Relationship between Active Sites and Polymerization Sites in α-Chymotrypsin*

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ABSTRACT: Weight-average molecular weights were determined by means of light-scattering and Archibald ultracentrifuge measurements at pH 6.1–6.2 in 0.2 ionic strength sodium phosphate buffered solutions of α-chymotrypsin at enzyme concentrations between 0.17 and 3%, in the presence of β-phenylpropionate, a competitive inhibitor, at molar inhibitor concentrations of 0.001, 0.004, and 0.04. Results of these experiments have been compared with predicted molecular weights, computed with the aid of reported values for the dissociation constant of the complex between β-phenyl propionate and enzyme in 0.1 ionic strength phosphate buffer at pH 7.8, and for the dissociation constants of the dimeric and trimeric forms of the enzyme in the buffer employed in the present study. These predictions were made on the basis of three distinct models, all assuming the same intrinsic free energy of binding of β-phenylpropionate to each site: (1) a single active binding site on monomer only; (2) a single site each on monomer, dimer, and trimer; and (3) one active site on monomer, two on dimer, and three on trimer molecules. The comparison shows unequivocally that under the conditions of these experiments, each polymer contains all the original binding sites of the parent monomer units in accessible form, *i.e.*, one site on the monomer, two sites on the dimer, and three sites on the trimer molecule, and that polymerization does not take place *via* the active sites of the monomer units.

A large number of investigators have recognized and measured the polymerization of α-chymotrypsin in aqueous solutions over a great range of pH and ionic strength (Schwert, 1949; Steiner, 1954; Massey *et al.*, 1955; Neurath and Dreyer, 1955; Tinoco, 1957; Rao and Kegeles, 1958.) It has been found possible to ascribe the ultracentrifuge behavior of this protein to a rapidly reversible reaction (Gilbert, 1955). Under the conditions of the present study, considerable evidence is on hand that the protein is in the form of coexisting monomeric, dimeric and trimeric species (Rao and Kegeles, 1958; Bethune and Kegeles, 1961).

Since chymotrypsinogen does not show similar ability to polymerize (Neurath and Dreyer, 1955), the region of the activated enzyme made accessible during the activation process appears to have some involvement in the polymerization. Thus, as the nature of the catalytically active site has been a little better understood, it has been tempting specifically to implicate the active serine and histidine residues in the polymerization as well (Egan *et al.*, 1957; Kézdy and Bender, 1965). Other attempts to investigate such a correlation with the aid of diisopropylphosphorylation of chymotrypsin at the active serine residue have indicated that the situation is by no means so simple (Schwert and Kaufman, 1951; Smith and Brown, 1952; Neurath and Dreyer, 1955). A study undertaken to investigate the effect of dimerization on the kinetics of substrate hydrolysis (Martin and Niemann, 1958) suggested also that the dimer could bind, but not hydrolyse the substrate. Their conclusions would perhaps have been modified had the presence of trimers, as well as all statistical factors in equilibrium and rate expressions involving polysubstituted enzyme, been taken into account.

The present study is the second of two independent attacks on the problem under similar buffer conditions, where the equilibrium constants for dimerization and trimerization are available. The kinetic study (Inagami and Sturtevant, 1965), using stopped-flow techniques up to 6% enzyme concentration, has concluded that the dimeric and trimeric forms of the enzyme must also possess catalytic activity. The present equilibrium study (see also Sarfare and Kegeles, 1965) shows that within experimental error the enzyme polymerizes identically in the presence or absence of the competitive inhibitor β-phenylpropionate (Kaufman and Neurath, 1949; Neurath and Gladner, 1951). This can be true only if β-phenylpropionate binds equally well to a site on monomer, to either of two sites on dimer, and to each of three sites on a trimer molecule. Thus the sites for binding of this inhibitor and the sites for protein polymerization are mutually exclusive in 0.2 ionic strength, pH 6.1–6.2 phosphate buffers.

