Chicken Avidin Exhibits Pseudo-catalytic Properties
BIOCHEMICAL, STRUCTURAL, AND ELECTROSTATIC CONSEQUENCES*

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Avidin and its bacterial analogue streptavidin exhibit similarly high affinities toward the vitamin biotin. The extremely high affinity of these two proteins has been utilized as a powerful tool in many biotechnological applications. Although avidin and streptavidin have similar tertiary and quaternary structures, they differ in many of their properties. Here we show that avidin enhances the alkaline hydrolysis of biotinyl p-nitrophenyl ester, whereas streptavidin protects this reaction even under extreme alkaline conditions (pH > 12). Unlike normal enzymatic catalysis, the hydrolysis reaction proceeds as a single cycle with no turnover because of the extremely high affinity of the protein for one of the reaction products (i.e. free biotin). The three-dimensional crystal structures of avidin (2 Å) and streptavidin (2.4 Å) complexed with the amide analogue, biotinyl p-nitroanilide, as a model for the p-nitrophenyl ester, revealed structural insights into the factors that enhance or protect the hydrolysis reaction. The data demonstrate that several molecular features of avidin are responsible for the enhanced hydrolysis of biotinyl p-nitrophenyl ester. These include the nature of a decisive flexible loop, the presence of an obtrusive arginine 114, and a newly formed critical interaction between lysine 115 and the nitro group of the substrate. The open conformation of the loop serves to expose the substrate to the solvent, and the arginine shifts the p-nitroanilide moiety toward the interacting lysine, which increases the electron withdrawing characteristics and consequent electrophilicity of the carbonyl group of the substrate. Streptavidin lacked such molecular properties, and analogous interactions with the substrate were consequently absent. The information derived from these structures may provide insight into the action of artificial protein catalysts and the evolution of catalytic sites in general.

The avidin-biotin system has gained great importance over the years both as a means of studying the biorecognition phenomenon and as a tool for general application in the biological sciences. This powerful approach has facilitated the localization, identification, and assay of an almost unlimited number of biological molecules (1, 2). Historically, the development and extensive application of the avidin-biotin system was a definitive breakthrough in the area of nonradioactive labeling and detection of biologically active molecules.

Chicken egg white avidin and its bacterial analogue streptavidin share a similarly high affinity (K_a ~ 10^{15} M^{-1}) for the vitamin biotin (3). Despite the relatively modest sequence homology (~30% identity and 40% overall similarity), the two proteins share the same tertiary fold, similar tetrameric quaternary structures, and a nearly identical arrangement of amino acid residues within the respective binding pockets (4–7).

On the other hand, these two proteins also differ from one another in many of their molecular properties. For example, streptavidin is a neutral nonglycosylated protein, as opposed to avidin, which is glycosylated and positively charged (pI ~ 10.5). Perhaps because of the differences in their respective molecular surfaces, the two proteins lack immunochimical cross-reactivity. Moreover, when introduced in vivo, avidin and streptavidin exhibit different profiles of tissue uptake, organ distribution, and blood clearance properties (8). Despite the architectural similarity of the respective biotin-binding pockets, minor differences appear to be evident. For example, streptavidin binds peptides containing a His-Pro-Gln (9) sequence motif, whereas avidin fails to recognize such peptides. In contrast, avidin recognizes a His-Xxx-His motif (10, 11), which is not recognized by streptavidin. Moreover, whereas both proteins bind the dye 2-(4'-hydroxyazobenzene)benzoic acid (HABA), the binding constant (K_b), although moderate in both cases, is about 20-fold stronger in avidin (3).

In an early communication (1), we reported yet another puzzling difference between avidin and streptavidin in their capacity to catalyze the hydrolysis of certain biotinyl ester derivatives. Specifically, avidin was found to augment the hydrolysis of biotinyl p-nitrophenyl ester (BNP), whereas streptavidin was found to strongly protect the same biotin derivative from hydrolysis.

