Avidin Can be Forced to Adopt Catalytic Activity

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The recently elucidated 3D structure of the complex between the biotin-binding, egg-white glycoprotein avidin and the azo dye HABA (4′-hydroxyazobenzene-2-carboxylic acid) has revealed that the dye binds to the protein as the hydrazoquinone tautomer. This interaction causes an instantaneous shift in the absorption spectrum from λ_{max} 348 nm (orange) of the free HABA to λ_{max} 500 nm (red) of the complexed tautomer.[1] In this communication we demonstrate that avidin can be forced to catalyze hydrolytic reactions on derivatives of HABA, in order to accommodate the azo dye in its tautomeric form at its preferred binding site. The overall reaction is described in Figure 1.

In order to check whether such a cleavage can indeed be accomplished, we prepared different derivatives of HABA in which the hydroxyl group of the phenol is blocked with different functional groups (Table 1). In this context, we prepared esters (R_1, R_2), carbonates (R_3, R_4), a carbamate (R_5), and the mesylate (R_6) derivatives of HABA.[2] We then examined whether, upon incubation with avidin, the red color of the avidin–HABA complex appears gradually but faster than the free HABA derivative in the absence of avidin. If so, this would indicate that avidin incorporates the HABA derivative in the binding pocket and causes its hydrolysis.

Figure 2 shows the absorption spectra of HABA and its acetyl derivative (acHABA) before and after incubation with avidin. As can be seen from the figure, HABA absorbs at 348 nm whereas acHABA absorbs at 322 nm and displays no absorbance at 500 nm.[3] The shift in color from 348 to 322 nm was found to be typical for the esterification of the phenol group in HABA. Upon addition of identical amounts of avidin, the absorption maximum of underivatized HABA undergoes an immediate shift to 500 nm. In contrast, the shift in the spectrum of acHABA is gradual and depends on the time of interaction and the concentration of avidin.

To determine whether accelerated hydrolysis is achieved in the presence of avidin, samples of the HABA derivatives were incubated with and without avidin. The reaction velocity of ester hydrolysis was examined by measuring the formation of the reaction product (HABA) as a function of time. This was accomplished spectroscopically at 500 nm. Background (autohydrolysis) was measured by the addition of avidin to solutions containing the desired HABA ester in buffer, followed by the immediate measurement of the avidin–HABA complex at 500 nm.

Figure 3 shows the kinetics of avidin-promoted acHABA hydrolysis. The plot shows a typical sharp burst followed by saturation, a characteristic property of enzymatic reactions. Acceleration rates and kinetic parameters[4] were examined for the substrates shown in Table 1.

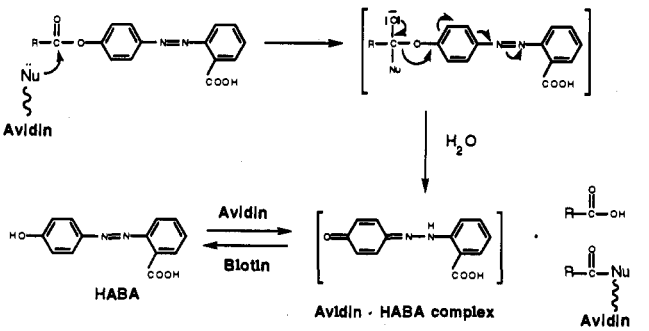

Figure 1. Proposed mechanism of enzymatic hydrolysis of HABA esters by avidin. The HABA derivative is bound at the binding site of the protein, and the phenol ester bond is attacked by an appropriate nucleophile (Nu). After cleavage of the bond, HABA is bound as the hydrazoquinone tautomer at the active site of avidin. The dye can be displaced from avidin by biotin. The catalysis can be formulated in enzymatic terms: $E + S \leftrightarrow [ES] \rightarrow [EP^*] + P$, where E represents avidin, S is the derivatized HABA, P* is the hydrazoquinone tautomer of HABA, and P is the underivatized HABA molecule.

Table 1. Kinetic Parameters for the Hydrolysis of HABA Derivatives by Avidin[a]

HABA derivative	K_m (mM)	v_{max} (nM s^{-1})	k_{cat} (10^{-5} s^{-1})	k_{uncat} (10^{-6} s^{-1})	k_{cat}/k_{uncat}
R_1 = MeCO	2.1 ± 0.3	54.7 ± 5.0	21.9 ± 2.5	1.1 ± 0.1	199
R_2 = EtCO	0.7 ± 0.2	21.1 ± 1.9	8.4 ± 0.9	2.4 ± 0.3	35
R_3 = EtOCO	1.0 ± 0.3	9.5 ± 2.1	3.8 ± 0.9	3.2 ± 0.4	12
R_3 = iBuOCO	2.6 ± 0.5	23.8 ± 3.9	9.5 ± 1.8	6.7 ± 0.7	14
R_5 = $MeSO_2$	*b*				
R_6 = Me_2NCO	*b*				

[a] A Milton Roy Spectronic 1201 spectrophotometer was used to measure absorption changes at 500 nm. Cells containing 4.5 mg of avidin in 0.9 mL of 0.1 M phosphate buffer, pH 7.3, were pre-equilibrated at 32 °C. The kinetic run was initiated for each substrate concentration by the addition of 100 μL of a solution of the designated HABA ester in methanol. [b] No hydrolysis was observed for derivatives R_5 and R_6.

