Acyl Transfer versus Catalysis with Avidin

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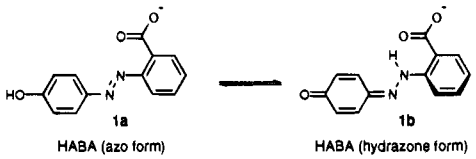
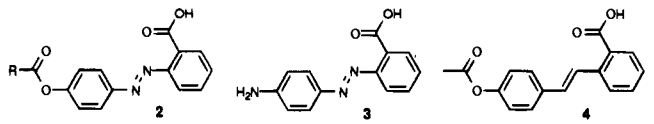
The importance and practical application of avidin, particularly in the avidin-biotin technology,[1] can never be overestimated. Nevertheless, the exact biological role of this ubiquitous protein is still unclear.[2] Two recently reported crystallographic studies of the complexes of 2-[(4′-hydroxybenzyl)azo]benzoic acid (HABA) with streptavidin[3] and with avidin[4] show that the HABA ligand, which adopts a planar conformation (azo form, **1a**; yellow, $\lambda_{max} = 350$ nm) in aqueous media, is distorted from planarity to the hydrazone tautomer **1b** (red, $\lambda_{max} = 500$ nm) upon binding to the protein. This raises an intriguing question: Can the ability of avidin to distort its ligand be utilized as a driving force in catalysis?



We reasoned, for example, that a similar distortion of HABA carboxylate **2** within the avidin binding site would enhance the carbonyl electrophilicity and promote nucleophilic attack either by a water molecule or by a nucleophilic residue of the host protein. While attack by water would result in avidin-catalyzed hydrolysis of **2**, attack by an avidin residue would lead to affinity labeling of the protein. Here we provide 3-fold evidence that **2** is indeed activated in the presence of avidin but the result is affinity labeling and not catalytic hydrolysis.[5]



A number of HABA carboxylates, **2a-c**, were prepared,[6] and their conversion to HABA, **1**, as a function of time was monitored by HPLC. Measurements of initial rates at pH ranging from 4.5 to 9.0 show that the reaction is significantly enhanced when avidin is added to the mixture. No rate enhancement is observed when both avidin and biotin (in a stoichiometric ratio) are added, indicating that the reaction occurs specifically at the biotin/HABA binding site.

The accelerated conversion of HABA acetate, **2a**, to **1** in the presence of avidin has been interpreted by Vetter et al. in terms of avidin-catalyzed hydrolysis of **2a**.[5] To verify that interpretation, we carried out the hydrolysis of **2a** in pH 6.8 at 20 °C, and monitored not only the formation of one product, **1** (by HPLC), but also the formation of the second product, acetic acid (using a pH-stat). The reaction that was carried out in the presence of avidin was compared with a "background" reaction where the effect of avidin was neutralized with equimolar amounts of biotin. Indeed, accelerated formation of **1** is observed in the biotin-free experiment as compared with the background reaction. The situation with respect to acetic acid, however, is exactly the opposite. While in the background reaction the two products are produced in equimolar ratio, in the biotin-free mixture less than 0.5 mol of acetic acid is produced per 1 mol of **1** formed (Figure 1). These observations are inconsistent with the notion of avidin-catalyzed hydrolysis of **2a** and are agreeable with an acyl-transfer reaction. Furthermore, as can be clearly concluded from the figure, the acetylated avidin cannot be considered as an unstable intermediate in the hydrolytic pathway. Judging from the unchanged concentration of acetic acid over long periods of time, that species is a very stable compound under the reaction conditions.

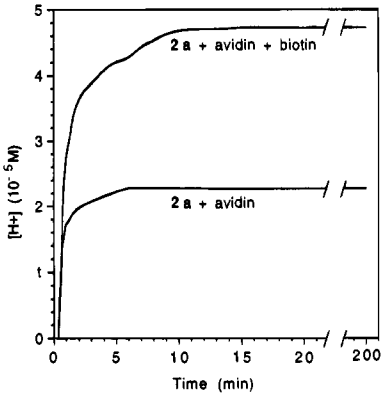


Figure 1. Amount of acetic acid produced in the hydrolysis of **2a**. Two reactions were carried out at 20 °C, pH 6.8, in two identical aqueous mixtures (10 mL each) containing phosphate buffer (2 mM), NaCl (2 mM), THF (2%), and avidin (1×10^{-4} M subunits). Biotin (1×10^{-4} M) was added to one of the mixtures. HABA acetate, **2a** (5×10^{-5} M), was added to these mixtures, and the pH was kept constant by automatic titration with NaOH (10 mM) using a pH-stat (Titrator 11, pH meter 26, Radiometer, Copenhagen). The amount of acetic acid formed was calculated from the amount of base added (after subtracting the amount needed to neutralize the benzoic acid function in both **2a** and **1**). The upper curve represents the amount of acetic acid released in the reaction mixture containing biotin. The lower curve represents the amount of acetic acid released in the biotin-free mixture. As can be seen from both curves, **2a** is consumed within the first 5 min of the reaction.