Experimental Section

Worthington Biochemicals Corp. crystalline α-chymotrypsin, lot no. CDI 6102-3 was used as received from the supplier. β-Phenylpropionic acid was used as ob-

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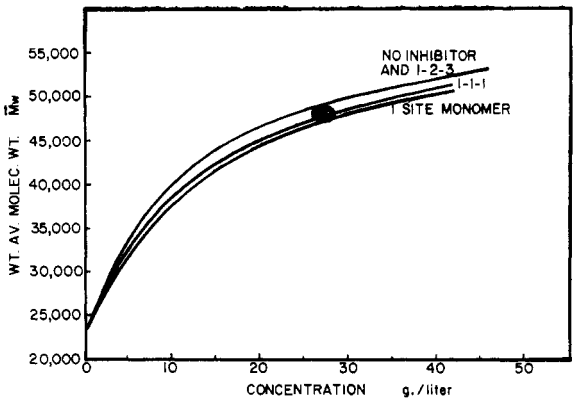

FIGURE 1: Weight-average molecular weights of α-chymotrypsin plotted *vs.* total protein concentration in grams per liter, in the absence of inhibitor and in the presence of 0.001 M β-phenylpropionate at pH 6.1 (see text). Curves are predicted according to various indicated combination mechanisms (see text), filled circle is light-scattering result in presence of inhibitor, and unfilled circle is light-scattering result in absence of inhibitor.

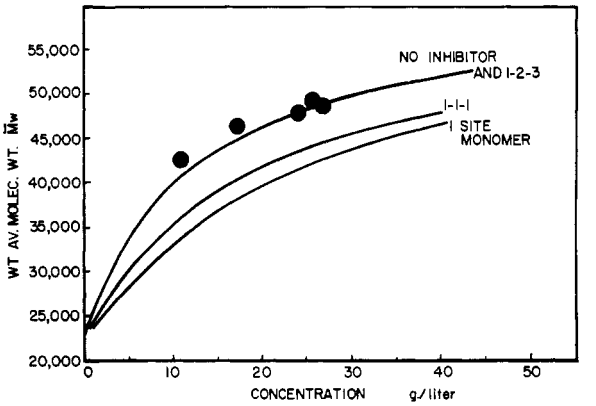

FIGURE 2: Weight-average molecular weights of α-chymotrypsin plotted *vs.* total protein concentration in grams per liter, in the absence of inhibitor and in the presence of 0.004 M β-phenylpropionate at pH 6.1 (see text). Curves are predicted according to various indicated combination mechanisms (see text) and filled circles are light-scattering results in the presence of inhibitor.

tained from Fisher Scientific Co. Light-scattering measurements were performed at 5461 A with a modified Brice-Phoenix Model 2000 photometer, generally following the procedures described by Timasheff *et al.* (1955). The required refractive index increments were determined on dialyzed enzyme samples interferometrically at 25° in an apparatus designed for chromatographic applications (Kegeles and Sober, 1952; Richard, 1955, 1958; Richard and Kegeles, 1959). Several separate interferometric determinations of the specific refractive index increment on solutions prepared by weight from dried protein samples yielded the value 0.00187/1 g/100-ml solution and this value was used to compute protein concentrations. The same value was found in sodium phosphate buffers at 0.2 ionic strength and pH 6.1 in the presence of 0.004 M β-phenylpropionate. Protein concentrations were also determined by light absorption at 282 mμ, using the value 2.075 for the specific absorption (Schwert and Kaufman, 1951). An α acid–glycoprotein preparation kindly supplied by Dr. Karl Schmid was measured with the Archibald ultracentrifuge method by C. L. Sia in these laboratories, and this protein preparation was used in turn to calibrate the light-scattering measurements for molecular weights. All solutions were prepared for turbidity measurements in water which had been double distilled without breaking the surface. All glassware was steamed and cleaned with freshly surface-distilled acetone. For turbidity measurements, enzyme solutions which had been dialyzed in buffer were mixed by calibrated pipettes with buffer solutions of the appropriate concentration of β-phenylpropionate (0.001 or 0.004 M). The buffer solutions used to dissolve both the enzyme and the inhibitor for turbidity measurements consisted of 0.029 M disodium hydrogen phosphate and 0.114 M sodium dihydrogen phosphate, ionic strength 0.2 and pH 6.1. Buffer solutions for ultracentrifuge measurements at a *total* inhibitor concentration of 0.04 M were carefully adjusted by titration with sodium hydroxide to match the previous buffers in ionic strength and pH before dissolving and dialyzing of the protein. The final composition of the buffer system used for the ultracentrifuge measurement was 0.0243 M disodium hydrogen phosphate, 0.0956 M sodium dihydrogen phosphate, 0.0385 M sodium β-phenylpropionate, and 0.0015 M β-phenylpropionic acid, ionic strength 0.207 and pH 6.1. All molecular weights at a total inhibitor concentration of 0.04 M were determined with the ultracentrifuge by means of the Archibald method (Archibald, 1947), as employed previously in this laboratory (Klainer and Kegeles, 1955, 1956; Rao and Kegeles, 1958). One absolute protein concentration was determined by light absorption, and this was used to calibrate areas under schlieren curves formed with a synthetic boundary cell (Kegeles, 1952), in terms of initial protein concentration. A value of 0.736 was used for the partial specific volume of α-chymotrypsin (Schwert and Kaufman, 1951). Centrifugation was usually continued for 60 min at 6166 rpm, and photographs were taken at 15, 30, 45, and 60 min after reaching full speed. Molecular weight values from both the top and bottom menisci were extrapolated to zero time and the results averaged and plotted against initial protein concentration.

