Consideration of Kinetic Data on α -Chymotrypsin in Terms of Oligomer Activities

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(Received 1 March 1968)

Though the pioneer work by Schwert (1949) revealed the existence of rapid association equilibria in α -chymotrypsin solutions there have been few reported attempts to define the reactivities of the individual oligomers towards substrate. However, from kinetic (Martin & Niemann, 1958; Inagami & Sturtevant, 1965) and molecular-weight (Sarfare, Kegeles & Kwon-Rhee, 1966; Morimoto & Kegeles, 1967) data it has now been established that reaction with substrate or inhibitor proceeds independently of polymerization under conditions where monomer, dimer and trimer coexist. Much less is known about the reactivity of a higher oligomer, probably hexamer (Gilbert, 1955; Nichol & Bethune, 1963), that coexists with monomer in slightly alkaline (pH 8) buffers of low ionic strength (I 0.01–0.05). The purpose of the present communication is to consider this question in the light of existing kinetic data on the hydrolysis of *N*-acetyl-L-tryptophan amide in 0.02M-tris-HCl buffer, pH 7.9 (Huang & Niemann, 1951; Foster & Niemann, 1955).

From the theoretical viewpoint selection of an amide substrate has the advantage that the low rate of product formation permits direct interpretation of the initial reaction velocity in terms of the reversible interaction between enzyme and substrate (Huang & Niemann, 1951). Kinetic data may therefore be compared with those calculated from eqn. (1), which describes the binding of a small solute, S, to a polymerizing protein system when the reactions are competitive (Nichol, Jackson & Winzor, 1967):

$$r_M = \frac{[A][S]pK_A(1 + K_A[S])^{p-1} + X[A]^n[S]qK_C(1 + K_C[S])^{q-1}}{[A](1 + K_A[S])^p + nX[A]^n(1 + K_C[S])^q} \quad (1)$$

The binding function, r_M , defines the number of moles of S bound per base mole of protein, and [A] and [S] are the molar concentrations of the free enzyme and free substrate respectively; p and q refer to the respective numbers of sites on monomer

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and polymer, K_A and K_C being the corresponding intrinsic association constants describing solute binding; X is the association constant for the reaction $nA \rightleftharpoons A_n$. For the case in which the binding of solute is not affected by polymerization substitution of the values np for q and K_A for K_C in eqn. (1) yields eqn. (2):

$$r_M = \frac{pK_A[S]}{1 + K_A[S]} \quad (2)$$

Such simplification of eqn. (1) is not possible in the event that $q \neq np$ or $K_A \neq K_C$ or both, but numerical solutions may be calculated for chosen values of [S] by first determining [A] from the following polynomial:

$$nXM_A(1 + K_C[S])^q[A]^n + M_A(1 + K_A[S])^p - \bar{c}_P = 0 \quad (3)$$

M_A is the molecular weight of monomer, and $\bar{c}_P$ the initial concentration of protein expressed on a weight scale.

In calculations of r_M designed to simulate the binding of *N*-acetyltryptophan amide to α -chymotrypsin in 0.02M-tris-HCl buffer, pH 7.9, the following parameters were assigned fixed values: M_A , 25 000 (Desnuelle, 1960); p , 1 (Balls & Jansen, 1952); K_A , 220 M⁻¹; n , 6; X , 1.3×10^{22} M⁻⁵. Of these values only the last three require comment. K_A was obtained as the reciprocal of the Michaelis constant reported by Foster & Niemann (1955) for the lower

enzyme concentration (0.65 mg./ml.); should polymerization affect the binding of substrate this value would approach more closely the correct K_A than that reported for the higher enzyme concentration (1.30 mg./ml.). In our evaluation of X from the molecular-weight data of Tinoco (1957) monomer and hexamer were assumed to be the only oligomers

Fig. 1. Effects of substrate concentration on the binding of *N*-acetyl-L-tryptophan amide to α -chymotrypsin in 0.02M-tris-HCl buffer, pH 7.9, as reflected in measurements of solute binding or enzyme kinetics (a) and the weight-average molecular weight, M_w , of the solution (b). Solid lines refer to theoretical relationships for experiments with an enzyme concentration of 1.30mg./ml. and models in which α -chymotrypsin hexamer is fully active (curve A) and inactive (curve B). Broken lines C and D refer to experimental binding data inferred from enzyme kinetics (see the text).

present in solutions of α -chymotrypsin under these conditions. An essentially identical estimate ($0.5 \times 10^{22} \text{M}^{-5}$) was obtained from the size of the slower-moving boundary observed in velocity sedimentation, but this procedure, based on eqn. (6) of Gilbert (1955), is of course subject to the same assumption.