The most convincing evidence for an acyl-transfer reaction is obtained from electrospray mass spectrometry experiments.[7] The main mass in the spectrum of avidin from egg white (Sigma No. A-9279) is 15 966 Da, which corresponds to an avidin subunit. The spectrum of a Sephadex-purified sample of avidin that has been treated with **2b** at pH 7.0 for 48 h[8] shows two main peaks, 15 966 and 16 050 Da, in a ratio of 46:54, respectively. The mass difference between these two species (84 mass units) corresponds to monoacylation (54% yield) of the avidin subunits with a valeroyl group. Similar analysis of avidin that has been treated with HABA heptanoate, **2c**, reveals

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(2) Green, N. M. Avidin-Biotin Technology. In *Methods in Enzymology*; Wilchek, M., Bayer, E. A., Eds.; Academic: San Diego, London, 1990; Vol. 184, p 51.

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(4) Livnah, O.; Bayer, E. A.; Wilchek, M.; Sussman, J. L. *FEBS Lett.* **1993**, *328*, 165.

(5) An independent effort to achieve catalysis with avidin was reported after this paper was submitted: Vetter, S.; Bayer, E. A.; Wilchek, M. *J. Am. Chem. Soc.* **1994**, *116*, 9369.

(6) Compounds **2a-c** were prepared by treatment of HABA with the appropriate anhydride in chloroform/pyridine at 25 °C for 24 h.

(7) We thank Dr. Gary Siuzdak of The Scripps Research Institute for carrying out these experiments using a Perkin-Elmer SCIEX API3 electrospray mass spectrometer. The spectrometer has been purchased by the Lucille P. Markey Charitable Trust and an NIH instrumentation grant (1 S10 RR07273-01).

(8) Avidin (15 mg/mL, 10^{-3} M) was reacted with **2b** (10^{-3} M) in water: DMF = 9:1 at 25 °C for 48 h and then excessively dialyzed against deionized water. The protein solution was then further purified on a Sephadex column and analyzed by electrospray mass spectrometry.

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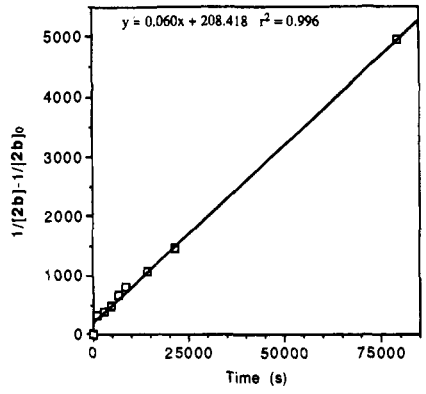

Figure 2. Determination of the second-order rate constant. Reaction was carried out using equal concentrations of reactants. Avidin (0.064 mM avidin subunits) and **2b** (0.064 mM) were mixed in [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane buffer (Bistris) (50 mM buffer, 50 mM NaCl, 1% DMF) at 25 °C and pH 7.0. The rate constant ($k = 0.060$ s^{-1} M^{-1} at 25 °C and pH 7.0) was calculated from the equation $1/S = 1/S_0 + kt$ where S is the molar concentration of **2b**.

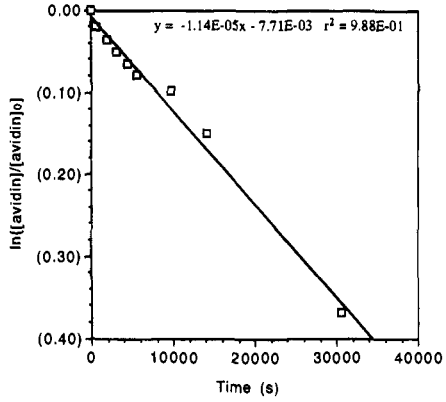

Figure 3. Determination of the pseudo-first-order rate constant. Reaction was carried out as described above in Figure 2 with the exception that the concentration of **2b** was now 0.640 mM and the concentration of avidin subunits 0.064 mM and the solution contained 10% DMF. The pseudo-first-order rate constant ($k = 1.14 \times 10^{-5}$ M^{-1} at 25 °C and pH 7.0) was calculated from the equation $\ln\{[\text{avidin}]/[\text{avidin}]_0\} = kt$. A second-order rate constant ($k = 0.020$ s^{-1} M^{-1}) was obtained either by dividing the pseudo-first-order k by the concentration of **2b** or by using the regular second-order rate equation $[\ln(S/A) - \ln(S_0/A_0)]/(S_0 - A_0) = kt$.

two species of 15 966 and 16 078 Da in a ratio of 75:25, respectively. This is consistent with monoacylation of the avidin subunits with a heptanoyl group (112 mass units). The incomplete acylation of avidin may be understood on grounds of the strong affinity of avidin to HABA ($K_d = 6 \times 10^{-6}$ M).[2] HABA, which is formed not only in the trans-acylation reaction, but also in the background hydrolysis, inhibits the acyl-transfer reaction via competitive binding to avidin. Indeed, excessive dialysis of the product mixture followed by treatment with fresh **2b** and repeating this cycle five times increases the yield of acylated avidin up to 80% (by mass spectral analysis).

