Structure-based Rational Design of Streptavidin Mutants with Pseudo-catalytic Activity*

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Introduction of enzymatic activity into proteins or other types of polymers by rational design is a major objective in the life sciences. To date, relatively low levels of enzymatic activity could be introduced into antibodies by using transition-state analogues of haptens. In the present study, we identify the structural elements that contribute to the observed hydrolytic activity in egg white avidin, which promote the cleavage of active biotin esters (notably biotinyl p-nitrophenyl ester). The latter elements were then incorporated into bacterial streptavidin via genetic engineering. The streptavidin molecule was thus converted from a protector to an enhancer of hydrolysis of biotin esters. The conversion was accomplished by the combined replacement of a “lid-like loop” (L3,4) and a leucine-to-arginine point mutation in streptavidin. Interestingly, neither of these elements play a direct role in the hydrolytic reaction. The latter features were thus shown to be responsible for enhanced substrate hydrolysis. This work indicates that structural and non-catalytic elements of a protein can be modified to promote the induced fit of a substrate for subsequent interaction with either a catalytic residue or water molecules. This approach complements the conventional design of active sites that involves direct modifications of catalytic residues.

Avidin and its bacterial analogue streptavidin share many similar biochemical and structural properties, including their remarkable affinity toward the vitamin biotin. The tremendously high affinity between the proteins and their ligand has been exploited as a powerful tool in numerous biotechnological applications.

Despite differences in the primary structure of the two proteins (~30% identity and 40% overall similarity) both have strikingly similar tertiary β-barrel topology and homo-tetrameric quaternary arrangement. Both proteins contain four biotin-binding sites wherein the interacting residues are remarkably similar. In addition to the binding residues, the contribution of a critical tryptophan (Trp-110 and Trp-120 in avidin and streptavidin, respectively), donated from an adjacent monomer, plays a pivotal role both in biotin binding and in the stability of the tetrameric structure (5–7). Avidin and streptavidin in their apo forms, include a disordered L3,4 loop (i.e. the loop connecting strands β3 and β4), thus exposing the biotin-binding site. Upon biotin binding, the “lid-like” loop adopts an ordered and closed conformation, thereby burying the biotin molecule in the binding site (8–11) and rendering the ligand almost completely inaccessible to the solvent.

In contrast to the latter structural and functional similarities, the two proteins differ in many other properties (2). One of the intriguing differences between avidin and streptavidin lies in their propensity to promote the hydrolysis of selected biotinyl ester derivatives. Specifically, avidin was found to enhance the hydrolysis of biotinyl p-nitrophenyl ester (BNP), whereas streptavidin was found to strongly protect the same biotin derivative from hydrolysis. It was also discovered that upon chemical modification of lysine residues in avidin, the hydrolysis of BNP is lost (12). In this context, we have recently determined the crystal structures of avidin and streptavidin in complex with biotinyl p-nitroanilide (BNA), the stable amide analogue of BNP (12). The structures of the avidin and streptavidin complexes with BNA revealed the structural and chemical factors that appear to contribute to the observed differences in hydrolytic potential toward BNP.

Three main determinants were noted that could account for the hydrolytic activity in avidin versus the protection afforded by streptavidin. (i) The disordered conformation of the L3,4 loop in avidin, which would permit solvent accessibility to the ester group of BNP as opposed to the closed conformation of the loop in streptavidin (similar to that in the streptavidin-biotin complex), in which the ligand is almost completely buried (Fig. 1). It is also noteworthy that the L3,4 loop in avidin is three residues larger than that of streptavidin, and its disposition in avidin is more susceptible toward derivatized biotin ligands, thus resulting in the observed disordered conformation (13). (ii) Arg-114 of avidin, which protrudes from the β8 strand, repels the BNP into a conformation that promotes its interaction with Lys-111 from an adjacent monomer. In streptavidin, the homologous position is occupied by a smaller residue, Leu-124, that allows the BNP ligand to adopt an alternative conformation that precludes interaction with Lys-121 (Fig. 2). (iii) In avidin, the formation of a hydrogen bond between the nitro group of BNP and Lys-111 increases the electron-withdrawing properties of the p-nitrophenol moiety, thus enhancing its leaving-group properties and promoting hydrolysis under conditions of relatively low pH values. In streptavidin, however, the BNP acquires a different conformation that prevents interaction with the critical lysine residue (Fig. 2).

