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# The structure of the complex between avidin and the dye, 2-(4′- hydroxyazobenzene) benzoic acid (HABA)

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The crystal structure of the complex formed between the egg-white biotin-binding protein, avidin, and the dye, 2-(4′-hydroxyazobenzene) benzoic acid (HABA), was determined to a resolution of 2.5 Å. The interaction of avidin with the benzoate ring of HABA is essentially identical to that of the complex formed between HABA and streptavidin (the bacterial analogue of the egg-white protein). This interaction emulates the definitive high-affinity interaction of both proteins with the ureido moiety of biotin The major difference between the avidin- and streptavidin–HABA complexes lies in their interaction with the hydroxyphenyl ring of the dye molecule; in avidin, two adjacent amino acid residues ($Phe^{72}$ and $Ser^{73}$), which are not present in streptavidin, form additional interactions with this ring These are suggested to account for the higher affinity of avidin for HABA. The characteristic red shift, which accompanies the interaction of both proteins with the dye, was traced to a proposed charge-transfer complex formed between the hydroxyphenyl ring of HABA and the indole ring of $Trp^{70}$ in avidin ($Trp^{79}$ in streptavidin). Comparison of binding site residues of two such similar proteins versus their markedly different affinities for two such different substrates should eventually contribute to a better design of biomimetic reagents and drugs.

Avidin; Streptavidin, Biotin-binding site, HABA, Biomimetics, Protein structure, X-ray crystallography

## 1. INTRODUCTION

The vitamin biotin binds to the egg-white glycoprotein avidin with the highest affinity constant ($K_d \sim 10^{-15}$ M) yet described for the noncovalent interaction between a protein and a biologically active molecule [1]. A binding site which exhibits such strong affinity might also be expected to interact with other molecules with lesser strength or specificity. Secondary interactions of this nature could involve target molecules with very similar or completely different structures. In this context, proteins with much lower affinity constants are known to possess such biomimetic interactions, e.g. coenzyme-binding proteins which adhere to textile dyes [2].

Avidin binds to a variety of different dyes and peptides, which have no apparant structural similarity to biotin [1,3,4]. Nonetheless, they appear to bind at the same site, since biotin readily displaces such biomimetics. The reverse, however, is not possible due to the much higher affinity constant of the avidin–biotin complex. One such biomimetic for avidin is the dye, 2-(4′-hydroxyazobenzene) benzoic acid (HABA). HABA has become important in avidin–biotin technology, since it can be readily used to determine the amount and biotin-binding activity of avidin in solution [5–7]. Upon binding to avidin, the color of HABA changes from yellow to red; the yellow color is restored upon displacement of the avidin-dye complex by biotin. Interestingly, streptavidin and bovine serum albumin also bind to HABA, but with a considerably lower affinity ($K_d$ of $1 \times 10^{-4}$ for streptavidin versus $6 \times 10^{-6}$ M for avidin [8]).

Recently, the three-dimensional structures of the avidin–biotin [9], streptavidin–biotin [10,11] and streptavidin–HABA [12] complexes have been described. We were interested in determining the structure of the complex of avidin with HABA, in order to determine which elements in the binding site participate in the interaction, and, in particular, to address the following questions: Do the same amino acid residues which interact with biotin also participate in the interaction with HABA? Are they homologous to those in streptavidin which interact with HABA? And why is the interaction stronger for avidin than for streptavidin?

Such studies may enable us to design other types of interacting molecules which could be useful for application in the avidin–biotin system [7,13,14] and contribute relevant information for biological interactions in general [15].

## 2. MATERIALS AND METHODS

The avidin–HABA complex was obtained by soaking HABA into avidin crystals [16]. Specifically, crystals of avidin were transferred to a 200 μl solution containing 205 μM of HABA in 25% PEG 1000, 15 mM citrate buffer (pH 5.4) and 10 mM sodium azide. The binding of HABA to avidin in the crystals could be viewed under the microscope

