Dimerization and Activity of Chymotrypsin at pH 4*

Keisuke Morimoto and Gerson Kegeles

ABSTRACT: Native and acetylated α-chymotrypsins were compared in polymerizability by means of Archibald ultracentrifuge measurements at pH 4, in 0.2 and 0.018 ionic strength acetate buffers. Although acetylation of the active serine was verified by inability to produce a burst of *p*-nitrophenol from *p*-nitrophenyl acetate, the acetylated enzyme could not be distinguished in molecular weight behavior from the native enzyme. These experiments have provided lack of any evidence, at protein concentrations up to 3.7%, for the existence of an intermolecular self-acylation, which has been implicated as impairing enzyme activity.

Because chymotrypsinogen does not parallel chymotrypsin in its ability to polymerize reversibly, it has been tempting to implicate the active site of the enzyme in the polymerization process (Egan *et al.*, 1957; Kézdy and Bender, 1965). Other work has cast doubts on the simple involvement of the active site (Schwert and Kaufman, 1951; Smith and Brown, 1952; Neurath and Dreyer, 1955). Kinetic studies (Martin and Nieman, 1958; Inagami and Sturtevant, 1965) appear to have borne out the lack of a dual function of the monomeric active site as a polymerizing site, but these have been contradicted by other kinetic studies (Kézdy and Bender, 1965). The latter workers have postulated an inactive intermolecular acyl enzyme dimer formed by the acylation of the active serine of either monomer unit by a carboxyl group of the other participating monomer. The previous molecular weight studies from these laboratories (Sarfare *et al.*, 1966) examined α-chymotrypsin in the presence and absence of the specific inhibitor β-phenylpropionate, in 0.2 ionic strength phosphate buffer at pH 6.1, and demonstrated that under these conditions the enzyme polymerization to dimers and trimers (Rao and Kegeles, 1958) takes place independently of the active site. The purpose of the present study is to reexamine this question under the rather different buffer conditions (acetate buffer, pH 4, 0.2 ionic strength) used in part by Kézdy and Bender in their studies. A preliminary report referring to a portion of these studies has appeared elsewhere (Morimoto and Kegeles, 1966).

Experimental Section

The quantitative studies of inhibition by β-phenylpropionate of α-chymotrypsin had been performed at pH 7.8 (Kaufman and Neurath, 1949; Neurath and Gladner, 1951). It did not now seem valid to extrapolate these results to pH 4. Instead, advantage was taken of the stability at pH 4 of acetylated chymotrypsin prepared by use of *p*-nitrophenyl acetate (Balls and Wood, 1956). The following slightly modified procedure was used. α-Chymotrypsin solution (5 ml of 2%) at pH 5 was mixed with 0.5 ml of 3.6% *p*-nitrophenyl acetate in acetone. The mixture was allowed to react at 20° for 45 min, and the solution was then clarified by 20-min centrifugation at 14,290 rpm at 5°. Dialysis against appropriate buffer then proceeded for 12–16 hr at 6°, prior to further examination. From this solution active enzyme could be regenerated by adjustment of the pH to 7 and the temperature to 20° for 10 min. The regenerated enzyme was redialyzed at 6° against the appropriate pH 4 buffer before examination. Enzyme activity was measured colormetrically at 330 mμ (Dixon and Neurath, 1957) after mixing the enzyme in pH 4 buffer with *p*-nitrophenyl acetate in a mole proportion of 1:3, the reaction proceeding at 20°. The molecular weights were measured with the Archibald method (Archibald, 1947; Klainer and Kegeles, 1955), using fluorochemical FC-43, perfluorotributylamine, to form a false cell bottom (Ginsburg *et al.*, 1956). The pH 4 buffers were made up to 0.2 or 0.018 ionic strength, using sodium acetate and acetic acid. The enzyme was Worthington three times crystallized, lot no. CDI 6102-3, and *p*-nitrophenyl acetate was obtained from K & K Laboratories. Absolute protein concentrations after dialysis were obtained spectrophotometrically at 282 mμ by using 2.04 as the absorbancy, determined from protein dried over phosphorus pentoxide and made up by weight to 1.0 g/l. in a 1-cm cell. All concentrations reported for centrifuge experiments were corrected from these initial values to take into account changes at the ends of the cell during centrifugation. The partial specific volume of the protein was taken as 0.736 (Schwert and Kaufman, 1951).

