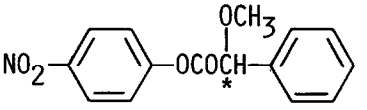
Tetrahedron Letters,Vol.23,No.15,pp 1593-1596,1982 0040-4039/82/151593-04$03.00/0
Printed in Great Britain

A HYDROLYTIC ENZYMELIKE BEHAVIOR OF BOVINE SERUM ALBUMIN IN HYDROLYSIS OF *p*-NITROPHENYL ESTERS

Toshio Kokubo,* Toshio Uchida, Shigeo Tanimoto, Masaya Okano, and Toyonari Sugimoto*
Institute for Chemical Research, Kyoto University, Uji, Kyoto 611, Japan

Summary: The investigation of hydrolysis of *p*-nitrophenyl acetate (NPA) catalyzed by bovine serum albumin (BSA) proved that the initial rate in the steady state follows a Michaelis-Menten equation. In the use of D- and L-*p*-nitrophenyl α-methoxyphenylacetates (D- and L-NPMA's) as an enantiomeric ester, the L-enantiomer is hydrolyzed about three times faster than the D-enantiomer, mainly due to stronger binding of the former enantiomer by BSA.

Several proteins, normally not classified as a hydrolytic enzyme, can catalyze hydrolysis of some esters under a physiological pH condition.[1] It has before been recognized that BSA is one of such proteins, although the mechanistic details of catalytic hydrolysis still remain unclarified.[2] In this communication we report the results of BSA-catalyzed hydrolysis of NPA and two enantiomeric esters of NPMA under the steady state condition ([BSA]<<[ester]), not quite attempted so far, at pH 8-9 where BSA shows remarkable hydrolytic activity.



D-, L-NPMA

The hydrolysis of *p*-nitrophenyl ester was followed spectrophotometrically by measuring the increasing absorbance of *p*-nitrophenolate anion at 402 nm. The reaction was initiated by injecting a small aliquot of dioxane solution of *p*-nitrophenyl ester to a phosphate or Tris-HCl buffer solution with BSA or without BSA. The concentration of BSA was kept constant at approximately 0.7 μM,[3] and those of NPA and NPMA[4] were varied over the range of about 50-200 μM and about 30-100 μM, respectively. All the kinetic studies were conducted at 25.0±0.1°C.

A mode of hydrolysis of NPA in the presence of a small amount of BSA exhibits biphasic behavior, as a representative example is shown in Figure 1. Immediately after initiating the

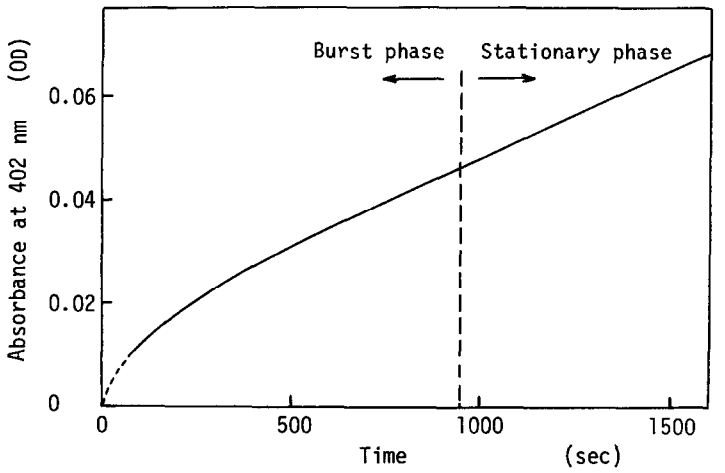


Figure 1. The BSA-catalyzed hydrolysis of NPA in a 1% dioxane-0.05 M phosphate buffer (pH 8.0) at 25.0°C; [BSA]=0.73 μM and [NPA]=67 μM.

reaction, rapid release of *p*-nitrophenolate anion was observed. As the reaction proceeded the rate decreased gradually, and the initial burst phase shifted to a stationary phase before a few percent of the ester was consumed. Then, the stationary state continued during the further consumption of at least 5% of NPA, and the rate is composed of both the rates for uncatalyzed hydrolysis and for catalytic hydrolysis related to the action of BSA.[5] When the reciprocal of the latter rate was plotted against the reciprocal of the concentration of NPA, a straight line was obtained, thereby indicating that the catalytic rate at the stationary state follows a Michaelis-Menten equation. Accordingly, this type of behavior can be interpreted as a manifestation of the reaction resembling hydrolytic enzyme-catalyzed hydrolysis in the following sequence with its associated kinetic parameters, K_m and k_{cat}: $\text{BSA} + \text{NPA} \overset{K_m}{\rightleftharpoons} \text{BSA·NPA} \xrightarrow[H_2O]{k_{cat}} \text{BSA} + \text{NP} + \text{A}$, where BSA·NPA represents the Michaelis complex, NP *p*-nitrophenol and A acetic acid. Table I shows the

Table I. The kinetic parameters obtained for the BSA-catalyzed hydrolysis of NPA at different pH values at 25°C.

pH	K_m (mM)	k_{cat} ($\times 10^{-3}$ sec^{-1})	k_{un} ($\times 10^{-5}$ sec^{-1})[c]
8.0[a]	0.19	3.4	2.48
8.5[b]	0.31	14	8.43
8.9[b]	0.63	45	17.3