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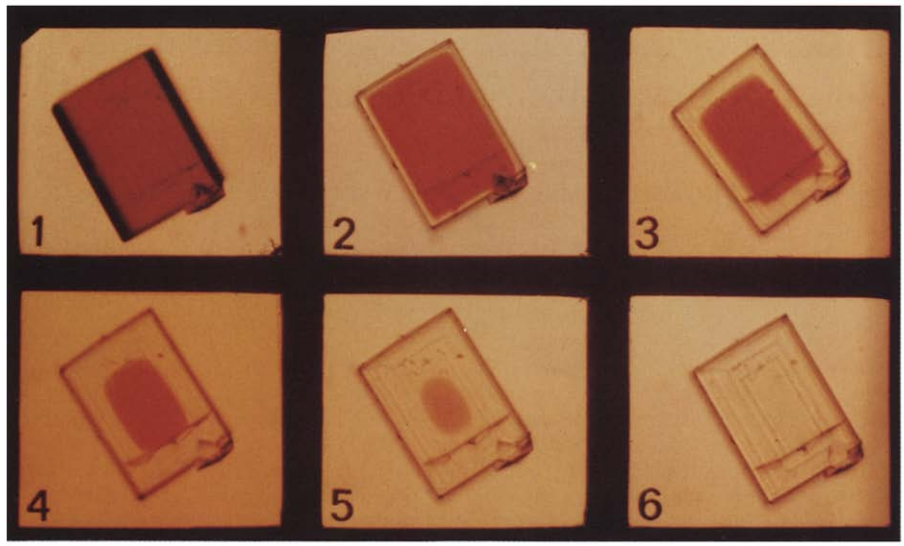


Fig. 1. Soaking biotin into HABA-dyed crystals of avidin as a function of time; (1) 0 h, (2) 0.5 h, (3) 1 h, (4) 2 h, (5) 3 h, (6) 4 h.

by the slow change in color. The process was completed over a period of 4 h, after which the crystals turned completely red. Subsequent addition of biotin lead to slow displacement of the dye from the binding site with concomitant decolorization of the crystals over the four-hour period (Fig. 1).

The crystallographic data were collected at room temperature on a Rigaku R-AXIS-II image plate (IP) system, mounted on an RU300 rotating anode operated at 5 kW, collimated by a set of Charles Supper French mirrors. The space group is $P2_12_12$ and the cell parameters were determined to be, $a$ = 72.09 Å, $b$ = 80 27 Å, $c$ = 43.32 Å, $\alpha = 90°$, $\beta = 90°$, $\gamma = 90°$ – very close to those of the ligand-free protein and the avidin–biotin complex [9]. The IP was set at 75 mm from the crystal, and frames were collected with 1.5° oscillation for a duration of 45 min per frame. A near-complete data set to the resolution of 2.5 Å was collected over two days (Table I).

The structure of the avidin–HABA complex was determined using X-PLOR [17,18], starting from the refined structure of the avidin–biotin complex [9] omitting the biotin and the water molecules. Initial rigid body refinement of the structure using two avidin monomers in the asymmetric unit as rigid bodies, showed some movement with the R-factor dropping from 34.6% to 30.8% at the resolution range of 10.0–5.0 Å. The structure was further refined using the slow-cooling protocol [19] and temperature factor refinement lowering the R-factor to 23.7% for the 8.0–2.5 Å. resolution date. During the refinement and the model building we observed that, as in the structure of the apo avidin, the loop between strands $\beta$3 and $\beta$4 had no defined electron density and appeared to be partially disordered In the avidin–HABA complex there was notable density for two additional amino acid residues, thus reducing the disordered section from residues 36–44 in avidin [9] to residues 38–44 in the avidin–HABA complex. Solvent molecules and HABA were added during model building and refinement. All residues of the avidin–HABA structure are within the allowed regions of the Ramachandran plot. Although non-crystallographic symmetry restraints were not used in refinement, the root-mean-square deviation of Cα between monomers is 0.31 Å. The results of the refinement are shown in Table I.

## 3. RESULTS AND DISCUSSION

The general fold of the avidin–HABA structure is very similar to that of the recently described avidin–biotin complex. HABA is a planar molecule, but when bound to avidin HABA loses its complete planarity and exists as its hydrazone tautomer (Fig. 2). As a result, the nitrogen atom proximal to the benzoate ring changes its geometry from a planar $sp^2$ to a tetrahedral $sp^3$. The HABA molecule loses the absolute conjugation of its $\pi$-electrons and now consists of two planar entities with a slight kink between them.

HABA occupies the biotin-binding site in avidin and, like biotin [9], displaces the five bound water molecules. Many of the characteristic interactions observed in the avidin–biotin complex are also present in the avidin–HABA complex. The position of one of the benzoate oxygen atoms of HABA corresponds to that of the ureido oxygen of biotin, and forms an identical network of three H-bonds with $Asn^{12}$, $Ser^{16}$ and $Tyr^{33}$ thus forming an oxyanion (Fig. 3). The other carboxylate oxygen of HABA forms H-bonds with $Ser^{16}$ and $Thr^{35}$ of avidin and an intramolecular H-bond with one of the HABA