Theoretical Predictions. The calculations of weight-average molecular weights are based on reported values for the dissociation constant of the complex between β-phenylpropionate and the enzyme (Kaufman and Neurath, 1949; Neurath and Gladner, 1951) and on reported values for the dissociation constants for the

dimer and trimer (Rao and Kegeles, 1958). Kaufman and Neurath reported a value of $K_I = 4.5(10)^{-3}$ moles/l., and more detailed work of Neurath and Gladner revised this to $K_I = 5.5(10)^{-3}$ moles/l. for the dissociation constant of the enzyme–inhibitor complex. As the experiments were performed at low enzyme concentrations, only the monomer is involved. The reported dissociation constant for dimer to monomer on the moles per liter scale is $k_2 = 9.65(10)^{-4}$, and the dissociation constant for a mole of trimer to a mole of monomer and a mole of dimer is $k_3 = 2.94(10)^{-4}$ on the moles per liter scale, both based on a monomer molecular weight of 23,000. Weight-average molecular weights M_W of uninhibited enzyme were calculated from these data by eq 1

$$M_W = [2.3(10)^4]^2[(E) + 4(E)^2/k_2 + 9(E)^3/k_2k_3]/C \quad (1)$$

Here (E) is the equilibrium concentration of monomer in moles per liter. The corresponding total concentration C of enzyme in grams per liter is given by eq 2

$$C = 2.3(10)^4[(E) + 2(E)^2/k_2 + 3(E)^3/k_2k_3] \quad (2)$$

A fine-grained table of M_W *vs.* C was computed by assigning successive values of (E) in eq 1 and 2, and the results are shown as the top curves in Figures 1, 2, and 3.

When inhibitor is added, eq 1 and 2 are modified to take account of the inhibited forms of the enzyme, and, in addition, one additional equation arises which states the conservation of total moles of inhibitor present in all forms. On the assumption that the inhibitor binds to a single site on a monomer molecule only, the equations become

$$M_W = [2.3(10)^4]^2[(E) + 4(E)^2/k_2 + 9(E)^3/k_2k_3 + (E)(I)/K_I]/C \quad (3)$$

$$C = 2.3(10)^4[(E) + 2(E)^2/k_2 + 3(E)^3/k_2k_3 + (E)(I)/K_I] \quad (4)$$

$$(I_0) = (E)(I)/K_I + (I) \quad (5)$$

Here (I_0) and (I) are the total initial inhibitor concentration and the equilibrium inhibitor concentration in moles per liter. For a given set of experimental values for C and (I_0), (3), (4), and (5) represent simultaneous restrictions on the roots (E) and (I). In order to solve these simultaneous equations for the pair of real, positive roots, an initial small value of (E) was assigned in eq 5 in a digital computer program, and successively incremented values of (I) were tried until the computed value of (I_0) became equal to the experimental value (0.001, 0.004, and 0.4 in the three sets of experiments). The final value of (I) was then inserted into eq 3 and 4 along with the chosen value of (E) to compute corresponding single values of M_W and C, providing one point along a curve of M_W plotted *vs.* C. The computation continued with successively incremented values of (E), and ended when C reached the predetermined value of 40 g/l. The results are shown as the lowest curves in Figures 1, 2, and 3.