Results

In Figure 1 are shown weight-average molecular weight values for native and acetylated chymotrypsin,

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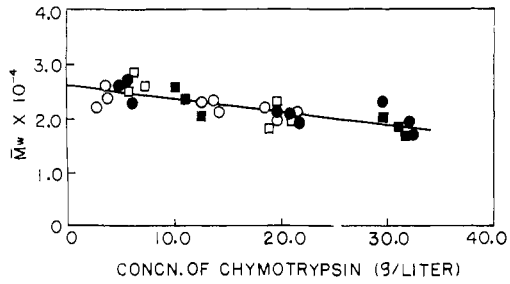

FIGURE 1: Weight-average molecular weight *vs.* concentration of chymotrypsin at ionic strength 0.018; open symbol: values from liquid-air meniscus; closed symbol: values from bottom of cell; (○) intact chymotrypsin; (□) acetyl chymotrypsin.

plotted against time-dependent concentrations measured at the menisci of the solution, for 0.018 ionic strength pH 4 buffer. The molecular weight at low concentration appears to approach that of the monomer and this seems to decrease with increasing concentration. In uni-univalent buffers, the isoelectric point is in the range 8.1–8.3 (Anderson and Alberty, 1948; Kubacki *et al.*, 1949). Thus at pH 4 there is strong electrostatic repulsion between protein molecules tending to produce only monomeric species. In addition, strong intermolecular repulsions cause the apparent molecular weight to decrease at increasing concentration, an effect already noted for other highly nonideal solutions (Kegeles *et al.*, 1957). The essential point concerning Figure 1, however, is that it is not possible to discern any systematic differences between the molecular weights of native and acetylated chymotrypsin, at protein concentrations from 0.2 to 3.2%, in 0.018 ionic strength buffer.

In Figure 2 are shown weight-average molecular weight values for native, regenerated, and acetylated chymotrypsin plotted against concentration at the menisci, for 0.2 ionic strength buffer at pH 4. At sufficiently low concentration, dissociation should cause

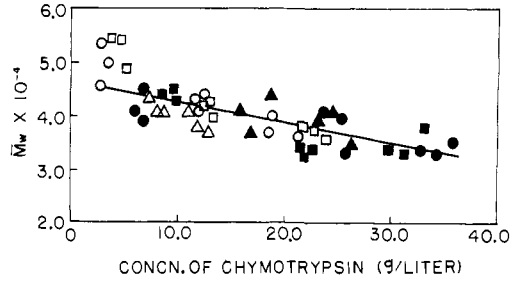

FIGURE 2: Weight-average molecular weight *vs.* concentration of chymotrypsin at ionic strength 0.20; open symbol: values from liquid-air meniscus; closed symbol: values from bottom of cell; (○) intact chymotrypsin; (□) acetyl chymotrypsin; (△) chymotrypsin recovered from acetyl chymotrypsin.

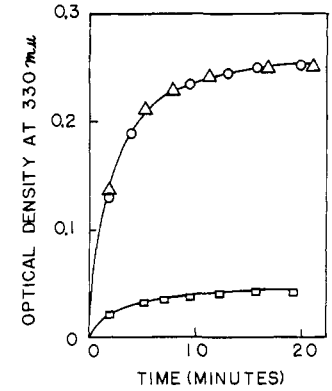

FIGURE 3: Liberation of *p*-nitrophenol at 0.20 ionic strength: (○) intact chymotrypsin; (□) acetyl chymotrypsin; (△) chymotrypsin recovered from acetyl chymotrypsin. Enzyme (1.0 ml of 4.1 g/l.) in buffer of pH 4.0 and 0.1 ml of 0.09 g/100 ml of *p*-nitrophenyl acetate in acetone were mixed. A cell of length 0.2 cm was used.

the molecular weight to approach that of the monomer (Schwert, 1949). The present results do not contain values at low concentrations of sufficient accuracy to observe the rather abrupt downturn of the curve. The drop of apparent molecular weight with increasing concentration again is caused by high mutual repulsion of charged molecules. However, the increased shielding of charge by the 0.2 ionic strength buffer permits association, apparently to the dimer. The major point of interest, again, is not the detailed interpretation of the shape of the curve, but the fact that it is impossible to distinguish, over this range of concentration, the molecular weight behavior of acetylated enzyme from that of native or regenerated enzyme.

