Self-Association of α -Chymotrypsin at Low Ionic Strength in the Vicinity of its pH Optimum

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The self-association of α -chymotrypsin and its di-isopropyl phosphoryl derivative in 1.03 sodium phosphate buffer, pH 7.9, was investigated by velocity sedimentation, equilibrium sedimentation and difference gel chromatography. No differences between the native and chemically modified enzyme were observed in the ultracentrifuge studies, and only a marginal (0.6%) difference in weight-average elution volume was detected by difference gel chromatography of 5 g/litre solutions on Sephadex G-75. From quantitative analyses of sedimentation velocity and sedimentation-equilibrium distributions obtained with iPr_2P (di-isopropylphosphoryl)-chymotrypsin, the polymerizing system is postulated to involve an indefinite association of dimer (with an isodesmic association constant of 0.68 litre/g) that is formed by a discrete dimerization step with equilibrium constant 0.25 litre/g. In addition to providing the best fit of the experimental results, this model of chymotrypsin polymerization at low ionic strength is also consistent with an earlier observation that dimer formation is a symmetrical head-to-head phenomenon under conditions of higher ionic strength (1.029, pH 7.9) where association is restricted to a monomer-dimer equilibrium. It is proposed that the dimerization process is essentially unchanged by variation in ionic strength at pH 7.9, and that higher polymers are formed by an entirely different mechanism involving largely electrostatic interactions between dimeric species.

Despite the probable irrelevance of the polymerization of α -chymotrypsin in the biological context, the self-association of this enzyme has served as a very useful model system for the development and refinement of techniques for the quantitative study of rapid polymerization equilibria. In buffers of moderate ionic strength (1.02-0.3), α -chymotrypsin undergoes rapid reversible dimerization at pH 4 (Winzor & Scheraga, 1964; Aune & Timasheff, 1971) and also in the vicinity of its pH optimum (Shiao & Sturtevant, 1969; Nichol *et al.*, 1972). The aim of the present investigation was to study quantitatively the association of α -chymotrypsin in the vicinity of the pH optimum (pH 8) but at lower ionic strength, conditions under which polymerization proceeds beyond the dimer stage (Massey *et al.*, 1955; Gilbert, 1955, 1959; Nichol & Bethune, 1963; Winzor & Scheraga, 1963; Ackers & Thompson, 1965; Pandit & Rao, 1974). Whereas the previous results were considered largely in the light of a possible two-state model, an indefinite association of monomer was invoked by Pandit & Rao (1974, 1975). In view of the symmetrical nature of α -chymotrypsin dimerization at higher ionic strength (Nichol *et al.*, 1972), a complete change in the mode of association at low ionic strength would be required for either type of model to be correct. The present paper reports a

physicochemical study designed to assess quantitatively the nature of α -chymotrypsin polymerization in 1.03 phosphate buffer, pH 7.9.

Experimental

Materials

Bovine pancreatic α -chymotrypsin (three-times-crystallized, freeze-dried and salt-free) was obtained from Sigma Chemical Co., St. Louis, MO, U.S.A., and iPr_2P -F (di-isopropyl phosphorofluoridate) from Aldrich Chemical Co., Milwaukee, WI, U.S.A. Other chemicals were of reagent grade, and glass-distilled water was used for the preparation of all buffers and solutions.

The major buffer used in this investigation was a sodium phosphate buffer, pH 7.9, 1.03, with the nominal composition 0.001 M-NaCl/0.009 M- Na_2HPO_4 /0.001 M- NaH_2PO_4 . A more concentrated (1.029) phosphate buffer (0.093 M- Na_2HPO_4 /0.007 M- NaH_2PO_4) with the same pH was also used in a few comparative experiments.

Methods

Enzyme inhibition by iPr_2P -F. A 25-fold molar excess of iPr_2P -F in acetonitrile was added to 50-

100mg of α -chymotrypsin in 10ml of *I*0.03 phosphate buffer, pH7.9, and the reaction mixture incubated at 4°C for 12h. This procedure differed from that used by Jansen *et al.* (1949) with respect to the larger molar excess of reagent (25-fold, cf. two fold) and the longer incubation period (12h, cf. 2h). Both of these changes were found to be necessary to decrease the residual enzymic activity towards *p*-nitrophenyl acetate to 0.2–0.3% of the activity observed with native enzyme.