Table I

Refinement results for the avidin–HABA complex

| | |
|---|---|
| Program used | X-PLOR |
| R-factor ($F_{obs} > 3\sigma$) | 18.9% |
| Resolution range | 8.0–2.5 Å |
| Number of unique reflections | |
| $F_{obs} > 0$ (%) | 8,610 (97.3%) |
| $F_{obs} > 3\sigma$ (%) | 7,312 (82.6%) |
| Current model | |
| 2 avidin monomers | 1,792 atoms |
| 2 HABA molecules | 36 atoms |
| 2 GlcNac | 28 atoms |
| waters | 108 molecules |
| RMS deviations from ideality | |
| Bond length | 0.013 Å |
| Bond angle | 1.87° |
| RMS deviation of Cα between the monomers | 0.31 Å |

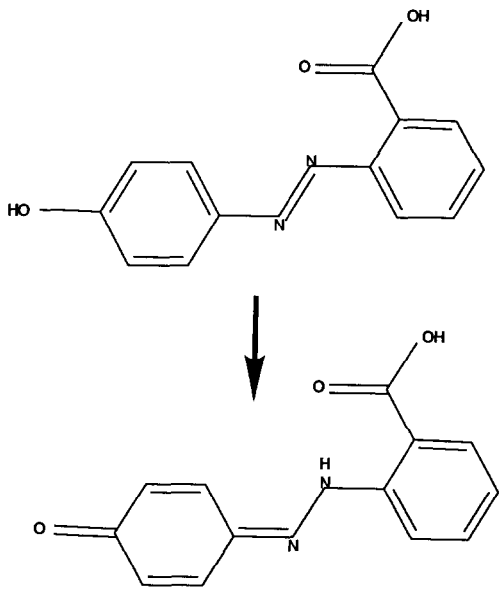


Fig. 2 The possible tautomers of HABA. The structure shown at top represents the free dye, and the structure below is formed and stabilized in the avidin–HABA complex.

nitrogens. The benzoate ring also exhibits hydrophobic interactions with three aromatic residues, i.e. Phe[79], Trp[97] and the Trp[110] from the adjacent monomer.

At the other end of the molecule, the hydroxyphenyl oxygen of HABA forms an H-bond with Ser[73] and interacts with the $\pi$-electron plane of the phenyl ring of Phe[72] which moves 0.9 Å in relation to its position in the avidin–biotin structure. Moreover, the hydroxyphenyl ring of the ligand is stacked upon the indole ring of Trp[70], thus forming a charge-transfer complex; the tryptophan acting as the donor and the hydroxyphenyl ring of HABA as the acceptor (Fig. 3).

When comparing the interactions of HABA with avidin and its bacterial analogue streptavidin, one can observe the similarities and differences in their respective interactions and interpret the observed variations in the dissociation constants. The similarities in the binding properties of HABA by the two proteins are manifested by the interactions of the oxygens in the benzoate ring which are completely analogous. In streptavidin [12], one of these oxygens interacts via three H-bonds with Asn[23], Ser[27] and Tyr[43] and the other interacts with Ser[27] and Ser[45] of the protein and via intramolecular interaction with the adjacent nitrogen. Although the interaction of the aromatic residues of streptavidin with HABA were not described in detail [12], by analogy with avidin, one can conclude that these hydrophobic interactions are analogous to those of the avidin–HABA complex. These would thus involve residues Trp[92] and Trp[108], as well as Trp[120] from the adjacent monomer, all of which interact with the benzoate ring of HABA. In addition, Trp[79] would presumably form a charge-transfer interaction with the hydroxyphenyl ring of the dye molecule.

The key difference in the interaction of HABA with avidin and streptavidin lies in two unique interactions between the hydroxyphenyl ring of the dye and avidin; i.e. the above-described interactions of the vicinal residues Phe[72] and Ser[73] of avidin with HABA – these interactions have no parallel in streptavidin (Table II). These two residues are located in the loop between strands $\beta$5 and $\beta$6 of avidin. The corresponding loop in streptavidin is four residues longer than that of avidin and has no contacts with HABA. The involvement of the Phe[72] and Ser[73] residues of this particular loop in the binding of HABA by avidin is highly reminiscent of their role in the binding of biotin, which is also considered to reflect the differences in affinity observed for forming the avidin–biotin and streptavidin–biotin com-