In order to ascertain that the acetylated enzyme had not lost its blocking acetyl groups from the active site serine by the time the ultracentrifuge experiments had been completed, pooled solutions of acetylated enzyme recovered from three centrifuge experiments, regenerated enzyme from this pool, and native chymotrypsin were allowed to act on *p*-nitrophenyl acetate as a substrate, under conditions described in the Experimental Section. In Figure 3 are shown these results at 0.2 ionic strength. In Figure 4 are shown comparative results for native enzyme only, at 0.018 and 0.2 ionic strength. The ordinate plotted represents the difference of the optical density of liberated *p*-nitrophenol and disappearing *p*-nitrophenyl acetate, the wavelength used being much more sensitive to the former (Dixon and Neurath, 1957). The activity of the recovered acetylated enzyme (Figure 3) is about 16% of the values for native and deacetylated enzymes, which are essentially the same. The calculated values for the absorbancy expected are 0.24 and 0.33 for Figures 3 and 4, on the basis of literature values for *p*-nitrophenol and *p*-nitrophenyl acetate, corresponding to initial liberation of 1 equiv of *p*-nitrophenol/equiv of protein monomer. These calculated values are the

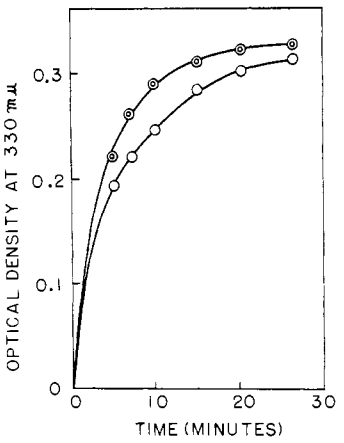

FIGURE 4: Liberation of *p*-nitrophenol: (○) at ionic strength 0.018; (◎) at ionic strength 0.20. Chymotrypsin (10.0 ml of 5.5 g/l.) in buffer of pH 4.0 and 0.5 ml of 0.24% *p*-nitrophenyl acetate in acetone were mixed. A cell of length 0.2 cm was used.

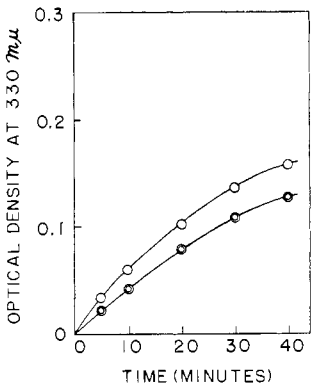

FIGURE 5: Liberation of *p*-nitrophenol: (○) at ionic strength 0.018; (◎) at ionic strength 0.20. Chymotrypsin (10.0 ml of 0.49 g/l.) in buffer of pH 4.0 and 0.5 ml of 0.02% *p*-nitrophenyl acetate in acetone were mixed. A cell of length 1.0 cm was used.

maximum possible absorbancies which could be expected if the initial reaction liberated 1 mole of *p*-nitrophenol/mole of enzyme present, calculated as monomer. Thus the comparisons are made in the stage of initial liberation of *p*-nitrophenol, rather than during turnover by the enzyme.

The specific effect of ionic strength on the rate of liberation of *p*-nitrophenol can be investigated at very high dilution of the protein, where it should exist primarily as monomer at either 0.018 or 0.2 ionic strength. The results are shown in Figure 5. Here it is noted that the enzyme at 0.018 ionic strength is actually slightly more active than at 0.2 ionic strength. Thus if the enzyme at higher protein concentration is primarily dimeric at 0.2 ionic strength and predominantly monomeric at 0.018 ionic strength, as suggested by Figures 1 and 2, then it is interesting to note in Figure 4 that the dimer would have to be at least as active an enzyme as the monomer.