In the current study, we describe the interaction of avidin and streptavidin with a number of ester and amide derivatives of biotin. Based on the three-dimensional crystal structures of avidin and streptavidin that harbor a model biotin-containing nitrophenyl analogue in their binding sites, we propose a mechanism that accounts for the observed differences in catalytic
properties. This study may shed light on the role of proximity and orientation in generating new catalytic interactions among selected submolecular components. The information bears general relevance to how catalytic sites are formed.

MATERIALS AND METHODS

Hydrolysis of Biotinyl Derivatives—BNP, nor-BNP, and homo-BNP were synthesized according to Bayer et al. (12). Avidin or streptavidin (16 nmol of subunit) was diluted in 0.9 ml of weak phosphate buffer (H11021 0.1 mM, pH 4). BNP or other derivative (15 nmol), diluted in 5 ml of dimethylformamide, was added to the desired protein solution and incubated for 5 min at 23°C. The appropriate pH value was then attained by the addition of 0.1 ml of a suitable buffer (0.1–1.0 M). The hydrolysis was determined by following the release of p-nitrophenol (ε400 = 17,500 cm⁻¹ M⁻¹).

Autohydrolysis of BNP was determined in a similar manner, except the weak buffer solution contained no protein. Hydrolysis of biotinyl-N-hydroxysuccinimide ester (in 0.1 M ammonium carbonate, pH 8) was observed spectrophotometrically (ε400 = 10,000 cm⁻¹ M⁻¹). Hydrolysis of other biotinyl and non-biotinyl derivatives was determined by TLC, whereby a solution of avidin, incubated with the desired substrate for various time intervals, was lyophilized to dryness, denatured in 99% formic acid, applied to a TLC plate, and developed with butanol-acetic acid-water (4:1:1). The biotin-containing spots were visualized using p-dimethylaminocinnamaldehyde spray (13).

Group-specific Modifications of Avidin—Lysine groups of avidin were acetylated using acetyl N-hydroxysuccinimide ester according to the previously described procedure (14). Tyrosine groups were nitrated using tetranitromethane as described (15). Carboxyl side chains of aspartic and glutamic acids were modified using glycine ethyl ester in the presence of a water-soluble carbodiimide (16). Arginine residues were modified selectively using phenyl glyoxal (17), and histidines were modified using diethyl pyrocarbonate (18).

Crystallization and Data Collection: The Streptavidin-BNA Complex—Biotinyl p-nitroanilide (BNA) was synthesized as described previously (19). The complex between core streptavidin and BNA was prepared by introducing several grains of biotinyl derivative into a 30-mg/ml solution of streptavidin containing 5% ethanol. The solubility of BNA in aqueous solutions is low, and the streptavidin-BNA complex was established by mixing the solution for 20 h at 37°C. Formation of the streptavidin-BNA complex was monitored via the orange-colored dye, HABA, which becomes red upon binding to avidin or streptavidin (20). A stock solution was prepared by diluting a saturated HABA solution 1:100 with water. The HABA stock (2 ml) was added to 2 ml of the streptavidin-BNA complex solution on a glass cover slide. The transformation of the solution from red to orange (usually after an incubation period of about 20 h) indicated that the biotin-binding sites of streptavidin were fully occupied by BNA. Crystals of the streptavidin-BNA complex were obtained by the vapor diffusion hanging drop method using a Hampton Research crystal screen I (21) at room temperature. The 1-ml reservoir solution contained 0.2 M sodium citrate, 0.1 M HEPES buffer (pH 7.5), and 20% isopropanol. A 3-µl drop contained 1.5 µl of the protein complex and 1.5 µl of the reservoir solution. An amorphous precipitation appeared after 3 days. Crystals grew from the precipitation after 3 months and reached a final size of 0.2 × 0.2 × 0.4 mm. The crystals belonged to the orthorhombic space group I222, with two streptavidin monomers in the asymmetric unit and cell dimensions a = 47.121 Å, b = 95.160 Å, c = 105.304 Å. Diffraction data...
were collected from a single crystal at room temperature on an RAXIS-IIc image plate mounted on a Rigaku RU300 rotating anode generator with short Charles Supper mirrors. Data were integrated and scaled using the DENZO/SCALEPACK programs (22, 23).

