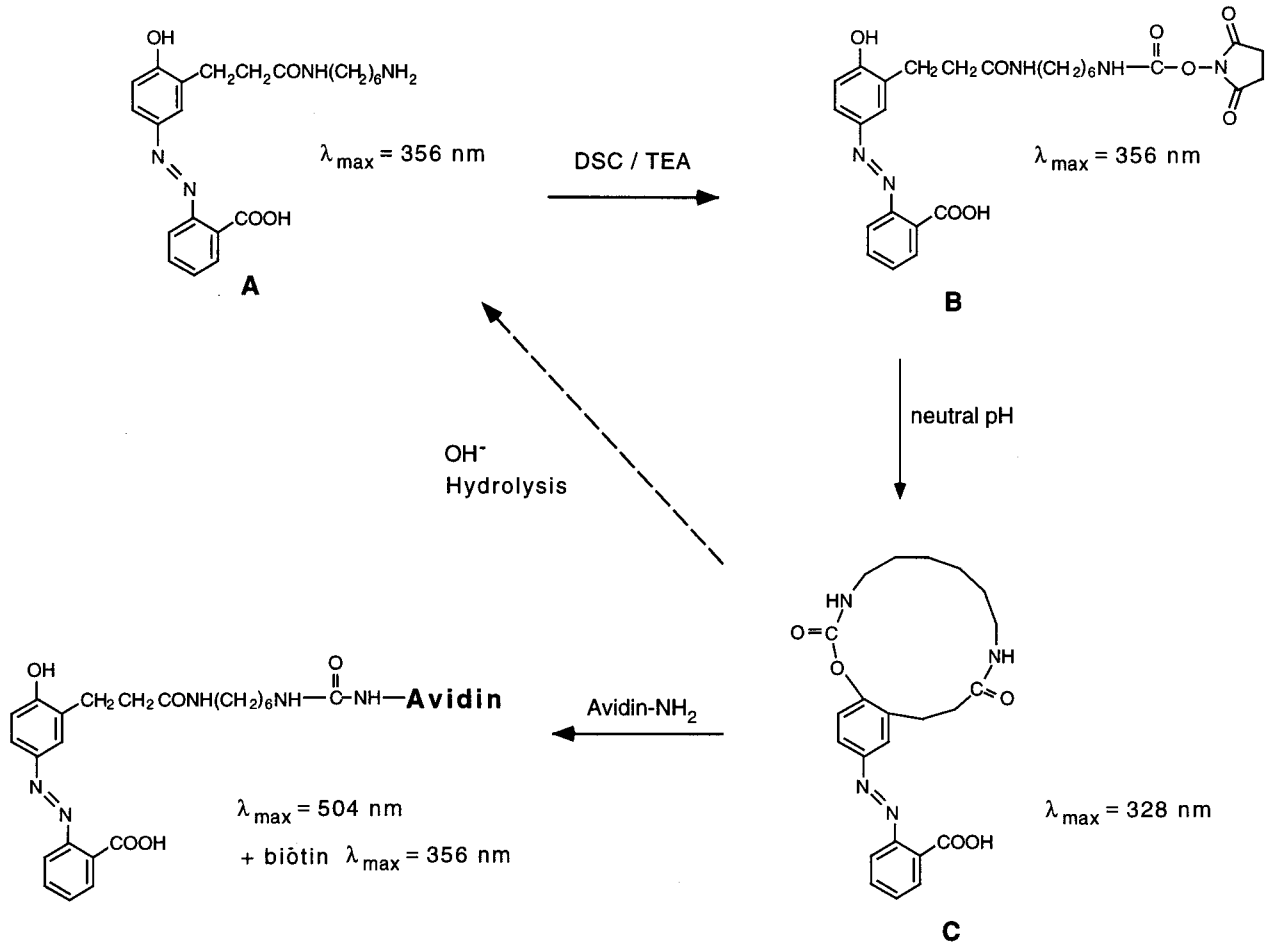

Figure 1. Synthesis and reaction of the cyclic HABA reagent. HABA, carrying an appropriate spacer arm (A), was activated to the corresponding linear *N*-hydroxysuccinimidocarbamate (B) using DSC. In neutral aqueous solution, compound B cyclizes and is transformed into the final cyclic labeling reagent (C), which is used for reaction with avidin. Basic conditions lead to the hydrolysis of the cyclic HABA, which reverts to the precursor (A).