Based on the structural and biochemical differences between the two proteins, we designed a series of streptavidin mutations. Our main objectives were to evaluate the structural factors that result in the pseudo-catalytic properties in avidin and to convert streptavidin from a protector of the BNP ligand

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1 The abbreviations used are: BNP, biotinyl p-nitrophenyl ester; BNA, biotinyl p-nitroanilide; PMSF, phenylmethylsulfonyl fluoride.
to one that enhances its hydrolysis. Consequently, Leu-124 in streptavidin was converted to Arg (mutant M1), and the relatively shorter L3,4 loop (residues 48–52) of streptavidin was replaced to emulate that (residues 38–45) of avidin (mutant M2). A third construct was generated to contain both modifications (i.e., the point mutation and the L3,4 loop exchange; mutant M3).

In this study, we thus elected to mutate streptavidin in a manner that would both sterically divert the substrate into the correct conformation for the hydrolytic reaction to occur and expose the substrate to the solvent. Importantly, throughout the design of the three mutations, the purported residue (Lys-121 in streptavidin) that drives the hydrolysis was not subjected to mutation. This work is the first instance in which rational mutagenesis of a protein has been employed for steric diversion of a substrate in a binding site to attain a preferred orientation that favors hydrolysis. This strategy represents an alternative approach for protein modification in the design of enzymes (14–16).

**MATERIALS AND METHODS**

**Construction and Expression of Mutants**—A pET-21a plasmid, containing an insert encoding for core streptavidin with favorable codon usage for expression in *Escherichia coli*, was kindly provided by P. Stayton. Site-directed mutagenesis of Leu-124 to Arg (mutation M1), was performed with primers P1 and P2 (Table I) using the QuikChange kit (Stratagene) according to the manufacturer’s instructions. The core streptavidin was used as a template for the exchange of the L3,4 loop of avidin. The desired exchange of the L3,4 loop (mutation M2) was achieved by PCR using two separate fragments: fragment 1 was formed by primers P3 and P6, and fragment 2 was produced using primers P4 and P5 (Table I) using the core streptavidin as a template. The combination of the L124R mutation of streptavidin (mutation M1 as template), combined with the primers used for modifying the L3,4 loop, generated the double mutant (mutation M3), in which the L124R mutation was obtained in combination with the modified L3,4 loop.

Streptavidin mutants were expressed in *E. coli* using the pET-21a expression vector (Novagen) according to the protocol essentially as reported previously (17). Starter BL21 cells, transfected with the various constructs, were grown in 2×/H11003 yeast-tryptone media at 37 °C until an A<sub>600</sub> of 0.6–0.8 was attained. Expression was induced with 0.4 mM isopropyl/β-D-thiogalactopyranoside for 6–8 h at 37 °C, after which the cells were harvested.

**Isolation and Purification of Streptavidin Mutants**—The cell pellet was resuspended in lysis buffer (20 mM Tris-HCl, pH 7.4, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 0.02% sodium azide) to 1/20 of the original culture volume. After mechanical disruption of the cells using a microfluidizer (model M-110 EHIS, Microfluidics Corp., Newton, MA), the lysate was centrifuged at 12,000 rpm for 20 min. For the isolation of the inclusion bodies, the insoluble fraction was washed once with 50 mM Tris-HCl (pH 8), 10 mM EDTA, 1.5 mM NaCl, 1 mM PMsF, and 0.5% Triton X-100 and four times in the same buffer solution without Triton X-100. For the L124R mutation, inclusion bodies were dissolved in 6 M guanidine hydrochloride (pH 1.5) and incubated for 1 h at 4 °C. The suspension was then centrifuged, and the supernatant fluids were dialyzed against 2 liters of dialysis buffer containing 50
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RESULTS

The extent of BNP hydrolysis was examined at several pH values. The rates of hydrolysis were performed at pH values ranging between 6.5 and 10.5, and the concentration of p-nitrophenol was measured as a function of time.

The absorbance values at 410 nm indicate p-nitrophenol release. All values were measured after 960 min of reaction. The error of absorption was 0.001 absorbance units; under the experimental conditions, the maximal amount of product formed was 7.03 nmol per 10 ml.

Hydrolysis Assay—BNP was dissolved in dimethylformamide (7 nmol per 10 μl) and added to a solution containing 16 nmol of subunit mutant in 0.5 ml. The assay for BNP hydrolysis was conducted as previously described (12, 20) with some modifications. Core streptavidin and avidin were also assayed for comparison of the activity of the given mutant. Protein solutions were incubated for 15 min at 25 °C. The pH value was initially adjusted to the approximate value by adding either 1 M phosphate buffer or 1 M Na2CO3, and the solution was then titrated with 5 N NaOH to the desired pH value. Hydrolysis was assayed versus time at different pH values and monitored by the release of p-nitrophenol at A410. The hydrolysis assays were performed at pH values ranging between 6.5 and 10.5, and the concentration of p-nitrophenol was measured as a function of time.

pH is raised, the hydrolytic activity for M1 increases slightly relative to that of streptavidin until pH 10.5, whereby the hydrolysis of BNP by mutant M1 is increased drastically to emulate that of avidin (Fig. 3).