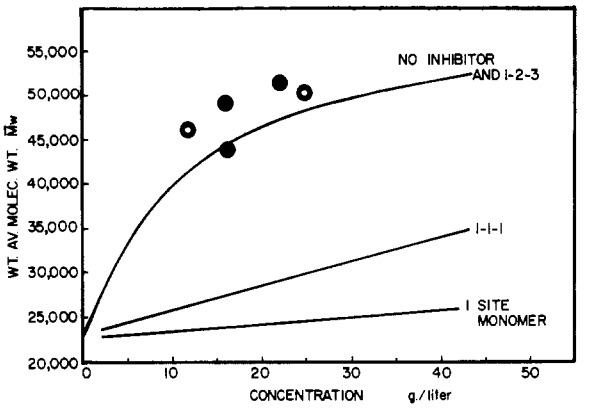

FIGURE 3: Weight-average molecular weights of α-chymotrypsin plotted *vs.* total protein concentration in grams per liter, in the absence of inhibitor and in the presence of 0.04 M β-phenylpropionate at pH 6.1 (see text). Curves are predicted according to various indicated combination mechanisms (see text), filled circles are Archibald ultracentrifuge results in the presence of inhibitor, and unfilled circles are Archibald ultracentrifuge results in the absence of inhibitor.

On the assumption that the inhibitor binds to a single site on a monomer molecule, a single site on a dimer molecule, and a single site on a trimer molecule, the equations become

$$M_W = [2.3(10)^4]^2[(E) + 4(E)^2/k_2 + 9(E)^3/k_2k_3](1 + (I)/K_I)/C \quad (6)$$

$$C = 2.3(10)^4[(E) + 2(E)^2/k_2 + 3(E)^3/k_2k_3](1 + (I)/K_I) \quad (7)$$

$$(I_0) = [(E) + (E)^2/k_2 + (E)^3/k_2k_3]((I)/K_I) + (I) \quad (8)$$

Equations 6, 7, and 8 were solved numerically in the same way as described above for the simultaneous solution of eq 3, 4, and 5, and the results are shown as the middle curves in Figures 1, 2, and 3, labeled 1–1–1 in the figures.

When inhibitor binds with the same intrinsic free energy to one site on a monomer molecule, either of two sites on a dimer molecule, and each of three sites on a trimer molecule, the relationships must be modified, in addition, to take account of all statistical factors (Inagami and Sturtevant, 1965). For example, when a dimer molecule containing inhibitor on both available sites dissociates into a molecule of free inhibitor and a molecule of dimer holding one molecule of inhibitor, there are two equivalent ways in which the dissociation can take place. On the other hand, once the dissociation has been completed, there is only one pathway for the recombination to the original saturated dimer molecule.

Hence, the dissociation constant for this process is twice the intrinsic dissociation constant for a monomer containing a molecule of inhibitor. The statistical factors for all forms of the inhibited enzyme are contained in the following relations

$$(E)(I)/(EI) = K_I \quad (9)$$

$$(E_2)(I)/(E_2I) = (1/2)K_I \quad (10)$$

$$(E_3)(I)/(E_3I) = (1/3)K_I \quad (11)$$

$$(E_2I)(I)/(E_2I_2) = 2K_I \quad (12)$$

$$(E_3I)(I)/(E_3I_2) = K_I \quad (13)$$

$$(E_3I_2)(I)/(E_3I_3) = 3K_I \quad (14)$$

The pertinent equations for the calculation of weight-average molecular weights at various enzyme and inhibitor concentrations then follow for this case

$$M_W = [2.3(10)^4]^2[(E)(1 + (I)/K_I) + (4(E)^2/k_2)(1 + (I)/K_I)^2 + 9((E)^3/k_2k_3)(1 + (I)/K_I)^3]/C \quad (15)$$

$$C = 2.3(10)^4[(E)(1 + (I)/K_I) + 2((E)^2/k_2)(1 + (I)/K_I)^2 + (3(E)^3/k_2k_3)(1 + (I)/K_I)^3] \quad (16)$$

$$(I_0) = ((E) + 2(E)^2/k_2 + 3(E)^3/k_2k_3)((I)/K_I) + (2(E)^2/k_2 + 6(E)^3/k_2k_3)((I)/K_I)^2 + (3(E)^3/k_2k_3)((I)/K_I)^3 + (I) \quad (17)$$