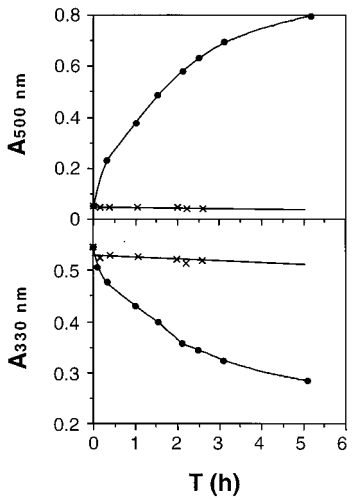

Figure 2. Reaction of avidin with the cyclic HABA derivative (C) in the presence (×) or absence (●) of biotin. Avidin was incubated with an equimolar (per subunit) concentration of the cyclic HABA in aqueous buffer at pH 8, and the changes in absorbance at 500 and 330 nm were followed spectrophotometrically.

in nature, can also be modulated in different ways to enhance the response, following the initial triggering by a hormone or another effector. The concept of chemical mimicry and the assembly of protein multilayers as demonstrated in this communication is appropriate for application in numerous fields, such as medicine, diagnostics, biosensors, nanotechnology, and artificial intelligence, thus expanding the scope of the avidin−biotin system.[10]

Experimental Section

Materials and Methods. Triethylamine (TEA) and *N*,*N*′-dicyclohexylcarbodiimide (DCC) were obtained from Merck (Darmstadt, Germany), *N*-BOC-1,6-diaminohexane was obtained from Fluka (Buchs, Switzerland), disuccinimidyl carbonate (DSC) was purchased from Calbiochem (La Jolla, CA), and 3(2-hydroxyphenyl)propionic acid and anhydrous hydrochloric acid solution in dioxane were purchased from Aldrich (Milwaukee WI). Avidin was provided by STC laboratories (Winnipeg, Manitoba, Canada), deglycosylated avidin (DG-avidin) by Belovo (Bastogne, Belgium), and streptavidin by Boehringer Mannheim (Mannheim, Germany). *N*-Hydroxysuccinimide, biotin, HABA, BSA, TPCK-treated trypsin, keyhole lympet hemocyanin (KLH) and all the other fine chemicals were obtained from Sigma (St. Louis, MO). Sepharose CL-4B and Sephadex G25 were purchased from Pharmacia Biotech AB (Uppsala, Sweden). Peroxidase-conjugated anti-rabbit IgGs were obtained from Jackson ImmunoResearch laboratories (West Grove, PA).

UV spectra were recorded with a Milton Roy spectronic UV−vis spectrophotometer, mod. 1201; HPLC analysis was carried out on a Vidac Protein and Peptides C_{18} column, using a Waters pumping system 600E, a Knaur variable-wavelength detector, and a Hewlett-Packard model 3390A integrator.

Mass spectra were measured on a VG-Platform II equipped with an electrospray ion source, a Gilson 215 autosampler, and a JASCO PU-

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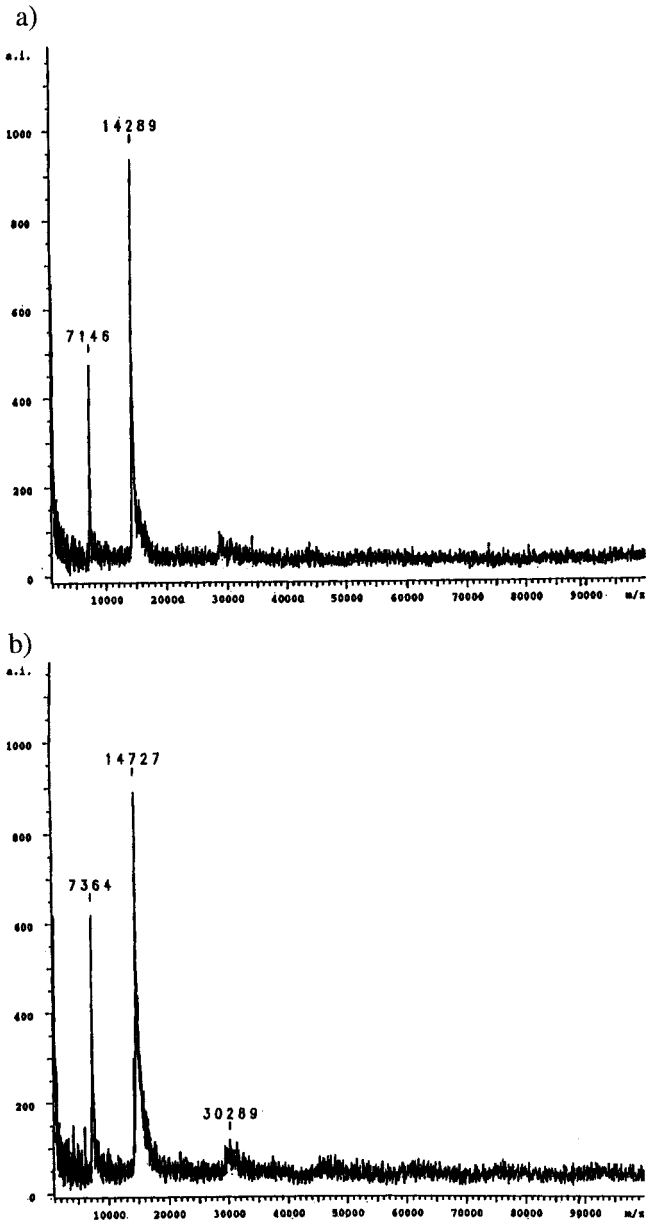