Crystallization and Data Collection: The avidin-BNA Complex—Crystals of “Lite” avidin (deglycosylated avidin (24)) were obtained by the vapor diffusion hanging drop method at 20 °C from a 4.2-mg/ml protein solution, mixed with an equal volume of reservoir solution containing 15% polyethylene glycol 2000 (PEG 2000) in 0.1M phosphate buffer (pH 6.3). These conditions were refined further to produce the largest possible avidin crystals using a reservoir solution consisting of 12% PEG 2000 in 0.1M imidazole malate buffer (pH 5.8). Each hanging drop contained 1.5 μl of protein solution and 1.5 μl of reservoir. The crystals reached a final size of 0.1 × 0.1 × 0.1 mm after 1 week. To enlarge the crystals, multiple macroseeding techniques were applied (25) until the crystals reached a final size of 0.3 × 0.2 × 0.1 mm. Crystals suitable for x-ray crystallographic analysis were initially soaked in a solution containing 1% HABA. The crystals immediately became red at their edges, and the red color continued to disperse throughout the crystals until they became completely colored. The HABA-dyed crystals were transferred to a 1% BNA solution, which became colorless within 24 h of soaking, indicating that the BNA replaced the HABA in the binding sites of avidin. Crystallographic data were collected at room temperature from a single crystal on an RAXIS-IV image plate mounted on a Rigaku ULTRAX 18S rotating anode generator with confocal Max Flux™ optics. The crystal belonged to the orthorhombic P2₁2₁2₁ space group with cell parameters of a = 72.27 Å, b = 80.36 Å, c = 43.40 Å, and an avidin dimer in the asymmetric unit. Data were integrated, reduced, and scaled using the DENZO/SCALEPACK program (22, 23).

Structure Determination and Refinement: The Streptavidin-BNA Complex—The structure was solved via molecular replacement methods using AMoRe (26) implemented in the CCP4 suite (27). The search model, with hydrogen atoms and the biotin molecule removed, was used...
in both monomeric and dimeric forms to validate the results. The
structure was then refined using rigid body refinement CNS at the
resolution range of 50–4.0 Å. The structure was further refined using
simulated annealing and individual temperature factor refinement
protocols in CNS, and after two cycles the BNA molecule was added into
the structure.

**Structure Determination and Refinement: The Avidin-BNA Complex**—The crystallographic data were of an identical symmetry and similar cell parameters as the avidin-biotin complex structure (25), and thus molecular replacement was not necessary. The structure was initially refined using rigid body refinement at the resolution range of 50–4 Å. The structure was then refined using the simulated annealing procedure followed by individual temperature factor refinement using CNS (28). The structure was built into electron density maps using the program O (29, 30). The BNA ligand was added to the structure after the second cycle. After 17 iterative cycles of refinement and model building, the refinement statistics reached the values summarized in Table II.

**RESULTS**

**Pseudo-enzymatic Properties of Avidin**—During the course of our studies on the biotin-binding site of chicken egg white avidin, we attempted to affinity label avidin using the active-ester derivative of biotin, biotinyl p-nitrophenyl ester. When BNP was added to avidin at pH 4 and the pH was increased, a rapid release of p-nitrophenol was observed (Fig. 1). However, tryptic digestion of heat-denatured avidin failed to yield any biotin-containing peptides. Moreover, denaturation of avidin using 70% formic acid served to release free biotin, thus indicating that simple hydrolysis of BNP occurred instead of covalent labeling of avidin by the reagent. The hydrolysis reaction thus resulted in the formation of biotin and free p-nitrophenol such that the biotin was retained almost irreversibly but non-
covalently in the binding site of avidin. The resultant avidin-
biotin complex could no longer serve to catalyze additional molecules of BNP. Other biotin derivatives, including several other biotinyl esters, were not affected (Table I). The avidin-mediated hydrolysis of BNP appeared to depend on a very precise interaction between a residue (or residues) at or near the biotin-binding site. By increasing or decreasing the length of the derivative by a single methylene group in the side chain, the hydrolysis was essentially prevented (Table I). Thus, norbiotin and homobiotin analogues of BNP failed to undergo substantial hydrolysis in the presence of avidin (Fig. 2).