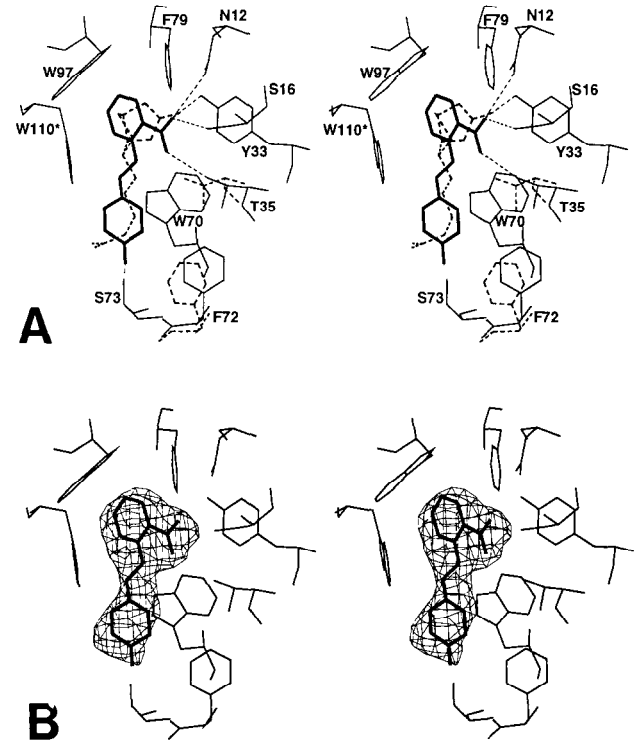


Fig. 3. Stereoscopic view showing the HABA molecule in the binding site of avidin. (A) The amino acid residues involved in forming the avidin–HABA complex. The H-bonds are shown in thin dashed lines. Biotin, Thr[35] and Phe[72] (displayed in dashed lines) from the avidin–biotin complex are superimposed to show the relative positions of the two ligands and the key differences in the positions of Thr[35] and Phe[72] in the binding sites of the two complexes. In the avidin–HABA complex, the phenyl ring of Phe[72] interacts with the hydroxyphenyl ring oxygen of the ligand and is situated 0.9 Å from its position in the complex with biotin, thus providing enough space to accomodate the hydroxyphenyl ring. Thr[35] of the avidin–HABA complex is located 1.5 Å closer to the ligand, compared with its position in the avidin–biotin complex (B) Electron density associated with HABA in the binding site of avidin. An unbiased difference map was calculated after omitting HABA and running the simulated annealing protocol in X-PLOR. The electron density map is contoured at 2.5 $\sigma$ above the mean density level.

Table II

The amino acid residues involved in the binding of HABA in avidin and the corresponding residues in streptavidin

| Interaction | Avidin | Streptavidin |
|---|---|---|
| H-bond | $Asn^{12}$ | $Asn^{23}$ |
| H-bond | $Ser^{16}$ | $Ser^{27}$ |
| H-bond | $Tyr^{33}$ | $Tyr^{43}$ |
| H-bond | $Thr^{35}$ | $Ser^{45}$ |
| Hydrophobic | $Trp^{70}$ | $Trp^{79}$ |
| Aromatic oxygen* | $Phe^{72}$ | – |
| H-bond | $Ser^{73}$ | – |
| Hydrophobic | $Phe^{79}$ | $Trp^{92}$ |
| Hydrophobic | $Trp^{97}$ | $Trp^{108}$ |
| Hydrophobic | $Trp^{110}$** | $Trp^{120}$** |

*The aromatic ring of $Phe^{72}$ interacts with the hydroxyphenyl oxygen of the HABA molecule.
**Residue from an adjacent monomer.

plexes. It is interesting to note that the relative *difference* in affinity exhibited by the two proteins for both biotin and HABA is very similar, but the consequences of such a difference in actual terms are much more significant for the low-affinity interaction with HABA vis-à-vis the high-affinity interaction with biotin.

The change in color from orange ($\lambda_{max}$ = 348 nm) to red ($\lambda_{max}$ = 500 nm), associated with the binding of HABA to avidin, seemed somewhat perplexing at first. The distortion in the planarity of the ligand and consequent reduction in the number of electrons in the conjugated system should have resulted in a blue shift [20], yet formation of the avidin-HABA complex produced a red shift. This apparent paradox can be explained by the interaction of the indole rings of $Trp^{70}$ in avidin ($Trp^{79}$ in streptavidin) with the hydroxyphenyl ring of HABA. The charge-transfer complex thus formed, enriches the HABA system with more electrons [20], thereby generating a red shift. In this regard, it was reported [21] that *p*-benzoquinone (which is analogous to the hydroxyphenyl ring of HABA) forms a charge-transfer complex with trypthophan which exhibits an absorbance peak at 500 nm and an extinction coefficient similar to those of the avidin–HABA and streptavidin–HABA complexes.

In this communication, the involvement of binding site residues critical to the avidin–HABA complex was compared with those contributing to the avidin–biotin complex and with those which form the corresponding complexes with streptavidin. These high-resolution images of the mode of binding of both high- and low-affinity substrates to two such similar proteins will enable the preparation of ligands with different affinities according to the requirements of a given experimental system or desired application.

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