Summary

These molecular weight studies indicate that there is no observed difference in the polymerizability of native or regenerated α-chymotrypsin and of α-chymotrypsin which has its active site serine group reversibly blocked by acetylation. Thus it must be assumed that acetyl chymotrypsin undergoes rapidly reversible polymerization at pH 4 in 0.2 ionic strength acetate buffer, as does the native protein, and that this polymerization does not involve the serine group of the active site. Moreover, the observed dimeric molecules have at least as much activity as the predominately monomeric form which appears to exist at 0.018 ionic strength at the same pH. Thus, these experiments provide lack of evidence for the existence of any very appreciable amount of self-acylated dimer present up to about 3.7% protein concentration in 0.2 ionic strength buffer. If any such species exists, it would have to be present in such a way that when the active serine is blocked, the amount of dimer is unaffected within the experimental error of the ultracentrifuge observation. These confirmatory results must be viewed in the further light of the fact that the postulated self-acylated dimer was already in strong direct contradiction to the first kinetic data at high concentration (Martin and Nieman, 1958) and is at variance with the more recent stopped-flow kinetic data which indicate enzyme activity in dimeric and trimeric enzyme (Inagami and Sturtevant, 1965), as well as of the finding that at pH 6.1 the site-specific binding of β-phenylpropionate is unaffected by polymerization (Sarfare *et al.*, 1966).

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The Activation of Bovine Procarboxypeptidase A. I. Isolation and Properties of the Succinylated Enzyme Precursor*

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ABSTRACT: Treatment of bovine pancreatic procarboxypeptidase A with succinic anhydride leads to spontaneous disaggregation into succinylated subunits. By means of gel filtration and chromatography, succinylated subunit I, the immediate precursor of succinylcarboxypeptidase A, has been isolated. The purified zymogen has a sedimentation coefficient of approximately 4 S and a molecular weight of 40,000–42,000.

In comparison to carboxypeptidase A, the zymogen is particularly rich in the dibasic amino acid residues. Tryptic activation results in an enzymatically active species having the properties of succinylcarboxypeptidase.

The study of activation of pancreatic zymogens, notably trypsinogen, chymotrypsinogen, and procarboxypeptidase B, has demonstrated that in each of these systems the formation of an enzymatically active species is dependent on the tryptic cleavage of a unique peptide bond in the amino-terminal region of these proteins (Neurath, 1964). This primary chemical event is believed to be the prerequisite for conformational changes which in turn give rise to the formation of the catalytic site. Analogous studies on the formation of carboxypeptidase A from its inactive precursors have been complicated by the fact that these zymogens exist as tightly bound aggregates of two and three different subunits, respectively (Brown *et al.*, 1961, 1963a), which together make up the protein known as procarboxypeptidase A (Keller *et al.*, 1956, 1958). This physical state of aggregation may be responsible for the fact that *in vitro* the activation of bovine procarboxypeptidase A proceeds very much slower than that of the other pancreatic zymogens (Neurath, 1964).

Disaggregation of procarboxypeptidase into its component subunit fractions can be accomplished by concentrated urea solutions, or in aqueous solutions at pH 10.5 (Brown *et al.*, 1963a). Under the latter conditions, however, the immediate precursor of carboxypeptidase A, "fraction I," becomes denatured and can only be identified by chemical analysis but not by enzymatic function. In the work reported herein, attempts were made to effect disaggregation and separation of the subunits by succinylation of the parent protein, procarboxypeptidase A-S6, with succinic anhydride. It has previously been shown that certain proteins such as hemerythrin (Klotz and Kerestes-Nagy, 1963), porcine heart aspartate transaminase (Polyanovsky, 1965), or rabbit muscle aldolase (Hass, 1964) undergo spontaneous disaggregation under these conditions, presumably through electrostatic repulsion between the negatively charged carboxylate groups which replace the positively charged ϵ-amino groups of lysyl residues. Moreover, Vallee *et al.* (1963) demonstrated that carboxypeptidase A retains its enzymatic function following succinylation and further, that

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