

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 ($k = 0.060 \text{ s}^{-1} \text{ M}^{-1}$ 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**.

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 ($K_d = 6 \times 10^{-6} \text{ M}$).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 $k = 0.060 \text{ s}^{-1} \text{ M}^{-1}$. A similar experiment with a different ratio of avidin to **2b** (1:1.6) affords a similar rate constant ($k = 0.064 \text{ s}^{-1} \text{ M}^{-1}$) (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 $k = 1.14 \times 10^{-5} \text{ s}^{-1}$).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 Ser⁷³ 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,

(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 \times 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 ($k = 0.020 \text{ s}^{-1} \text{ M}^{-1}$) 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.

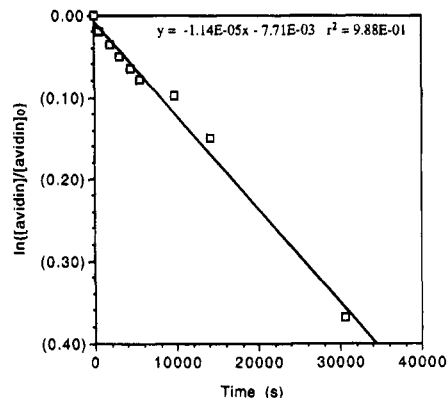


Figure 3. Determination of the pseudo-first-order rate constant. Reaction was carried out as described above in Figure 2 with the exception that the concentration of **2b** was now 0.640 mM and the concentration of avidin subunits 0.064 mM and the solution contained 10% DMF. The pseudo-first-order rate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

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.

Acknowledgment. We thank D. M. Hilvert, R. M. Ghadiri, and J.-L. Reymond of Scripps, T. Baasov of Technion, S. Hoz of Bar-Ilan University, and D. E. Cane of Brown University for helpful comments on the paper.

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.

JA9432877

(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*-butyldimethylsilyloxy)benzaldehyde. Treatment with aqueous NaOH (1 N) followed by acetylation with acetic anhydride in pyridine afforded **4**.