Kinetics of the chymotrypsin catalyzed oxygen exchange of N-acetyl-3:5-dibromo-L-tyrosine

Enzyme-catalyzed oxygen exchange reactions have been found by RITTENBERG AND SPRINSON[1] and by DOHERTY AND VASLOW[2] for chymotrypsin, and by BENTLEY AND RITTENBERG[3] and STEIN AND KOSHLAND[4] for other enzymes. Quantitative rate measurements are of interest in comparison with those for other substrates and also the particular simplicity of these reactions makes rate studies attractive.

For the system, chymotrypsin-N-acetyl-3:5-dibromo-L-tyrosine, equilibrium binding data are available[2] and the equilibrium constants, K_E, may be compared with the kinetically obtained Michaelis constant, K_M, for the exchange reaction

$$R-C(=\,^{16}O)-\,^{16}O^- + E \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} R-C(=\,^{16}O)-\,^{16}O^- \cdot E$$

$$R-C(=\,^{16}O)-\,^{16}O^- E + H_2{}^{18}O \xrightarrow{k_3} R-C(=\,^{18}O)-\,^{18}O^- + E + H_2{}^{16}O$$

Replacement of the second oxygen occurs in similar steps.

Identity of the two numbers would make it likely that the complexes studied by the two methods are the same: a larger value of the Michaelis constant could indicate either that non-active complex was measured by the dialysis method or that k_3 is significant in comparison with k_{-1}. Under the latter assumption and condition, k_1 and k_{-1} can be explicitly calculated. The Michaelis constant for the isotope exchange reaction is

$$\frac{k_{-1} + k_3}{k_1}$$

as in ordinary reactions.

The rate constant and the Michaelis constant are obtained from the first order equation for the exchange reaction,

$$\ln\left(1 - \frac{X}{X_0}\right) = \frac{-k_3 E t}{K_M + S}$$

X_0 = ^{18}O fraction in water = final fraction
X = ^{18}O fraction in substrate
E = enzyme concentration
S = substrate concentration
t = time

which is obeyed if the isotope enrichment and the difference in Michaelis constants for the isotopically different substrates, *i.e.*,

$$X_0\left(\frac{K_M^{18O}}{K_M^{16O}} - 1\right)$$

is small. These equations will be discussed in a detailed report to appear later.

The measurements have been carried out using methods described in detail by LINDERSTRØM-LANG and co-workers[5]. The enzyme and substrate solutions in ^{18}O enriched water are mixed at 0 time, aliquots of 200, 300, or 400 μl containing 5 or 10 mg of substrate are taken at various times, frozen in dry ice and lyophilized. After thorough drying, 15 μl or 25 μl of ordinary water are added to the residues with more enzyme and the solutions allowed to stand 24 hours. The solutions are then cryosublimated onto borax and glycerine, again cryosublimated and the densities measured in the gradient tubes. The borax removes volatile acids formed in the experiment and the glycerine prevents dusting.

The results of experiments at 20° at four different substrate concentrations are shown in Fig. 1 where $\ln(1 - X/X_0)$ is plotted as a function of time. Turnover constants $-S/E\ t \ln(1 - X/X_0) = k_3 S/(K_M + S)$ are calculated for each point and there ciprocal of the average for each experiment plotted against reciprocal concentration in Fig. 2. From the straight line obtained by least squares treatment the slope and intercept of the curve have been obtained following LINEWEAVER AND BURK[6]. Several experiments shown (open circles) have not been used in the averaging. These were from a new sample of substrate which did yield results consistent with the others after further purification. Two other experiments using a different and possibly impure water sample and a doubtful enzyme preparation gave erratic results and were also excluded.

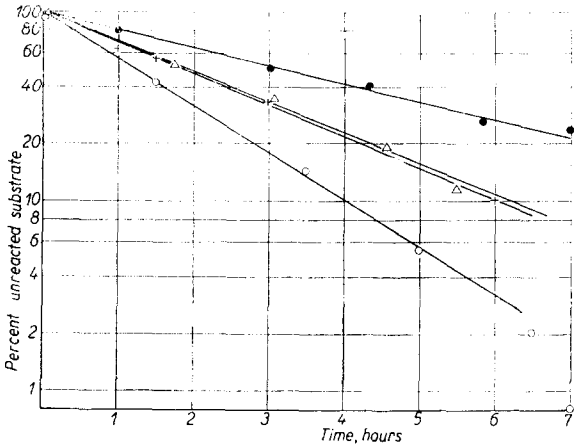

Fig. 1. ● Substrate 0.117 M, enzyme $2.10 \cdot 10^{-4}$ M
+ Substrate 0.0588 M, enzyme $2.08 \cdot 10^{-4}$ M
△ Substrate 0.0407 M, enzyme $1.63 \cdot 10^{-4}$ M
○ Substrate 0.0228 M, enzyme $2.10 \cdot 10^{-4}$ M

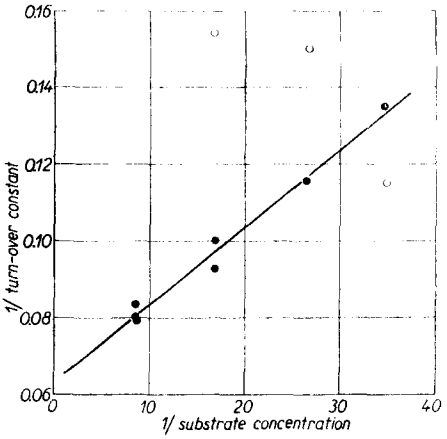

Fig. 2.

Another group of experiments was carried out at 30° C and 0.117 molar substrate concentration using mainly the method described in reference[5] and one experiment of three points using the present method.

At 20° C and pH 7.2 the Michaelis constant is found to be 0.032 ± 0.003 and the equilibrium constant 0.034 ± 0.004. The difference is well within experimental error and thus the complex measured by the equilibrium method appears to be identical with that of the Michaelis theory. Within these limits of error, k_{-1} is at least 7 times k_3.

The rate constant k_3 at 20° C is 316 gram atoms oxygen/mole enzyme/hour[8], each substrate molecule having two exchangeable oxygens. At 30° C and 0.117 M the turnover constant is 380. Using the equilibrium constant[7], 0.05, as the Michaelis constant, k_3 at 30° C is 540 and the apparent heat of activation, 10^3 cal. The rate of hydrolysis of N-acetyl-3:5-dibromo-L-tyrosine amide is available for comparison only at 40° C so it is necessary to calculate the rate constant for exchange at 40° C. This is 920 as compared with 660 for amide hydrolysis. For a number of amides hydrolyzed by chymotrypsin, the rate constants recalculated to these units lie between 350 and 1300 at 25° C[9].

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