When BNP was combined with bacterial streptavidin, the protein failed to hydrolyze the ester. In fact, the observed autohydrolysis of BNP was protected by streptavidin up to pH 12 (Fig. 3). This observation indicates that there is a hitherto undetected qualitative difference in or near the binding do-
 mains of avidin and streptavidin. In an attempt to identify which amino acid(s) are involved in the observed avidin-mediated hydrolysis, avidin was subjected to group-specific modification reagents prior to interaction with BNP (Fig. 4). Using this approach, the direct involvement of tyrosine, arginine, and aspartic and glutamic acids could be excluded. Modification of histidine appeared to provide partial protection of BNP hydro-
lysis. Acetylation or succinylation of lysine groups in avidin, however, appeared to confer complete protection against hydro-
lysis of BNP. Fully acetylated or succinylated avidin prepara-
tions were still capable of binding biotin and BNP. This result suggested that one or more lysine groups may be in-
volved in the hydrolysis of the biotinyl active-ester derivative.

In contrast, lysine does not appear to serve as a nucleophile in the hydrolysis of BNP, because repeated efforts failed to yield a peptide of avidin with covalently bound biotin. Therefore, we searched for an alternative explanation that would account for the promotion of BNP hydrolysis by avidin and its protection by streptavidin. We therefore decided to crystallize avidin and streptavidin with appropriate biotin-containing derivatives. Because of the rapid hydrolysis of BNP by avidin, we synthesized a comparable chemically stable analogue, biotinyl p-ni-
troanilide, for this purpose, and crystallized the two proteins in its presence.

**Structure Determination of Avidin- and Streptavidin-BNA Complexes**—Core streptavidin (31) complexed with BNA was determined via molecular replacement techniques, using both monomeric and dimeric streptavidin (Protein Data Bank code 2IZF) as search models (32). The initial electron density maps \(F_o - F_c\) and \(3F_o - 2F_c\) calculated after rigid body refinement in the absence of BNA, provided a clear indication of the location of the BNA molecule (Fig. 5A). The structure was further refined using simulated annealing with slow cooling protocol in CNS (28) with data from 50.0 to 2.4 Å (see Table II). The \(F_o - F_c\) and \(3F_o - 2F_c\) densities were scaled anisotropically (B11 = –4.44 Å², B12 = 0.0 Å², B22 = 0.68 Å², B13 = 0.0 Å², B23 = 0.0 Å², B33 = 3.76 Å²), and
a bulk solvent correction was applied (33). The structure was built into electron density maps using the graphics program O (29). The model of the streptavidin-BNA complex consists of residues 15–135 for monomer 1 and residues 14–135 for monomer 2 with 143 solvent molecules. The model was refined to a resolution range of 50–2.4 Å with a crystallographic R value of 17.7% (Rfree value of 25.0%).

Crystals of the deglycosylated avidin (Lite avidin) complexed with BNA belonged to the same space group with similar cell parameters to those of the native avidin and its complex with biotin (Protein Data Bank code 2AVI) (4). The 2AVI model (without biotin, carbohydrate, and solvent molecules) was initially refined using rigid body protocols in CNS (28). The electron density maps calculated from the model indicated the location of the ligand and remaining GlcNac sugar moiety. Difference maps showed significant negative electron density for the segment of the L3,4 loop (connecting the β3 and β4 strands) as taken from the model of the avidin-biotin complex (Fig. 5B), indicating that the loop either displays a significantly different conformation or is completely disordered. Further refinement using simulated annealing protocols at 2.0 Å resolution (28) indicated that the L3,4 loop has an open conformation compared with the biotin complex; three residues in each monomer (39 to 41) had no clear electron density and were thus not modeled into the structure. The Fobs were scaled anisotropically (B11 = 2.26 Å², B12 = 0.0 Å², B22 = 0.36 Å², B13 = 0.0 Å², B23 = 0.0 Å², B33 = 1.90 Å²), and a bulk solvent correction was applied (33). The structure was built into electron density maps using the graphics program O (29). The structure of the avidin-BNA complex thus consists of residues 3–38 and 42–123 for monomer 1, and residues 2–38 and 42–123 for monomer 2. The structure contains 82 solvent molecules and was refined to a resolution of 2.0 Å with a crystallographic R value of 20.4% (Rfree value 23.4%). The coordinates of avidin-BNA (1IJ8) and streptavidin-BNA (1I9H) complexes are available at the Protein Data Bank, Research Collaboratory for Structural Bioinformatics.