Inhibited enzyme [iPr_2P (di-isopropylphosphoryl)-chymotrypsin] was then subjected to zonal gel chromatography on Sephadex G-75 to remove the excess of iPr_2P -F and also to remove any autolysis fragments. A column (2.4cm $\times$ 17cm) of Sephadex G-75 was equilibrated with *I*0.03 phosphate buffer (pH7.9), and 50–10ml of concentrated iPr_2P -chymotrypsin (5–10mg/ml) applied to the column. This combination of high protein concentration and low ionic strength was used to ensure that non-autolysed solute migrated considerably faster than monomeric chymotrypsin, and hence that even large autolysis fragments would be removed by discarding the trailing fourth of the eluted zone. Any large autolysis fragments that retained full ability to polymerize would, of course, have been included in the iPr_2P -chymotrypsin sample used for physico-chemical characterization. However, the results of Pandit & Rao (1974) indicate that the autolysis fragments are essentially non-associating systems.

Measurement of protein concentration. Concentrations of native and iPr_2P -modified α -chymotrypsin solutions were determined either spectrophotometrically at 280nm or refractometrically at 546nm. In the former procedure, the value of 20.1 for the extinction coefficient ($A_{1\%}^{1cm}$) of native α -chymotrypsin (Egan *et al.*, 1957) was assumed to apply also to iPr_2P -chymotrypsin. Refractometric measurements of concentration were based on a specific refractive increment of 1.86×10^{-4} litre/g for α -chymotrypsin (Sarfare *et al.*, 1966; Aune & Timasheff, 1971) and its iPr_2P derivative.

Polyacrylamide-gel electrophoresis. As a check on the purity of α -chymotrypsin and iPr_2P -chymotrypsin samples, and also on the likely rate of fragment production by autolysis, solutions that had been obtained by the above zonal-gel-chromatographic step were subjected to sodium dodecyl sulphate/urea/polyacrylamide-gel electrophoresis. These experiments were performed essentially by the procedure of Weber & Osborn (1969), except that 5M-urea was included in all buffers (Sender, 1971). After electrophoresis for 6h at 4mA per gel, the gels were stained with 0.25% Coomassie Blue in methanol/acetic acid/water (5:1:5, by vol.) for 4h, before destaining by extensive washing in the same solvent medium.

The two bands expected on the basis of the

composition of reduced α -chymotrypsin, which has polypeptide chains with mol.wts. of 11000 and 13000, were indeed observed with iPr_2P -chymotrypsin and also with freshly chromatographed α -chymotrypsin. Whereas storage of the iPr_2P -chymotrypsin solutions for 24h under the conditions prevailing in equilibrium-sedimentation experiments (*I*0.03 phosphate, pH7.9, and 20°C) had no effect on its electrophoretic behaviour, exposure of native α -chymotrypsin to these conditions for even 5h led to pronounced autolysis, which was detected by the appearance of an additional band in the gel-electrophoresis pattern.

Ultracentrifugation. Sedimentation experiments were carried out at 20°C in a Spinco model E ultracentrifuge fitted with electronic speed control. Speeds in the range 7200–24000 rev./min were used in equilibrium experiments, and velocity runs were performed at either 52000 or 60000 rev./min. The schlieren optical system was used for velocity sedimentation, whereas equilibrium experiments were recorded as Rayleigh interferograms. Resultant photographic records were measured on a Nikon two-dimensional comparator.

Velocity runs were analysed in terms of $\bar{s}$, the weight-average sedimentation coefficient (Goldberg, 1953), which was obtained from a plot of $\log \bar{r}$ versus t , where $\bar{r}$ denotes the mean boundary position at time t . The plateau concentration corresponding to each $\bar{r}$ was determined from the loading concentration by applying the usual correction factor for radial dilution, and the average of these plateau concentrations taken as $\bar{c}$, the total protein concentration to which $\bar{s}$ refers. Equilibrium-sedimentation experiments at low (Van Holde & Baldwin, 1958) or high (Yphantis, 1964) angular velocities were analysed in terms of the $\Omega(r)$ function (Milthorpe *et al.*, 1975). Buffer densities were measured with a 50ml pycnometer, and relative viscosities with an Ostwald viscometer having a flow time of 4min for water. The value of 0.736ml/g for the partial specific volume of α -chymotrypsin (Schwert & Kaufman, 1951) was assumed to apply also to the iPr_2P derivative.