Figure 3. MALDI MS of DG-avidin (a) before and (b) after reaction with cyclic HABA. DG-avidin shows a *m*/*z* value of 14 287−14 290, corresponding to a single subunit of the tetrameric protein. The reaction product showed a single peak at *m*/*z* 14 727−14 734 with an increase in mass (about 440 units) that is consistent with the molecular weight of the HABA derivative.

980 HPLC pump. Electrospray ionization was done in both the positive (M + 1) and the negative (M − 1) modes, and the exact molecular weight is given here. Samples were dissolved in water:acetonitrile (1:1).

HABA-$(CH_2)_2$CONH$(CH_2)_6NH_2$ (A). 3′-(6-Aminohexylaminocarbonylethyl)-4′-hydroxyazobenzene-2-carboxylic Acid. 3-(2-Hydroxyphenyl)propionic acid (**1**) (0.997 g, 6 mmol) was dissolved in CH_2Cl_2 (21 mL), and *N*-hydroxysuccinimide (0.828 g, 7.2 mmol) and DCC (1.485 g, 7.2 mmol) were added to the solution cooled in an ice bath. After 3.5 h, the solution was filtered to remove the dicyclohexylurea, and N^1-BOC-1,6-diaminohexane (1.52 g, 6 mmol) was added, while stirring, to the dichloromethane solution followed by 835 μL (6 mmol) of TEA. The reaction was stirred overnight at room temperature and evaporated to dryness. The residue was redissolved in ethyl acetate and washed sequentially with diluted $NaHCO_3$, diluted citric acid, and water. The product was then dried over Na_2SO_4 and evaporated to dryness. Diethyl ether (30 mL) was added to the resulting oil, and the precipitated impurities were removed by filtration. The product was isolated after solvent evaporation: yield 1.92 g (90%); R_f = 0.55 ($CHCl_3$:MeOH, 9:1).

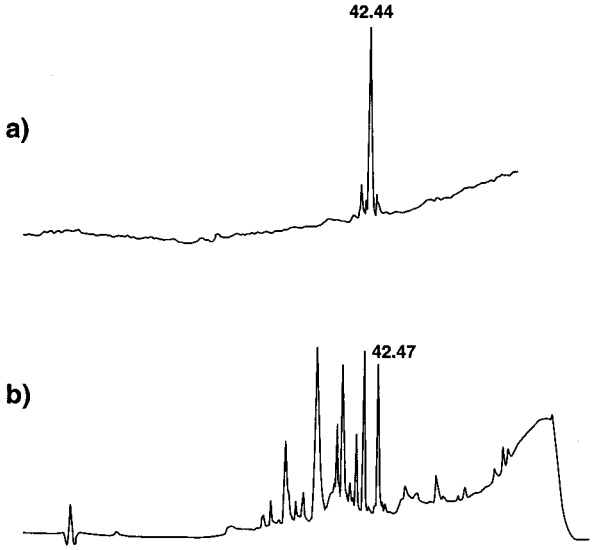

Figure 4. HPLC analysis of the tryptic digest of HABAylated avidin on a Vidac RP-18 column. Detection was carried out at (a) 356 nm and (b) 220 nm, corresponding to the adsorption maxima of HABA and the peptides.