In both the avidin- and streptavidin-BNA complexes, the overall fold of the monomers and their tetrameric arrangements are similar to those of the native and biotin-complexed proteins described earlier (4–7). Each monomer is constructed of eight anti-parallel β-strands, which form a classical β-barrel with the biotin-binding site at the wide end of the barrel. The main structural difference between the avidin and streptavidin
monomers lies in the size and conformation of five of the hairpin loops between the β-strands.

The quaternary structures of avidin and streptavidin can be regarded as a dimer of dimers. The quaternary contact areas of avidin and streptavidin are very similar and consist of three intermonomeric interface regions. The interface previously defined as 1–2 (4) is crucial for the high affinity binding of biotin and other ligands. In this 1–2 interface, a tryptophan residue (Trp-110 in avidin and Trp-120 in streptavidin) is contributed from one monomer to the biotin-binding site of its neighbor, thus forming a tight hydrophobic cage (4) that contributes an essential component for biotin binding. Trp-110 in avidin and Trp-120 in streptavidin retain their conformation upon biotin binding. Site-directed mutagenesis studies of Trp-110 in avidin (34) and Trp-120 streptavidin (35) have substantiated the significance of this particular residue to the high affinity toward biotin and its contribution to the stability of the quaternary structure (36, 37).

The Streptavidin-BNA Complex with Reference to Other Available Streptavidin Structures—There is an extensive amount of structural information available for streptavidin. There are currently 84 streptavidin structures available in the Protein Data Bank. All of the known structures of core streptavidin, in both native and mutant forms, complexed with biotin, HABA, and short peptides, were compared with the BNA complex. Analysis of the compiled data revealed a striking diversity in the conformation of the L3,4 loop (i.e. residues 47–52) that connects strands β3 and β4. In the streptavidin-BNA complex, the L3,4 loop is in the closed conformation, which is similar to that observed in other core streptavidin structures (Fig. 6, A and C). A consensus conformation of the streptavidin L3,4 loop in the closed position can be defined by taking into account several of the available solved structures in the native and ligand-complexed state. In some cases, however, streptavidin structures, in the absence of ligand or complexed with short peptides, bear an L3,4 loop that is either in an open conformation or in a partially or completely disordered state. Thus, in ligand-free streptavidin, the loop is usually disordered. Likewise, when the site contains a relatively large ligand (e.g., a peptide), the loop is in an open conformation because of steric considerations. However, when biotin or a small ligand occupies the streptavidin binding site, L3,4 is essentially sealed to attain a consensus conformation, covering the ligand like a lid. The ligand is thus buried and is almost completely unavailable to solvent molecules (Fig. 6A).

In the streptavidin-biotin complex, the L3,4 loop exhibits a closed conformation, which contributes a single H-bond between the main chain amide nitrogen of Asn-49 and one of the biotin carboxylate oxygens. The other carboxylate oxygen forms an H-bond with the Oγ of Ser-88. In the streptavidin-BNA complex (Fig. 7C), the loop remains in the closed state despite