For the purpose of illustration, values were calculated for two models, the first involving a polymer with full activity, i.e. $q = 6$ and $K_C = K_A$, and the second an inactive polymer, i.e. $q = 0$ and $K_C = 0$. Results of these calculations are shown in Fig. 1(a), which presents the data in double-reciprocal form. From eqn. (2) it is evident that the first model yields the same linear plot (curve A) for both enzyme concentrations used by Huang & Niemann (1951). This line also describes adequately the data calculated for the second model and the lower enzyme concentration ($\bar{c}_p = 0.65 \text{mg./ml.}$), a result that justifies our use of 220M^{-1} for K_A . However, an increase in the value of $\bar{c}_p$ to 1.30mg./ml. leads to pronounced upward curvature of the double-reciprocal plot for the system with inactive hexamer (curve B); experiments at these two concentrations of α -chymotrypsin therefore suffice theoretically to distinguish between the two selected models. Though Foster & Niemann (1955) mention no evidence of curvature of the Lineweaver & Burk (1934) plot at the higher enzyme concentration the reported value of the Michaelis constant, namely, $5.0 \pm 0.5 \text{mM}$, is slightly higher than that on which

K_A is based; line C in Fig. 1(a) represents the equivalent experimental binding curve, the limits of experimental error being indicated by lines A and D. Clearly distinction between a model in which hexamer is fully active and one in which it is inactive places stringent demands on the accuracy of the assessed experimental error; differentiation between less extreme models, in which the polymer possesses some activity, would be impossible.

An alternative approach to the problem of investigating the reactivity of the hexamer involves the measurement of the weight-average molecular weights of solutions containing α -chymotrypsin and various concentrations of a competitive inhibitor (Sarfare *et al.* 1966). Theoretical relationships pertaining to the higher enzyme concentration and an inhibitor, S, characterized by the same intrinsic binding constant as *N*-acetyl-L-tryptophan amide, are shown in Fig. 1(b). By adopting this method it is clear that distinction between the model with independent sites for binding and polymerization (curve A) and that in which the hexamer is inactive (curve B) would present no difficulty; further, distinction between less extreme models should also be feasible.

The foregoing considerations demonstrate the inability of existing kinetic data on the hydrolysis of *N*-acetyl-L-tryptophan amide by α -chymotrypsin in 0.02M-tris-HCl buffer, pH 7.9, to define the activity of the hexamer coexisting with the monomeric enzyme under these conditions. In addition, the demonstrated theoretical advantage of molecular-weight measurements over kinetic for this purpose is believed to apply generally to investigations of oligomer activity.

- Balls, A. K. & Jansen, E. F. (1952). *Advanc. Enzymol.* **13**, 321.
 Desnuelle, P. (1960). In *The Enzymes*, 2nd ed., vol. 4, p. 93. Ed. by Boyer, P. D., Lardy, H. & Myrback, K. New York and London: Academic Press Inc.
 Foster, R. J. & Niemann, C. (1955). *J. Amer. chem. Soc.* **77**, 1886.
 Gilbert, G. A. (1955). *Disc. Faraday Soc.* **20**, 68.
 Huang, H. T. & Niemann, C. (1951). *J. Amer. chem. Soc.* **73**, 1541.
 Inagami, T. & Sturtevant, J. M. (1965). *Biochemistry*, **4**, 1330.
 Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.
 Martin, R. B. & Niemann, C. (1958). *J. Amer. chem. Soc.* **80**, 1473.
 Morimoto, K. & Kegeles, G. (1967). *Biochemistry*, **6**, 3007.
 Nichol, L. W. & Bethune, J. L. (1963). *Nature, Lond.*, **198**, 880.
 Nichol, L. W., Jackson, W. J. H. & Winzor, D. J. (1967). *Biochemistry*, **6**, 2449.
 Sarfare, P. S., Kegeles, G. & Kwon-Rhee, S. J. (1966). *Biochemistry*, **5**, 1389.
 Schwert, G. W. (1949). *J. biol. Chem.* **179**, 655.
 Tinoco, I., jun. (1957). *Arch. Biochem. Biophys.* **68**, 367.