[a] A 1% dioxane-0.05 M phosphate buffer (v/v). [b] A 1% dioxane-0.05 M Tris-HCl buffer (v/v). [c] The first-order rate constant for the hydrolysis of NPA in the absence of BSA.

kinetic parameters obtained for the catalytic hydrolysis at pH 8.0, 8.5 and 8.9 together with the pseudofirst-order rate constant, k_{un} for the uncatalyzed hydrolysis at the same pH values. Because base-catalyzed hydrolysis proceeded very rapidly above pH 9, it was difficult to determine the kinetic parameters correctly in this pH region. There is a tendency of increasing K_m with raising pH: 0.19, 0.31 and 0.63 mM at pH 8.0, 8.5 and 8.9, respectively, all of which are comparable with those obtained by some hydrolytic enzymes.[6] On the other hand, k_{cat} is larger by only a factor of 140-270 than k_{un} in the investigated pH region, where larger rate-acceleration is obtainable at higher pH. Thus, these results obviously demonstrate that very small effect on the rate-acceleration at this k_{cat} step makes BSA to be "a poor hydrolytic enzyme."

Furthermore, the enantioselectivity of the BSA-catalyzed hydrolysis was investigated by using both the enantiomeric esters of D- and L-NPMA's. For each ester the catalytic rate followed the same Michaelis-Menten equation to that observed for NPA. The K_m and k_{cat} values obtained for the hydrolysis at pH 8.0 are listed in Table II. The k_{cat} for D-NPMA is slightly larger than that for the L-NPMA, while the K_m values are significantly different between both the esters, of which the former enantiomer showed a larger value. Thus, on the basis of comparison of k_{cat}/K_m values between the two esters, which represent an index of susceptibility to hydrolysis, it is concluded that BSA exhibits higher hydrolytic activity for the L-enantiomer by about three times than for the D-enantiomer.

Table II. The kinetic parameters obtained for the BSA-catalyzed hydrolysis of D- and L-NPMA's in a 5% dioxane-0.05 M tris-HCl (pH 8.0) buffer (v/v) at 25°C.

	K_m(mM)	k_{cat}($\times 10^{-2}$ sec^{-1})[a]	k_{cat}/K_m($M^{-1}sec^{-1}$)
D-NPMA	0.28	3.0	110
L-NPMA	0.063	2.0	320

[a] The first-order rate constant, k_{un}, for the hydrolysis of each enantiomeric ester in the absence of BSA was $3.11\times10^{-4}sec^{-1}$.

It should be of considerable interest in connection with studies on generation and molecular evolution of proteins that BSA, actually having essential functions of carrying various kinds of organic compounds and metal ions and of maintaining colloid osmotic pressure of blood in biological

system, also catalyzes hydrolysis of ester with full characteristics of some hydrolytic enzymes , *i.e.*, rate-acceleration, turnover of catalyst and enantioselectivity, although all the effects are fairly inferior to those of the enzymes.

References and notes

1. (a) E. Breslow and F. R. N. Gurd, *J. Biol. Chem.*, 237, 371 (1962); (b) S. B. Tove, *Biochim. Biophys. Acta*, 57, 230 (1962); (c) J. I. Harris, B. P. Meriwether, and J. H. Park, *Nature*, 198, 154 (1963); (d) L. Polgár, *Acta Physiol. Acad. Sci. Hung.*, 25, 1, 303 (1964); (e) C. E. Wilde and R. G. O. Kekwick, *Biochem. J.*, 91, 297 (1964).
2. (a) J. J. Bruno and H. J. Ringold, *Fed. Proc.*, 28, 840 (1964); (b) J. T. Tildon and J. W. Ogilvie, *J. Biol. Chem.*, 247, 1265 (1972).
3. The used BSA was Pentex Bovine Albumin Monomer Standard (Lot No. p336) from Miles Laboratories Ltd.. The concentration of BSA was determined by comparing the measured absorbance at 280 nm with the extinction coefficient based on dry weight measurement ($E_{280}^{1\%}$=6.53). The molecular weight of BSA was taken as 66,200 (P. Q. Behrens, A. M. Spiekerman, and J. R. Brown, *Fed. Proc.*, 34, 591 (1975)).
4. NPA was obtained from Koch-Light Laboratories Ltd. and used without further purification. D- and L-NPMA's were prepared according to the procedure described by Moss *et al.* from each optically pure mandelic acid and purified by recrystallization from ether-hexane (R. A. Moss and W. L. Sunshine, *J. Org. Chem.*, 39, 1083 (1974)).
5. The catalytic rate (v) was determined by the following equation:

$$v = v_{obs} - k_{un}[NPA]$$

where v_{obs} is the observed rate and k_{un} is the pseudofirst-order rate constant for the uncatalyzed hydrolysis.
6. J. B. Jones and J. F. Beck, *Application of Biochemical Systems in Organic Chemistry (J. B. Jones, C. J. Sih, and D. Perlman, eds)*, *John Wiley & Sons, New York*, p. 112 (1976).
7. (a) T. Sugimoto, T. Kokubo, Y. Matsumura, S. Tanimoto, and M. Okano, *Bioorg. Chem.*, 10, 104 (1981); (b) T. Sugimoto, T. Kokubo, J. Miyazaki, S. Tanimoto, and M. Okano, *ibid.*, 10, 311 (1981).

(Received in Japan 25 December 1981)