**TABLE II**

| Data collection and refinement statistics of avidin- and streptavidin-BNA complexes |
|-----------------------------------------------|
| **Streptavidin-BNA** | **Avidin-BNA** |
| **Space group** | I222 | P21 21 2 |
| **Resolution** | 50.0–2.4 | 50.0–2.0 |
| **Unique reflections** | 3847 | 17510 |
| **Redundancy** | 2 | 5.5 |
| **Rmerge** | 9.8 (26.2) | 5.2 (27.9) |
| **Completeness (%)** | 90.7 (94.2) | 98.7 (98.1) |
| **Average I/σ** | 10.9 (3.7) | 30.7 (4.6) |
| **Number of protein atoms** | 1818 | 1871 |
| **Number of ligand atoms** | 50 | 50 |
| **Number of solvent atoms** | 134 | 82 |
| **Number of carbohydrate atoms** | None | 28 |
| **R-factor (F > 1σ)** | 17.7% | 20.4% |
| **Rfree** | 25.0% | 23.4% |
| **rmsd** | None | 28 |
| **Bond length** | 0.006 Å | 0.006 Å |
| **Bond angle** | 1.2° | 1.35° |
| **Ramachandran plot (PROCHECK)** | | |
| **Favored** | 85.80% | 92.10% |
| **Allowed** | 11.30% | 7.90% |
| **Generously allowed** | 2.90% | 0.00% |
| **Disallowed** | 0.00% | 0.00% |

* Rmerge = Σ[I(I−〈I〉)]/ΣI.

A comparison of contact surfaces between avidin and biotin or BNA as ligands with those of streptavidin. The contact surfaces are color-coded based on chemical nature of the interaction; green and blue represent hydrophobic and polar interactions, respectively. A and B represent the biotin complexes of streptavidin and avidin, respectively. Note the complete entombment of biotin in both binding sites. It is also clear that the L3,4 loop (arrows), which embraces the biotin molecule in both proteins, is notably larger in avidin (B) than in streptavidin (A). BNA, however, adopts different binding conformations upon binding streptavidin (C) and avidin (D). The L3,4 loop in streptavidin (C, arrow) remains in a conformation similar to that of the biotin complex (A). In contrast, the L3,4 loop in the avidin-BNA complex adopts a partially disordered "open" conformation (D, asterisks), which facilitates accessibility of BNA to solvent compared with its protection from solvent by the closed conformation of the analogous loop in the streptavidin complex (C).
the additional p-nitroanilide moiety of the biotin derivative. The H-bond between the main chain Asn-49 and the amide oxygen of BNA is thus maintained. A second H-bond between Ser-88 Oy and the BNA amide nitrogen (which emulates the position of the carboxylate oxygen of the underivatized biotin) is maintained as well. The closure of the loop as well as the binding conformation of BNA results in the near complete entombment of the ligand in streptavidin (96%, 307 of 319 binding conformation of BNA compared with those described above for biotin. First, a conserved H-bond between one of the ureido nitrogens of biotin and the Thr-35 Oy was lost (Fig. 7D). In addition, the open L3,4 loop in the avidin-BNA complex did not permit interaction of the three H-bonds formed between the closed loop and the biotin carboxylate oxygen in the avidin-biotin complex (Fig. 7B). On the other hand, the two H-bonds of the second biotin carboxylate oxygen, now replaced by the amide nitrogen of BNA, maintained analogous H-bonding interactions with Ser-73 Oy and Ser-75 Oy (Fig. 7, B and D). The anilide ring of BNA was thus positioned toward the open and partially disordered L3,4 loop.

Although the BNA molecule is buried substantially in the binding site of avidin, a significant area of its anilide ring is available to the solvent, due to the open conformation of the loop. The contact surface of BNA with avidin is 286 Å² of the total surface of 321 Å². Thus, about 11% of the total BNA structure, notably 28% of the p-nitrophenyl moiety, is available to the solvent (Fig. 6D). Significantly, one of the nitro-group oxygens of the anilide ring is situated in the vicinity of the Lys-111 Nε located in the adjacent avidin monomer (Figs. 7D and 8).

### DISCUSSION

The respective BNA ligands of the avidin and streptavidin complexes exhibit different conformations. Upon superposition of the two BNA molecules, one can clearly observe the similarity of the positions of the biotinyl moieties, whereas the anilide rings are situated in different directions with an angle of −50° between them (Fig. 8). The respective conformations of Lys-111 in avidin and Lys-121 in streptavidin are comparable in the two complexes. Another important difference between the two...
structures is the substitution of a critical arginine residue in avidin instead of a leucine in streptavidin. The position of Arg-114 from strand H9252 in avidin appears to be a driving force that governs the different conformations of the anilide moiety in the two structures (Fig. 8).