Difference gel chromatography. The layering technique of Gilbert (1966) has been used to compare the extents of association of α -chymotrypsin and its iPr_2P derivative. A column (2.4cm $\times$ 7.5cm) of Sephadex G-75 was equilibrated at 4°C with *I*0.03 phosphate, pH7.9, at a flow rate of 13.8ml/h. After application of 15ml of iPr_2P -chymotrypsin (4.95g/litre) in the same buffer, the column was eluted with 15ml of native α -chymotrypsin (also 4.95g/litre) in *I*0.03 phosphate buffer. Finally the column was eluted with buffer.

Difference gel chromatography (Baghurst *et al.*, 1971) has also been used to compare the elution volumes of monomeric iPr_2P -chymotrypsin in *I*0.03 and *I*0.29 phosphate buffers, pH7.9. To a column

(3.0cm $\times$ 22cm) of Sephadex G-75 equilibrated with 1.0.29 phosphate buffer (pH 7.9) and thermostatically controlled at 4°C was added 160ml of iPr_2P -chymotrypsin (0.08g/litre) dissolved in the low-ionic-strength (1.0.03) phosphate buffer. The eluate, maintained at a flow rate of 72ml/h throughout the application of solute and subsequent elution with 1.0.03 buffer, was monitored spectrophotometrically at 280nm. A low concentration of iPr_2P -chymotrypsin was used to ensure that more than 97% of the enzyme derivative was monomeric under both sets of conditions.

Results and Discussion

Qualitative investigations of α -chymotrypsin association

The aim of the present study was to examine the association of α -chymotrypsin rather than of its iPr_2P derivative, which has been used merely to overcome the difficulties of interpretation that autolysis introduces into quantitative studies of α -chymotrypsin in the vicinity of its pH optimum. It has therefore been necessary to establish the extent to which the polymerization of α -chymotrypsin is affected by inhibition with iPr_2P -F. Velocity sedimentation and difference gel chromatography have been used for this purpose.

The dependencies of $\bar{s}_{20,w}$, the weight-average sedimentation coefficient, on concentration for α -chymotrypsin and iPr_2P -chymotrypsin in 1.0.03 and 1.0.29 phosphate buffers (pH 7.9) are shown in Fig. 1, from which two observations can be made at this stage. First, there is no obvious difference between the sedimentation properties of the enzyme ($\bullet$) and its iPr_2P derivative ($\blacksquare$). The scatter of experimental points is attributed to uncertainty in the values of $\bar{s}_{20,w}$ due to the absence of a well-defined baseline in schlieren patterns obtained with slowly migrating solutes in single-sector cells. Secondly, the value of 2.7S obtained for $\bar{s}_{20,w}^0$ of monomeric chymotrypsin in 1.0.03 buffer is significantly higher than that of 2.4S for the enzyme in phosphate buffer of higher ionic strength. The latter value is in agreement with earlier studies by Schwert & Kaufman (1951), Dreyer *et al.* (1955), Egan *et al.* (1957), Neet & Brydon (1970) and Aune & Timasheff (1971). Further, the higher estimate for $\bar{s}_{20,w}^0$ of monomeric chymotrypsin in buffers of low ionic strength (pH approx. 8) has also been reported (Massey *et al.*, 1955; Nichol & Bethune, 1963). This difference in sedimentation behaviour of monomer in the two environments implies a difference in the extent of solvation and/or buffer-ion binding under conditions of high and low ionic strengths. Phosphate certainly binds to α -chymotrypsin, as is evident from the shift in isoelectric point from pH 8.2 in univalent buffers (Ander-

son & Alberty, 1948; Kubacki *et al.*, 1949) to pH 6.2 in 1.0.2 phosphate buffer (Rao & Kegeles, 1958). Thus at pH 7.9 α -chymotrypsin may well be essentially isoelectric in 1.0.03 phosphate but bear a sizeable net negative charge in 1.0.29 phosphate buffer of the same pH.