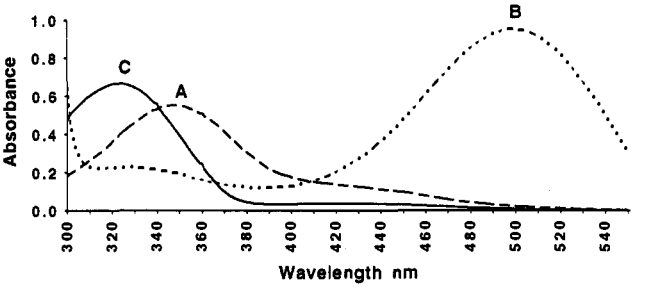

Figure 2. Absorption spectra of HABA (A), the avidin–HABA complex (B), and acHABA (C). The respective dye molecule or the avidin–HABA complex was brought to a concentration of 3×10^{-5} M in 0.1 M phosphate buffer, pH 7.3. The spectra were measured with a Milton Roy Spectronic 1201 spectrophotometer.

HABA esters of carboxylic and carbonic acids were enzymatically hydrolyzed by avidin. In contrast, the carbamate and sulfonate derivatives were stable to hydrolysis under these conditions. The acceleration level above background was about 200-fold for acHABA and lower for the other derivatives.

To check whether the hydrolysis involves the binding site of avidin, the reaction with acHABA was performed in the presence of increasing amounts of biotin. The data indicate that the extent of hydrolysis was dependent on the concentration of biotin. As the amount of biotin increased, the extent of hydrolysis was reduced proportionately (Figure 4). This corroborates the

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(2) The carboxylic acid esters were prepared by reaction of HABA with the carboxylic acid anhydride and basic catalysis by 4-(dimethylamino)pyridine. The carbonates and the mesylate derivative were synthesized from the corresponding chloroformate and methanesulfonyl chloride, respectively, in a Schotten–Baumann reaction. Helfreich, B.; Papalambrou, P. *Justus Liebigs Ann. Chem.* **1942**, *552*, 235–241. The *N,N′*-dimethylcarbamate was synthesized from HABA and the carbamoyl chloride in pyridine. Wheatley, W. B.; Fitzgibbon, W. E.; Stiner, G. F.; Cheney, L. E. *J. Am. Chem. Soc.* **1957**, *79*, 747–749.

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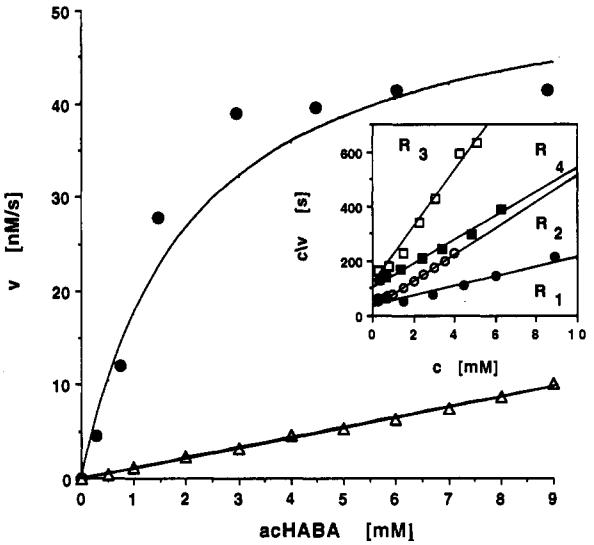

Figure 3. Kinetics of avidin-induced hydrolysis of HABA derivatives. The Michaelis–Menten plot of the measured hydrolysis of acHABA (dots) and the theoretical curve are shown. The rate of autohydrolysis (v_{uncat}) is directly proportional to the acHABA concentration (connected triangles). The kinetic parameters for the different HABA derivatives (see Table 1) were obtained from the Hanes plot (insert), and the data were subjected to linear regression analysis.