Table 1. Amino Acid Sequence of the HABAylated Peptide Obtained after Proteolytic Digestion of the HABAylated Avidin and HPLC Purification[a]

	cycle no.																
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
amino acids	S	S	V	N	D	I	G	D	D	?	?	A	T	R	V	G	−
amino acids 101−117	S	S	V	N	D	I	G	D	D	W	K	A	T	R	V	G	V

[a] The sequence was determined using automatic Edman degradation, starting with 100 pmoles of peptide. Trp could not be identified probably because of oxidation during the process.

Anthranilic acid (0.750 g, 5.45 mmol) and $NaNO_2$ (0.377 g, 5.45 mmol) were dissolved in water (15 mL) and cooled using an ice bath, and 1.5 mL of concentrated HCl was added. After 10 min, the solution was slowly added dropwise to a solution of the product (1.62 g, 5.45 mmol in 15 mL) dissolved in methanol:0.5 M KOH (1:1). The pH was controlled and adjusted to 8 using HCl and KOH.

After 20 min, methanol was removed by evaporation, the solution was acidified to pH 3−4 with diluted citric acid, and the solid product was extracted with ethyl acetate. The organic solution was washed with water, dried over Na_2SO_4, and evaporated to dryness.

The product was then dissolved in dry dioxane, and HCl-saturated dioxane was added. After 1 h, the product, precipitated as the hydrochloride salt, was isolated by filtration, washed with diethyl ether, and dried in a desiccator: yield 1.48 g (60%); R_f = 0.13 ($CHCl_3$:MeOH, 85:15); hygroscopic product, softens at about 120 °C, decomposes at about 200 °C; MS *m*/*z* 412 (calcd mass 412 g/mol); $\epsilon_{356\ nm}$ = 13 000 M^{-1} cm^{-1}.

HABA-$(CH_2)_2$CONH$(CH_2)_6$NHCOOSu (B). 3′-[6-(Succinimidyloxycarbonylamino)hexylaminocarbonylethyl)]-4′-hydroxy-azobenzene-2-carboxylic Acid. DSC (410 mg, 1.6 mmol) was dissolved in CH_3CN (16 mL). Compound A (358 mg, 0.8 mmol) was dissolved in DMF (2.4 mL) and slowly added (8 × 300 μL) to the DSC solution while stirring. After each addition, 2 equiv (1.6 mmol) of TEA was added. Five minutes after the last addition of the HABA derivative, 20 mL of 1 N HCl was added. The product, crystallized as a fine powder, was isolated by filtration, washed with diluted HCl, and then dried in a desiccator: yield 402 mg (90%); R_f = 0.63 ($CHCl_3$:MeOH, 85:15); mp 143−145 °C; MS *m*/*z* 553 (calcd mass 553 g/mol); $\epsilon_{356\ nm}$ = 13 000 M^{-1} cm^{-1}.

Cyclic HABA (C). A concentrated DMF solution of B (10 mg/mL) was diluted with 2−5 volumes of PBS, and the pH was adjusted

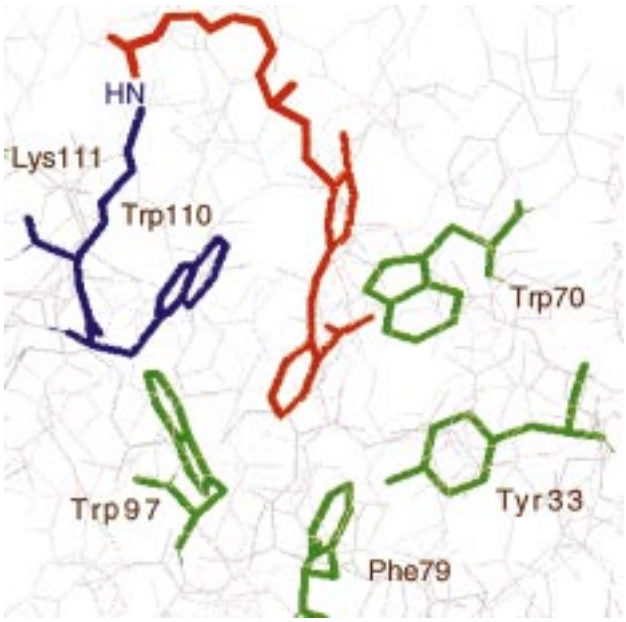

Figure 5. Schematic description of the affinity-labeled binding site of avidin. The figure is based on the known 3D structure of the avidin−HABA complex (5); the extended side chain of HABA was added manually. Trps-97 and -70, Phe-79, and Tyr-33 are from one monomer (shown in green); Lys-111 and Trp-110 (shown in blue) are from the adjacent symmetry-related monomer; the HABA derivative, which binds to the ϵ-amino group of Lys-111, is shown in red.