Finally, the above conclusions are strongly supported by kinetic data, none of which agree with Michaelis–Menten kinetics (as HABA acetate, **2a**, was found to be too reactive in water at room temperature, all kinetic studies were more conveniently carried out with HABA valerate, **2b**).[9] They are, however, consistent with second-order kinetics, first-order in avidin and first-order in **2b**.[10] In one experiment we use equal concentrations of **2b** and avidin subunits (Figure 2). Data from this experiment (at pH 7.0, 25 °C) are in agreement with the second-order model with a calculated rate constant $k = 0.060$ s^{-1} M^{-1}. A similar experiment with a different ratio of avidin to **2b** (1:1.6) affords a similar rate constant ($k = 0.064$ s^{-1} M^{-1}) (data not shown). Use of **2b** in large excess with respect to avidin (Figure 3) generates conditions pseudo-first-order in avidin (pseudo-first-order rate constant $k = 1.14 \times 10^{-5}$ s^{-1}).[11]

At this point we can only speculate on which of the protein residues is acylated in this reaction. Crystallographic data of the avidin–HABA complex show close proximity, and even hydrogen bonding between the Ser^{73} oxygen and the phenolic oxygen of HABA.[4] Therefore, one may propose that the acyl group is transferred to that serine oxygen, thus forming an ester function. Nevertheless, treatment of our avidin valerate with a basic buffer (pH 11, 25 °C, 14 h) followed by purification on a Sephadex column and mass spectral analysis showed only a minor increase in the intensity of the 15 966 Da peak relative to the 16 050 Da peak. Moreover, attempts to react valeroyl-avidin with amine nucleophiles, including **3**, phenylhydrazine, and hydroxylamine at pH 9.0, 37 °C, did not produce any detectable amounts of the corresponding valeramides. Again, this could be explained by product inhibition, with the approach of the nucleophile to the binding site being hindered by competitive binding of either HABA or the hydrophobic chain of the valerate ester.

An interesting question is whether acylation of avidin occurs merely due to proximity to the protein's nucleophilic residue or whether there is indeed any specific activation of the substrate. To check this, we used stilbene **4**,[12] which is a stable analog of the azo tautomer of **2a**. Attempts to react **4** with avidin did not produce any detectable phenolic product, suggesting that only an activated form of **2** leads to productive encounter with avidin.

In conclusion, we have shown here that the enhanced consumption of HABA carboxylates **2** in the presence of avidin does not reflect its avidin-catalyzed hydrolysis but a specific, bimolecular acyl-transfer reaction. Our 3-fold evidence is based on (a) experiments with a pH-stat, (b) electrospray mass spectrometry, and (c) second-order kinetics. Crystallographic studies with avidin valerate aimed to identify the acylated protein residue are underway. Although no specific transfer of the acyl group from acylated avidin to another nucleophile has yet been achieved, we predict that avidin-catalyzed acyl-transfer reactions would be possible when the problem of product inhibition is solved. One obvious application of these findings would be the kinetic resolution of chiral esters. Work is currently in progress to achieve these goals with avidin as well as with streptavidin.

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Supporting Information Available: Figures showing second-order and pseudo-first-order reaction kinetics for **2b** and avidin and the determination of pK_a values of **1**, **3b**, and **3e** and second-order rate constants (6 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, can be ordered from the ACS, and can be downloaded from the Internet; see any current masthead page for ordering information and Internet access instructions.

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(9) Reactions were carried out by mixing **2b** (5–50 μM) with avidin (1.5 μM) at 25 °C, pH 7.0 (50 mM [bis(2-hydroxyethyl)amino]tris-(hydroxymethyl)methane buffer (Bistris), 50 mM NaCl, 1% DMF). Measurements of initial rates (up to 5% conversion) were carried out with a Hitachi L6200A HPLC using an L4200 UV–vis detector operated at 330 nm, a D6000 analyzer, and a reversed-phase Supelcosil LC-18 column (25 cm × 4.6 mm). A Lineweaver–Burk analysis of the data ($1/V$ vs $1/S$) does not fit a linear model.

(10) Espenson, J. H. *Chemical kinetics and reaction mechanisms*; McGraw-Hill: New York, 1981; pp 16–30.

(11) The smaller value of the second-order rate constant ($k = 0.020$ s^{-1} M^{-1}) derived from this experiment may be attributed to the higher proportion of cosolvent used here (10% DMF) as opposed to 1% DMF employed in all other experiments.

(12) Stilbene **4** was prepared from methyl *o*-toluate by benzylic bromination with *N*-bromosuccinimide followed by substitution with triethyl phosphite and olefination with 4-[(*tert*-butyldimethylsilyl)oxy]benzaldehyde. Treatment with aqueous NaOH (1 N) followed by acetylation with acetic anhydride in pyridine afforded **4**.