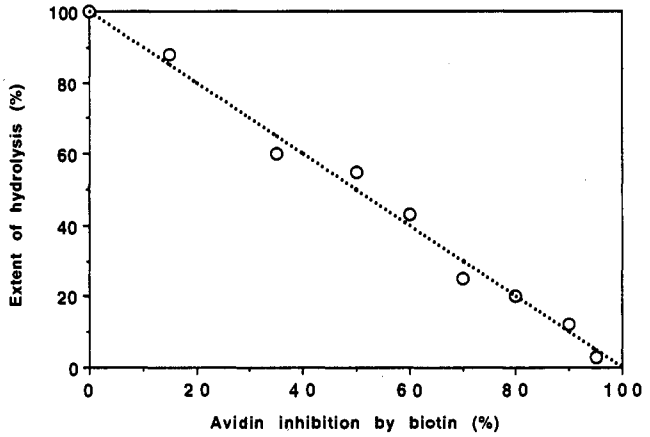

Figure 4. Inhibition by biotin of avidin-catalyzed HABA ester hydrolysis. Samples containing 4.5 mg/mL avidin and 5 mM acHABA in phosphate buffer (0.1 M, pH 7.3) were treated with increasing amounts of biotin, and ester hydrolysis over background was measured (dots). The decrease in hydrolysis is directly proportional to the increase of avidin inhibition by biotin (dashed line).

involvement of the binding site of avidin in the catalytic process. Other proteins, notably bovine serum albumin (BSA), did not accelerate the reaction, despite the fact that BSA also binds HABA.[5]

It was shown in previous studies that avidin also hydrolyzes biotinyl *p*-nitrophenyl ester (BNP).[6] In the case of BNP there was only one cycle of hydrolysis, since the strong binding ($K_d \sim 10^{-15}$ M) of biotin (the hydrolyzed product) to avidin prevents further turnover. On the other hand, the observed K_d ($\sim 6 \times 10^{-6}$ M) of the avidin–HABA complex is more appropriate for turnover. It was therefore of interest to determine whether turnover would be observed for the HABA esters and whether hydrolysis would continue until the ester was completely consumed.

A method to study turnover was thus established. For this purpose, avidin (4.5 mg/mL in phosphate buffer, 0.1 M, pH 7.3) was first treated with an equimolar concentration of underivatized HABA. The desired substrate (HABA ester) was then added to a concentration of 5 mM, and product–substrate exchange was estimated. To estimate the exchange, samples were taken at intervals over 48 h, and free avidin was added to assess the concentration of HABA by immediately measuring the absorbance at 500 nm. Samples of acHABA, either in buffer alone or in the presence of biotin-blocked avidin, were used as controls.

From these experiments, a slow turnover could be demonstrated, probably due to product inhibition, since the reaction product (HABA) binds much better to avidin than its ester derivatives. No loss in the enzymatic activity of avidin was observed. Interestingly, the hydrolysis rate and turnover of acHABA were found to be higher for streptavidin than for avidin (unpublished results). This is not particularly surprising, since the affinity of HABA to streptavidin is much lower than to avidin, and the dissociation constant (ca. 10^{-4} M) is in the range commonly observed for most enzymes.[7]

To study the mechanism of the hydrolysis, we prepared a radioactive derivative of acHABA, in which the acetyl group was labeled with ^{14}C. A 3-fold excess was reacted with avidin for 10 h, following which the protein solution was dialyzed exhaustively at pH 2.5 and the radioactivity was measured. Surprisingly, about 0.3 acetyl group was found to be *covalently* bound per subunit of avidin. To determine which residues may have been modified, the pH of the dialysis was raised to 10.5. This treatment resulted in a loss of radioactivity, indicating that the acetylated residue(s) may be serine, threonine, and/or tyrosine, but not lysine. Tyrosine can also be excluded, since there is only one tyrosine in the protein which is located near the carboxylic group of HABA.[1b]

From the 3D structure of the HABA–avidin complex, we can speculate that the residue which most probably undergoes modification is Ser-73, which forms a hydrogen bond with the phenol ring of HABA. Nevertheless, we cannot yet exclude Ser-75, Thr-40, or Thr-38, all of which are adjacent to the phenol ring of HABA and form hydrogen bonds with the carboxyl group of biotin.[1a] Efforts to determine which residue or residue(s) are modified are currently in progress. Preliminary results have indicated that the acetylated avidin was still capable of binding biotin and HABA.

In summary, this study is the first example of catalytic activity which can be conferred on a known noncatalytic binding protein by designing a chemically altered ligand. In this regard, we could predict (on the basis of the known 3D structure of the avidin–HABA complex) the type of substrate required for accelerated hydrolysis by the native protein. We were also able to elucidate the probable mechanism of action (Figure 1). The resultant "avzyme" is thus in contrast to the "abzymes" where a substrate or a transition state analog thereof is first constructed, following which an antibody with catalytic activity is then screened.[8]

Finally, our studies have confirmed, by chemical methods, the predicted mode of binding of HABA to avidin, i.e., that the anthranilic portion of HABA is recognized by avidin. This finding may insinuate yet another enigmatical involvement of avidin and related biotin-binding proteins in nature, over and above its unusually strong binding of biotin. In this context, anthranilic acid plays an important role in tryptophan biosynthesis and degradation, and avidin or its relatives may, in some manner, intervene in these processes.

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