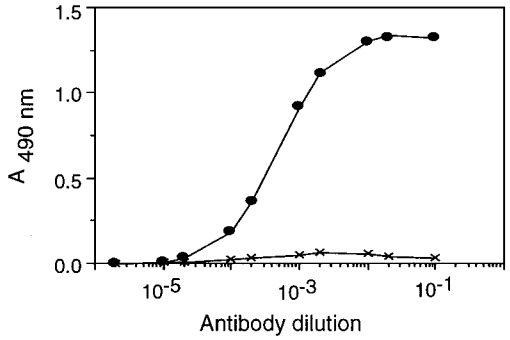

Figure 6. ELISA assay using the affinity-purified anti-HABA antibody. Plates were coated with affinity-labeled avidin, the desired dilutions of antibodies were applied with (●) or without (×) biotin, and the plates were assayed using a secondary antibody−enzyme conjugate.

to 8 with small additions of 4% $NaHCO_3$. Transformation of the active carbamate into the cyclic HABA derivative was verified by TLC and UV spectrophotometry (shift from 356 to 328 nm). Under the conditions used, total conversion was obtained after 24 h at room temperature. The solution was acidified, extracted with ethyl acetate, evaporated to dryness, and crystallized from ethyl acetate: $R_f = 0.71$ ($CHCl_3$:MeOH, 85:15); mp 176−178 °C; MS *m*/*z* 438 (calcd mass 438 g/mol); $\epsilon_{328\,nm} = 12\,000\ M^{-1}\ cm^{-1}$.

Affinity Labeling of Avidin, DG-Avidin, and Streptavidin. Compound C was added to an avidin solution (native, DG-avidin, or streptavidin) in PBS. The reaction was monitored by UV at 500 and 330 nm. The combined increase of the absorption at 500 nm and the decrease at 330 nm, or the ratio between them, were used as parameters to follow the reaction. When no further change was observed, the modified protein was purified from excess reagent by gel filtration on a Sephadex G25 column, using PBS as the eluant. Several reactions were carried out, varying the protein concentration (0.8−3 mg/mL) and the ratio between the reagent and the protein of between 1 and 10 cyclic HABAs per monomer. Biotin inhibition of the affinity labeling was carried out under the same conditions. The molecular absorbance coefficient ($\epsilon_{504\,nm} = 35\,000\ M^{-1}\ cm^{-1}$) was established for the

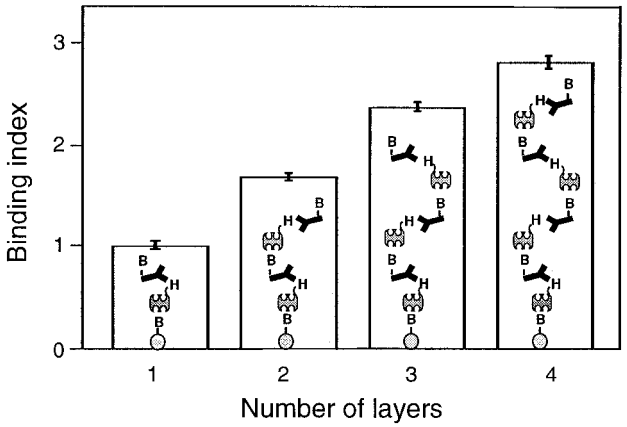

Figure 7. Artificial cascade: formation of layers generated by consecutive addition of HABAylated avidin and biotinylated antibodies. ELISA plates were coated with biotinylated-BSA (○-B), to which HABAylated avidin (other symbols see Figure 1) and biotinylated anti-HABA antibody were added. The cycle was continued several times. Formation of the layers was detected using a secondary antibody−enzyme conjugate. The signal of the first layer was set as binding index = 1.

covalently HABAylated avidin, which was similar to that of the noncovalently labeled avidin using a large excess of HABA.

Characterization of Labeled Avidin. Matrix-Assisted Light Desorption Ionization (MALDI). Precise determination of the number of HABA molecules per avidin subunit was assessed by MALDI-TOF spectrometry on DG-avidin in the native and the affinity-labeled form and either with or without preincubation with biotin. The samples were provided for the analysis in a salt-free lyophilized form, and they were immobilized on a sinapinic acid matrix for laser-induced ionization.