Difference gel chromatography on Sephadex G-75 has been used to provide more critical assessments of both these points. Fig. 2(a) presents the elution profile obtained by the layering technique of Gilbert (1966), which provides a sensitive index of the identity or otherwise of the elution volumes of native enzyme and iPr_2P -chymotrypsin in 1.0.03 phosphate, pH 7.9. The slight 'hump' at the junction between modified and native chymotrypsin solutions (both 4.95g/litre) implies a slightly faster migration rate of native enzyme under these conditions, which are conducive to considerable polymerization (Fig. 1). From the area of the hump the difference in weight-average elution volume is 0.08ml, or 0.6% of the elution volume (13.0ml for iPr_2P -chymotrypsin and 12.9ml for native enzyme). Since such a difference is beyond the limits of detection

Fig. 1. Concentration-dependence of the weight-average sedimentation coefficients of α -chymotrypsin and iPr_2P -chymotrypsin in 1.0.03 and 1.0.29 sodium phosphate buffers, pH 7.9

Solutions of α -chymotrypsin ($\bullet$) or iPr_2P -chymotrypsin ($\blacksquare$) were subjected to velocity sedimentation at 20°C and either 60000 or 50000 rev./min. The letters (A–D) refer to four possible models of chymotrypsin polymerization at 1.0.03 that are listed in Table 1, and the curves denote concentration-dependencies of $\bar{s}_{20,w}$ predicted by eqn. (4a), and either eqn. (4b) (solid line) or eqn. (4c) (broken lines).

Fig. 2. Difference gel chromatography of α -chymotrypsin and iPr_2P -chymotrypsin in sodium phosphate buffers, pH 7.9

(a) Comparison of the elution volumes of concentrated (4.95 g/l) solutions of α -chymotrypsin and iPr_2P -chymotrypsin in 1.03 buffer by the layering technique of Gilbert (1966); application of iPr_2P -chymotrypsin (15 ml) was terminated by application of α -chymotrypsin (15 ml), which was in turn followed by elution of the column (2.4 cm $\times$ 7.5 cm) of Sephadex G-75 with 1.03 buffer. (b) Comparison of the elution volumes of iPr_2P -chymotrypsin in 1.03 and 1.029 buffers: iPr_2P -chymotrypsin (0.08 g/litre, 160 ml) in 1.03 buffer was applied to a column (3.0 cm $\times$ 22 cm) of Sephadex G-75 equilibrated with 1.029 buffer.

by most experimental methods of studying extents of association, iPr_2P -inhibited and native α -chymotrypsin will be considered to polymerize indistinguishably. Although this result refers specifically to association at 4°C, a temperature chosen to minimize autolysis of native enzyme, the enthalpy of association is essentially zero (Steiner, 1954; Nichol *et al.*, 1972) and hence the equilibria are temperature-insensitive.

Fig. 2(b) presents the results of a difference-gel-chromatography experiment designed to compare the Stokes radii of monomeric iPr_2P -chymotrypsin in the phosphate buffers of high and low ionic strength. Comparison of Fig. 2(b) with Fig. 1 and eqn. (2) of Baghurst *et al.* (1971) shows that the difference between the two plateau concentrations $\bar{c}^a$ and $\bar{c}^b$

reflects a faster migration rate, or smaller elution volume, of monomeric iPr_2P -chymotrypsin at the higher ionic strength. The elution volume of chymotrypsin at the higher ionic strength is 77.3 ml, and that pertaining to migration at low ionic strength is 82.2 ml. It was therefore decided to determine whether this difference in elution volume is compatible with the 0.3 S difference in $s_{20,w}^0$ (Fig. 1).

From Table 4 and Fig. 7 of Andrews (1970) a plot was constructed of Stokes radius versus elution volume for a series of globular proteins. In solutions of high ionic strength (where $s_{20,w}^0 = 2.4$ S) the Stokes radius of α -chymotrypsin is 2.09 nm (Andrews, 1970) and hence a value of 1.86 nm is predicted for a monomer with $s_{20,w}^0 = 2.7$ S because of the inverse relationship between sedimentation coefficient and Stokes radius. On the basis of these two Stokes radii and the calibration curve, the difference between elution volumes predicted by the Andrews (1970) data is 8%, which compares favourably with the experimentally observed difference of 6%. Consequently, the difference-gel-chromatography results (Fig. 2b) substantiate the finding by sedimentation-velocity experiments (Fig. 1) that iPr_2P -chymotrypsin is more compact in buffer of low ionic strength at pH 7.9.

