THE PEPSIN CATALYSED HYDROLYSIS OF BIS-P-NITROPHENYL SULFITE

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SUMMARY: The reaction of pepsin with bis-p-nitrophenyl sulfite has been studied at pH 2.2 and 25°C by observing the production of p-nitrophenol spectrophotometrically. Under the condition where the pepsin concentration is much greater than the ester concentration the observations suggest that the p-nitrophenol is not liberated in a single kinetic process.

Certain organic sulfite esters are good substrates for pepsin and are useful in probing the catalytic mechanism of this enzyme (1,2). Equation 1 incorporates the essential features of most proposed mechanisms for the pepsin catalysed hydrolysis of sulfite esters and peptides (3-5).

$$E + S \rightleftharpoons ES_1 \longrightarrow ES_2 \longrightarrow E + P_2 + P_1 \qquad (1)$$

^{18}O tracer experiments with sulfite esters provide evidence for a covalent E-S intermediate in the sulfite esterase reaction (6).

In this study we have investigated the pepsin catalysed hydrolysis of bis-p-nitrophenyl sulfite (BNPS) under the conditions of substrate concentration greater than enzyme concentration, [S] >> [E], and substrate concentration much less than enzyme concentration, [S] << [E]. A report by May and Kaiser (4) described the observation of monophasic kinetics under the condition [E] >> [S]. They observed the p-nitrophenol to be liberated in a single first order process. The results reported here under very similar conditions suggest that there are two steps in the production of the nitrophenol.

MATERIALS AND METHODS

BNPS was made by the method of Robinson and White (7). Pepsin was obtained from Worthington Biochemical Corporation.

Steady state kinetic runs, [S] >> [E],were carried out at 25°C in 0.05 M phosphate buffer containing 5% CH_3CN at pH 2.2. Appearance of p-nitrophenol was monitored at 325nm. ($\Delta\varepsilon$ for the reaction = 3×10^3). Enzyme concentrations ranged between 1×10^{-6}M and 2×10^{-7}M; BNPS concentration varied from 1×10^{-4}M to 5×10^{-5}M. Due to the very rapid non-enzymatic rate of substrate hydrolysis ($k \cong 0.05$ sec^{-1}) it was not possible to determine k_{cat} and K_M independently but only as the ratio k_{cat}/K_M with the assumption that K_M >> [S]. Kaiser and May quote a value of 8.32×10^{-4}M for K_M at pH 2.

Experiments with [E] >> [S] were carried out using a Gibson-Durrum stopped flow instrument, and the appearance of p-nitrophenol was monitored at 325nm. The mixed solution was in 0.05 M phosphate buffer, 5% CH_3CN, at a pH of 2.2 at 25°C. Enzyme concentrations were varied between 1.24×10^{-4}M and 3.9×10^{-5}M, and substrate was either 5×10^{-6}M or 1×10^{-5}M.

RESULTS AND DISCUSSION†

The steady state experiments gave a value of 4×10^4 M^{-1} sec^{-1} for k_{cat}/K_M. Figure 1 shows reproductions of some typical oscilloscope traces. The reaction appears to be biphasic with an initial rapid release of p-nitrophenol being followed by a slower release of a relatively smaller amount. Due to the very rapid non-enzymatic hydrolysis of BNPS only a small part of the total amount of the nitrophenol is detected in a stopped flow experiment with the instrument available in the standard configuration. If it is assumed that the observed reaction curve represents two kinetic processes and the first order rate constant, k_{OBS}, for the faster step corresponds to that related in the equation derived from equation (1)

$$k_{OBS} = \frac{k_{cat}[E]}{K_M + [E]}$$

we calculate that k_{cat} = 129 sec^{-1} and $K_M = 1.37 \times 10^{-3}$M and therefore k_{cat}/K_M = 9.42×10^4 M^{-1} sec^{-1}. These values compare favorably with those of May and Kaiser.

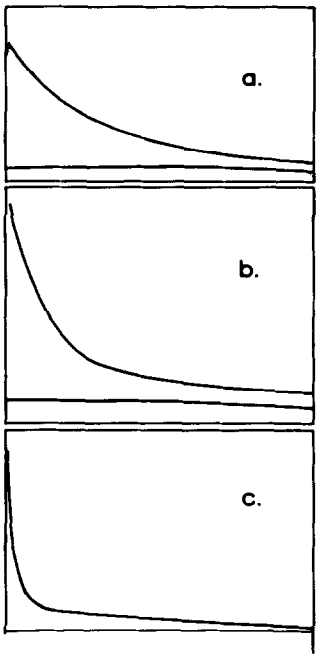

Figure 1. Diagram of oscilloscope traces. [Pepsin] = 4.73×10^{-5}M, [BNPS] = 5×10^{-6}M, pH 2.2, 0.05 M phosphate, 5% CH_3CN, T = 25.0°C. Percent transmission (ordinate) versus time.
(a) Primarily faster step being observed. Total transmission change about 9%. Total time 1 sec.
(b) Primarily first step being observed but some of slower step discernible. Total transmission change about 8%. Vertical scale magnified twofold over that in (a). Total time 2 sec.
(c) Fast step virtually complete within early part of sweep. Slower step observed for remainder of trace. Estimate of total change in transmission for the slower step is about 1 to 1.5%. Vertical scale magnification fourfold over that in (a). Total time 10 sec.

The slower reaction is difficult to characterize since the total absorbance change is very small and consumption of substrate non-enzymatically is approaching a comparable rate. Consequently quantitative apportionment of the p-nitrophenol to each step is not possible. The second step has a rate constant of the order of 0.1 sec^{-1}.

The results are consistent with the ^{18}O work which postulates the involvement in the mechanism of a covalent E-S compound; a carboxylic-sulfurous acid anhydride. Such compounds have been partially characterized and show extreme sensitivity to water (8). Because of the problems mentioned above it is not pos-

sible to assign stoichiometric values in the observed reaction and thereby show definitively whether a covalent intermediate is or is not involved in the reaction. It is clear however, that the reaction is more complex than previously proposed (4). It is not likely that the observations are an artifact due to the nature of the buffer system involved since essentially the same kinetics were observed in a glycine buffer.

There is a possibility that product p-nitrophenol interferes with the quantitative production of itself from BNPS in a discrete kinetic process. Substituted phenols are rather weak inhibitors of pepsin, K_I in the range 0.5 - 2 x 10^{-2}M at pH 2 and 37°C (9). In the reaction of pepsin with bis-phenylsulfite no inhibition by p-nitrophenol at the concentration levels employed in this work can be detected. Therefore this explanation seems unlikely, but it cannot be ruled out since the effect itself is very small.

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