At pH values below 8.0, mutant M2 still exhibits a protective effect vis-a`-vis BNP hydrolysis, although under more basic conditions the activity of this mutant is dramatically increased to levels of hydrolysis similar to those observed for avidin. The level of hydrolytic activity for the double mutation (M3) drops sharply at pH below 7.5 compared with that of avidin, which still exhibits substantial hydrolysis. The observed hydrolysis by avidin below pH 7 represents a minimal value since the pK of the p-nitrophenyl leaving group is around 7. Above pH 7.5, the extent of BNP hydrolysis exhibited by M3 is even higher than that of avidin.

The rates of hydrolysis were examined at pH 10 and 10.5 (Fig. 4), and the results confirmed the above-described tendencies. Streptavidin protects the hydrolysis of BNP even after overnight incubation. The point mutation M1 is only slightly more active than streptavidin at pH 10 (Fig. 4), but its activity increases drastically at pH 10.5 to levels of hydrolysis comparable to avidin. The hydrolytic activity of the loop mutation M2 is markedly higher than that of M1 at pH 10, and the course of hydrolysis

![Figure 3](image-url)  
**Fig. 3.** The hydrolytic activity of the different mutants compared with that of avidin and streptavidin at various pH values. The absorbance values at 410 nm indicate p-nitrophenol release. All values were measured after 960 min of reaction. The error of absorption was 0.001 absorbance units; under the experimental conditions, the maximal amount of product formed was 7.03 nmol per 10 ml.

![Figure 4](image-url)  
**Fig. 4.** Hydrolytic activity of the different mutants compared with that of avidin and streptavidin at pH 10.0 (A) and 10.5 (B) versus time. The error levels are the same as in Fig. 3.

| Primer name | Primer sequence |
|-------------|-----------------|
| P1          | 5'-GGG TGG AAA TCC ACC CGT GTT GGT CAC GAC ACC-3' |
| P2          | 5'-GGT GTC GTG ACC AAC ACC GGT GGA TTT CCA CGC-3' |
| P3          | 5'-GGT CGG CGG CAA GGT TTT ATT AG-3' |
| P4          | 5'-ACC GCT ACC TCC AAC GAA AAA AAG AAG TCT GTG ACC GGT GTG TAC-3' |
| P5          | 5'-TTT TTC TTC GGT GGA GCT ACG CCT GAC ACC TTC GTA GGT ACC GG-3' |

**Table I**

Sequence of primers used in PCR and site-directed mutagenesis reactions

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The hydrolytic activity of the loop mutation M2 is markedly higher than that of M1 at pH 10, and the course of hydrolysis...
increases gradually and reaches a maximum after overnight incubation, whereby the final values observed are approximate those measured for avidin. Although the extent of BNP hydrolysis by M2 indeed resembles that of avidin, the rate of reaction is much slower. The initial rate of hydrolysis for the combined mutant M3 is much higher at pH 10 than that of M2 but lower is much slower. The initial rate of hydrolysis for the combined M2 indeed resembles that of avidin, the rate of reaction those measured for avidin. Although the extent of BNP hydrolysis by M2 markedly increases the catalytic activity at pH 8 and higher (Figs. 3 and 4), similar to that observed for avidin. The increased activity of the double mutant relative to M2 implies that disorder in the L3,4 loop indeed promotes hydrolysis by water. Moreover, the stereochemistry of the p-nitrophenyl group by Arg-124 toward Lys-121 is also important for further enhancement of the reaction and for lowering the pH of hydrolysis, which results from the formation of a hydrogen bond.

In this study, we examined experimentally our previously reported structure-based hypotheses regarding the main determinants for pseudo-catalytic activity of egg-white avidin. Based on the x-ray structures of avidin and streptavidin complexed with BNA, we designed a functionally active hydrolytic site in streptavidin. In doing so, we succeeded in converting the streptavidin binding site from its protection of the BNP ligand from pseudo-enzymatic hydrolysis to a strong promoter of hydrolysis. The present study demonstrates clearly that if the structure and mode of catalytic action of a protein is known, the structural elements responsible for catalysis can be transferred to a structurally similar but non-catalytic protein. In this context, local alterations (e.g. point mutations or major modifications in loop regions) are permissible, while the scaffold (i.e. tertiary structure) of the protein must remain intact. Such an approach, in which catalytic properties have been induced in a binding protein, has only been attempted successfully in the past for antibodies (21–23). In the present study, we have now demonstrated that catalytic properties can also be incorporated into other protein systems.

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