Sedimentation-equilibrium studies of iPr_2P -chymotrypsin association

The results of four sedimentation-equilibrium experiments, two at high speed and two at low speed, on iPr_2P -chymotrypsin in 1.03 phosphate, pH 7.9, are summarized in Fig. 3(a). In this plot the thermodynamic activity of monomer has been obtained by means of the $\Omega(r)$ analysis (Milthorpe *et al.*, 1975); a monomer mol.wt. of 25000 (Hartley, 1964) has been used. A typical extrapolation for the evaluation of the monomeric activity associated with a particular total concentration $\bar{c}(r_F)$ is shown in Fig. 3(b), where the results of two equilibrium experiments are correlated. Since the results presented in Fig. 3(a) are to be analysed on the basis of ideal thermodynamic behaviour for iPr_2P -chymotrypsin in the concentration range examined, some justification of this approximation seems appropriate.

In quantitative studies of self-association, thermodynamic activities and concentrations have usually been interrelated by assuming (Adams & Fujita, 1963) that $\ln \gamma_j(r) = jBM_1\bar{c}(r)$, where $\gamma_j(r)$ denotes the activity coefficient of j -mer in a solution with total weight concentration $\bar{c}(r)$ of solute with monomer molecular weight M_1 . Non-ideality is assumed to be described in this manner so that activity coefficients cancel in the expressions for the various association equilibrium constants. This procedure has been shown (Ogston & Winzor, 1975) to

be a reasonable approximation even in instances where the assumption may not be justified theoretically. However, B then becomes an empirical constant designed to improve the closeness of correspondence between the experimental and theoretical dependence of $c_1(r)$ on $\bar{c}(r)$. Theoretically, the magnitude of B must be such that $\alpha_{11} < B < \alpha_{nn}$, where α_{11} and α_{nn} are the non-ideality coefficients expressing non-chemical self-interaction of monomer and highest polymer respectively. In the present context a reasonable magnitude of BM_1 would be 2×10^{-3} litre/g by considering α_{jj} as exclusion co-volume terms (Ogston & Winzor, 1975), but such a value would be reflected as less than a 1% difference between $c_1(r)$ and $a_1(r)$ for the highest $\bar{c}(r)$ studied in Fig. 3(a). Since such a discrepancy is within the order of experimental uncertainty, we have considered B to be indistinguishable experimentally from zero. It is noteworthy that Pandit & Rao (1974) concluded from experiments that $B \approx 0$ for α -chymotrypsin under fairly similar conditions (pH 8.3, I 0.05).

Analysis of the results presented in Fig. 3(a) is complicated inasmuch as the results are incompatible with the existence of a monomer-single-higher-polymer system ($nA \rightleftharpoons A_n$); in this sense the results parallel those of Pandit & Rao (1974, 1975). Ultimately, the results have been tested for conformity with four models (Table 1). Two involve discrete polymerization steps, for which the monomer concentration $c_1(r)$ and total concentration $\bar{c}(r)$ are related by:

$$\bar{c}(r) = \sum_{j=1}^n K_j [c_1(r)]^j \quad (1)$$

where $K_1 = 1$ by definition, and all other K_j values have the dimensions $l^{j-1} \cdot g^{1-j}$. Two models encompassing indefinite associations have also been tested. The first of these entailed isodesmic association of monomer, for which the relationship between $c_1(r)$ and $\bar{c}(r)$ is:

$$\bar{c}(r) = c_1(r) / [1 - k_1 c_1(r)]^2 \quad (2)$$

where k_1 (litre/g) is the molar isodesmic association constant divided by the monomer molecular weight

Fig. 3. Analysis of equilibrium sedimentation experiments on iPr_2P -chymotrypsin in 1.0.03 sodium phosphate buffer, pH 7.9

(a) Composite plot of the monomeric activity $a_1(r)$ as a function of total concentration $\bar{c}(r)$ of iPr_2P -chymotrypsin from four experiments, each denoted by a different symbol; curves C and D are theoretical relationships for the respective models listed in Table 1. (b) Representative plot of the extrapolation involved in determining the activity of monomer by means of the $\Omega(r)$ analysis (Milthorpe *et al.*, 1975): the ordinate intercept equals $a_1(r_F)/\bar{c}(r_F)$, the reference total concentration being fixed at 1.50 g/litre in each of the two experiments ($\bullet$, $\blacksquare$).

Table 1. Equilibrium constants for various models of iPr_2P -chymotrypsin polymerization in 1.0.03 phosphate, pH 7.9
The equilibrium-sedimentation results presented in Fig. 3(a) are analysed in terms of four models of association; isodesmic association constants, denoted as k_i , have the dimensions litre/g.