Tryptic Digestion. HABAylated avidin is stable against proteolytic digestion. Consequently, HABAylated DG-avidin in PBS solution (1.7 mg/2.125 mL, 100 nmol) was boiled for 2 h prior to protease treatment. The denaturation could be followed by the color change in the solution from red to yellow. The denatured protein became insoluble in PBS and was isolated by ultracentrifugation. TPCK-treated trypsin (60 μg), dissolved in 370 μL of $NaHCO_3$ (0.2 M, pH 8), was then added to the insoluble protein, and the mixture was incubated at 37 °C with gentle stirring. A molar ratio of 20 avidin versus trypsin was used. After 24 h, the colored solution was separated from the insoluble protein by ultracentrifugation and the solution was used for HPLC analysis and purification. Concentration was determined by UV spectrophotometry ($\epsilon_{356\,nm} = 13\,000$ for compound A).

Isolation of the Affinity-HABAylated Peptide. The solution obtained from proteolytic digestion was isolated by RP-HPLC using a Vidac Protein and Peptide C_{18} column, with H_2O/0.05% TFA and CH_3CN/0.05% TFA as eluants and a flow rate of 0.5 mL/min. After injection, the column was washed for 5 min with H_2O/TFA (0.05%). Then, a linear gradient from 0 to 60% CH_3CN/TFA (0.05%) over a 35-min period was applied, followed by a washing step with increasing amounts of CH_3CN up to 90%. Peak identification was carried out by UV detection either at 220 or 356 nm. The purification was carried out in several steps by loading 10 μL of the digest each time. This amount was calculated to correspond to approximately 550 pmol of HABAylated peptide. The peak showing absorbance at both 356 and 220 nm was collected and analyzed for the amino acid sequence.

Amino Acid Sequence of the Affinity-HABAylated Peptide. Sequence analysis was carried out by repetitive Edman degradation using a Procise 491 instrument from Perkin-Elmer/Applied Biosystem Division and the manufacturer's recommended chemistry and software.

Other HABAylated Proteins. HABAylated proteins (KLH and BSA) were prepared using the succinimidyl carbamate reagent B. The HABA reagent (2.5 mg), dissolved in DMF, was added to the protein solutions (2−10 mg/mL) in 0.1 M $NaHCO_3$ and allowed to react for 2 h at room temperature. Products were purified by size exclusion chromatography, and the degree of substitution was estimated by UV spectroscopy ($\epsilon_{356\,nm}$ of 13 000 $M^{-1}\ cm^{-1}$ for HABA).

Anti-HABA Antibodies. A KLH-HABA (0.5 mg) conjugate carrying ~50 molecules of HABA/protein was used for immunization. The protein was dissolved in Freund's adjuvant and was injected subcutaneously into rabbits. The antibodies were purified using an affinity column, prepared by coupling tyramine to *N,N′*-disuccinimidyl carbonate-activated Sepharose,[11] followed by diazotization with anthranilic acid.

Interaction of Anti-HABA Antibodies with HABAylated Avidin. Red avidin was adsorbed to microtiter plates, and its interaction with the antibodies was checked in the absence and the presence of biotin. Unoccupied binding sites were blocked using 0.1% gelatin. Biotin (10 μM) was added to displace the HABA moiety from the binding site. The HABA-specific antibody (0.4 μg/mL) was added and incubated for 2 h. Bound antibody was detected using peroxidase-conjugated goat anti-rabbit antibody.

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Construction of Multilayers. Biotinylated BSA was adsorbed to 96-well microtiter plates overnight. Unoccupied binding sites were blocked using 0.1% gelatin. For the building of the protein multilayers, HABAylated avidin (1 μg/mL) was added, followed by biotinylated anti-HABA antibodies (10 μg/mL, containing about 15 molecules of biotin/antibody). Each layer of anti-HABA antibodies was detected using peroxidase-conjugated goat anti-rabbit antibody.

Acknowledgment. This work was supported by the Israel Science Foundation, funded by the Israel Academy of Science and Humanity, and by MINERVA, Deutsch-Israelische Wissenschaftliche Zusammenarbeit, Germany. Mass spectral analyses were kindly carried out by Dr. E. H. Gur (Peptor, Rehovot, Israel) and MALDI analyses by Dr. Laura Orsatti (Mass spectrometry laboratories of the C.N.R, Padova, Italy). M.M. and H.H. contributed equally to this work.

JA982817M