Originally, before we solved the structures of the BNA complexes, the paradoxical pseudo-catalytic effects of the two proteins were puzzling, as reflected by the hydrolysis of BNP in avidin and its protection in streptavidin. The crystal structures of the avidin-biotin and streptavidin-biotin complexes failed to shed light on the subject. In view of the latter structures, we had assumed that the explanation would lie in the abundance of H-bonding interactions with the biotin carboxylate in the avidin complex versus those of streptavidin. In this context, we postulated that the extra H-bonds in the avidin-biotin complex might play a stabilizing role for a putative transition state during basic hydrolysis of BNP.

The avidin-BNA structure as detailed in the present work changed this conception. First, the open conformation of the flexible L3,4 loop together with the presence of the critical Arg-114 allows partial exposure of the BNA molecule (notably, the anilide group) (Fig. 6D). The open conformation of the loop and the availability of BNA to solvent would permit alkaline attack via a hydroxide ion (Fig. 9). The nitro group of BNA is thus placed in an ideal position for interaction with Lys-111 from the adjacent monomer. By analogy, Lys-111 of the avidin-BNP complex would be expected to increase the electron-withdrawing properties of the p-nitrophenyl moiety, thereby promoting hydrolysis at relatively low pH.

In streptavidin, the protection of BNP hydrolysis up to pH 12 can be attributed to the fact that BNA is almost completely buried in the binding site and the L3,4 loop is in the closed conformation. This particular arrangement would hinder solvent molecules from entering the site and promoting hydrolysis (Fig. 6C). Of course, the anilide (nitrophenyl) group would be inaccessible to the decisive Lys-121. Presumably, at very high pH values (>12), structural alteration of streptavidin would occur, especially in the flexible loop regions, thus exposing the ligand to solvent.

Most natural esterases and peptidases possess a consensus triad consisting of a serine, histidine, and aspartate in their respective catalytic sites (38). In some cases, the serine is replaced by threonine and aspartic acid by glutamic acid. In any case, a conserved consensus triad, comprising a hydroxyamino acid, a histidine, and an acidic amino acid, invariably characterizes the active sites of such hydrolases. In stark contrast, none of the known artificial protein catalysts, notably catalytic antibodies, exhibit such a triad, and an alternative molecular architecture, often comprising a histidine with the involvement of other types of functional residues (such as tyrosine and/or arginine), was discovered by x-ray crystallography (39–41).

The first generation of catalytic antibodies, prepared by using a transition-state analogue of p-nitrophenyl phosphonate...
(40), served to accelerate the hydrolysis of the corresponding activated p-nitrophenyl ester. The crystal structure of the catalytic antibody-hapten complex also showed that the phosphonate group is situated in a hydrophobic pocket near the entrance of the combining site and that the negatively charged hapten forms multiple interactions with charged and polar residues (42). From these structural studies, it was assumed that active esterase hydrolysis is attained by direct attack of hydroxide ions on the scissile carbonyl group. Because of the charge on the phosphonate and in light of the results of the present study, a better model hapten for crystallographic analysis of p-nitrophenyl ester hydrolysis by catalytic antibodies would have been the corresponding p-nitroanilide.

Our study shows that the status of the pseudo-enzymatic action of avidin on BNP emulates that of catalytic antibodies. Hence, BNA is situated in a hydrophobic pocket of avidin, and the hydrolysis of the p-nitrophenyl ester is assisted by a critically placed lysine residue that interacts directly with the nitro group. This interaction increases the electrophilicity of the carbonyl carbon, thus favoring the propensity of the leaving group to withdraw from the pocket and paving the way for hydrolysis by an incoming hydroxide ion.

Until now, most of the artificial protein catalysts described in the literature have been catalytic antibodies. However, with the advent of combinatorial libraries of proteins, we can envisage the development of other types of protein catalysts. Consequently, it will be difficult to propose a general statement regarding enzymatic activity of proteins without both solving the three-dimensional structure of each individual protein complexed with the corresponding model substrate and analyzing the molecular interactions between them.

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