As before, eq 15, 16, and 17 are solved simultaneously by numerical computer methods, assigning initial small values to (E) and (I), successively incrementing (I) until the right-hand side of eq 17 equals the assigned experimental value of (I_0), and then inserting the chosen value (E) and the final value of (I) into eq 15 and 16 to obtain a single point on the curve of M_W *vs.* C. The results of this computation are shown as the top curves in Figures 1, 2, and 3. It should be pointed out that these results coincide exactly with those for uninhibited enzyme, and that this exact superposition is also completely independent of the concentration of inhibitor and the dissociation constant K_I.

Discussion

As shown in Figures 1, 2, and 3, when only the monomer can bind inhibitor, the competition between the inhibitor and the polymerized forms of the enzyme for monomer depresses the weight-average molecular weight. This depression, which is hardly apparent at an inhibitor concentration of 0.001 M, becomes tremendously accentuated at 0.04 M β-phenylpropionate, as would be expected intuitively in accordance with the law of mass action. Although an intuitive prediction is more difficult in the case where a molecule of inhibitor binds to a single site on each of the three forms of the enzyme, the detailed calculations described above also predict a depression of the weight-average molecular weight which becomes accentuated with increasing inhibitor concentration, as shown in Figures 1, 2, and 3. On the other hand, when a molecule of inhibitor binds with the same intrinsic free energy to one site on a monomer molecule, either of two sites on a dimer molecule, and each of three sites on a trimer molecule, this mechanism, indicated by 1–2–3 in Figures 1, 2, and 3, predicts exactly the same weight-average molecular weight as for uninhibited enzyme. At 0.001 M inhibitor, Figure 1, there is so little difference between all the curves that experimental inaccuracy allows no decision to be made between the possible mechanisms. At 0.004 M inhibitor, Figure 2, the light-scattering experimental results lead to a very strong preference for the 1–2–3 mechanism. In Figure 3, the experimental ultracentrifuge results demonstrate unequivocally that both the binding of inhibitor to monomer alone and the binding of no more than a single molecule of inhibitor to either monomer, dimer, or trimer molecules are mechanisms which disagree with experiment far beyond permissible experimental error, and that the 1–2–3 mechanism is the only one possible, of those considered.

More complicated mechanisms could be proposed to fit the experimental data, in which the intrinsic free energy of binding of inhibitor to enzyme would differ, depending on whether the site exists on a monomer, or on a dimer or a trimer molecule. However, there are no *a priori* reasons for expecting the binding to be much more favorable in the case of the polymerized forms of the enzyme. On the other hand, mechanisms which assume the binding of inhibitor to sites on polymers to be much less favorable than in the case of monomer would always predict a sizeable depression, at high inhibitor concentrations, in the weight-average molecular weight. Thus, this research would, in addition to ruling out the extreme cases already considered where there is *no* binding to certain sites on polymer, tend also to rule out all cases in which polymerization in any way *seriously decreases* the binding of inhibitor.

A reasonable explanation of the data appears to be that the binding of competitive inhibitor to active sites on the enzyme is completely independent of whether these sites exist on a monomer, dimer, or trimer molecule under the buffer conditions chosen and that, therefore, the sites on the enzyme responsible for the substrate binding steps in enzymatic catalysis are mutually exclusive of the sites responsible for protein polymerization.

On this basis, it is possible that the acceptable choices of mechanisms selected by Inagami and Sturtevant (1965) to explain their data for the catalytic behavior of the enzyme at high concentrations in similar buffers could be narrowed down somewhat. The most likely mechanism suggested, judging from their results and those of the present research, is one whereby substrate is bound equally well to every possible site, *i.e.*, one site on monomer, two on dimer, and three on trimer, but in which acylation of the enzyme proceeds more favorably in the case of sites on monomer than in the

cases of sites on polymeric forms of the enzyme.

It is interesting to note that the moist crystal, containing two molecules in the unit cell, has been found to retain enzyme activity (Kallos, 1964).

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