Model	Species (j)	Equilibrium constants K_j ($l^{j-1} \cdot g^{1-j}$)
A	1, 2, 4	$K_2 = 0.21$, $K_4 = 0.092$
B	1, 2, 4, 6	$K_2 = 0.21$, $K_4 = 0.089$, $K_6 = 0.00061$
C	1, 2, 3, 4, 5, ...	$k_1 = 0.15$
D	1, 2, 4, 6, 8, ...	$K_2 = 0.25$, $k_1 = 0.68$

M_1 (Van Holde & Rossetti, 1967). In the second model with indefinite association, monomeric iPr_2P -chymotrypsin was considered to undergo discrete dimerization, with K_2 defined as in eqn. (1), and the dimer to undergo isodesmic association. [Isodesmic association, as defined by Van Holde & Rossetti (1967), is an indefinite association of a species such that all molar equilibrium constants are identical.] For this model the relevant expression (Adams *et al.*, 1975) is:

$$\bar{c}(r) = c_1(r) \left(1 + \frac{K_2 c_1(r)}{\left\{ 1 - \frac{K_2 k_i [c_1(r)]^2}{2} \right\}^2} \right) \quad (3)$$

where k_i is again the molar isodesmic association constant divided by M_1 . Basically, three smoothed experimental $[c_1(r), \bar{c}(r)]$ points, namely [1.00, 1.30], [1.50, 2.42] and [2.00, 4.30] (all in g/litre), were used to guide the curve-fitting, except for the system involving isodesmic association of monomer (model C in Table 1); for this system the reported k_i is the value that yielded the smallest standard deviation between theory and experiment. The equilibrium constants reported in Table 1 have not been assigned standard errors, since a more rigorous treatment of thermodynamic non-ideality than that used is required for such values to provide a reliable indication of the likely uncertainty in the values of the equilibrium constants.

Of these models, that involving isodesmic association of dimer (model D) was found to provide the best description of the results, the solid line in Fig. 3(a) representing the theoretical relationship for a system with $K_2 = 0.25$ litre/g and $k_i = 0.68$ litre/g. This model, with discrete dimerization followed by isodesmic association of dimer, is clearly preferred to isodesmic association of monomer, for which the broken line represents a system with $k_i = 0.15$ litre/g. Indeed, on the basis of the magnitudes of the standard deviations between theory and experiment, a monomer-dimer-tetramer system, or a monomer-dimer-tetramer-hexamer system, provides a closer description of the experimental data. However, both of these models (Table 1) were surpassed from this viewpoint by model D. Thus in contrast with the conclusion drawn from the previous molecular-weight studies of α -chymotrypsin at relatively low ionic strengths (Pandit & Rao, 1974, 1975), the present investigation implicates a chymotrypsin-polymerization scheme with dimer rather than monomer as the species undergoing isodesmic association. In neither investigation did Pandit & Rao (1974, 1975) consider this preferred isodesmic model, and it is noteworthy that description of their results in terms of the other isodesmic system required use of a negative value of B at the higher ionic strength (I 0.1). On the basis that the second virial coefficient is largely an exclusion-

volume factor, only positive values of B are meaningful physically, and hence the negative value deduced from curve-fitting may well be taken to indicate the use of an erroneous model of association (Milthorpe *et al.*, 1975).

Predicted sedimentation-velocity behaviour of the various models of iPr_2P -chymotrypsin association

Although the sedimentation-equilibrium results have indicated an order of preference for the various models of iPr_2P -chymotrypsin polymerization in I 0.03 phosphate, pH 7.9, further confirmation of model D (Table 1) has been sought from the sedimentation-velocity experiments. In order to evaluate the predicted dependence of $\bar{s}_{20,w}$ on $\bar{c}$, the assumption has usually been made (e.g. Gilbert, 1963) that:

$$s_j = s_j^0 (1 - g\bar{c}) \quad (4a)$$

$$s_j^0 = s_1^0 j^{2/3} \quad (4b)$$

where g is assumed constant (approx. 0.008 litre/g) for all polymers and s_1^0 denotes the sedimentation coefficient ($s_{20,w}^0$) of monomer obtained by extrapolation of Fig. 1 to zero concentration. However, none of the models yielded a reasonable fit of the experimental data, as is evident from Fig. 1, where, for example, the theoretical relationship for models A and B is shown as a solid line, the differences between these two models being insufficient to yield distinguishable dependence of $\bar{s}$ on $\bar{c}$. Consequently, consideration of all polymers as spheres seems ill-founded. As an alternative extreme, polymerization has been considered to be an end-to-end process with the monomer spherical, such that the dimer approximates to a prolate ellipsoid with an axial ratio of 2, the trimer, 3, and so forth. Frictional ratios (f/f_0) for the various polymeric species could then be calculated from the Perrin (1936) equation, and s_j determined from eqns. (4a) and (4c) with $s_1^0 = 2.7S$.

$$s_j^0 = (s_1^0 j^{2/3}) / (f/f_0) \quad (4c)$$

Results of these calculations of $\bar{s} = \sum (c_j s_j) / \sum c_j$ are also presented in Fig. 1 for the four models listed in Table 1, and clearly the agreement between prediction and experiment must be considered good for models A, B and D, especially in view of the use of the same g value for species with widely different axial ratios; the summations were extended to j values of 12 and 24 for the isodesmic models C and D respectively, to decrease the contribution of subsequent polymers to less than 0.01% of the total concentration.

In addition to providing information about the mean rate of migration, velocity sedimentation also gives a measure of the sedimentation-coefficient distribution within the sample. An attempt has been

Fig. 4. Velocity sedimentation pattern for iPr_2P -chymotrypsin in 1.03 sodium phosphate buffer, pH 7.9

The schlieren pattern was photographed after centrifugation of a solution of iPr_2P -chymotrypsin (3.8 g/litre) for 100 min at 20°C and 60000 rev./min: because of radial dilution the plateau concentration is 3.3 g/litre in this exposure. Sedimentation is from left to right.

Fig. 5. Theoretical sedimentation-velocity patterns for four possible models of iPr_2P -chymotrypsin polymerization in 1.03 sodium phosphate buffer, pH 7.9

The four profiles (a-d) represent the asymptotic shapes of sedimentation velocity patterns predicted (Gilbert, 1963) for the corresponding models (A, B, C and D respectively) of chymotrypsin association listed in Table 1: a plateau concentration of 3.3 g/litre has been used in each instance to permit comparison of the theoretical patterns with their experimental counterpart (Fig. 4).

made to correlate the observed pattern (Fig. 4) with the theoretical asymptotic profiles obtained by application of the Gilbert (1955, 1959, 1963) theory to the present models; the relevant expressions are eqns. (16) and (19) of Gilbert (1963) and the present eqn. (4c) for the various s_0^2 values. The normalized schlieren patterns for all four models are presented

in Fig. 5 for $\bar{c} = 3.3$ g/litre, a concentration appropriate to Fig. 4. An obvious difficulty encountered in comparing Figs. 4 and 5 is the presence of diffusional effects in the former and their absence from the latter. However, it seems reasonable to consider that Fig. 5(d), which is the theoretical pattern for the model (D) favoured by the equilibrium-sedimentation analysis, resembles most closely the velocity pattern. In agreement with an earlier observation (Winzor & Scheraga, 1963), the experimental pattern exhibits a maximum in the vicinity of 3 S and, although skewed, shows no signs of a partially resolved faster-migrating peak. Although diffusion tends to mask bimodality (Cox, 1971; Gilbert & Gilbert, 1973), it should be noted that the faster peak ($s \geq 4.5$ S) is the major feature of the distributions for the other three models.

Concluding remarks

Quantitative analyses of sedimentation-velocity and sedimentation-equilibrium distributions obtained with iPr_2P -chymotrypsin in 1.03 phosphate, pH 7.9, have led to postulation of the self-associating system as an isodesmic association of dimer ($k_1 = 0.68$ litre/g) that is formed by a discrete dimerization step ($K_2 = 0.25$ litre/g) from monomer. In this connexion it seems pertinent to note (a) that the dimerization constant is of very similar magnitude to the value of 0.17 litre/g observed for α -chymotrypsin (Nichol *et al.*, 1972) and iPr_2P -chymotrypsin (Winzor *et al.*, 1977) at the same pH (7.9) but higher ionic strength (1.02), where association is effectively restricted to a monomer-dimer equilibrium, and (b) that dimerization of α -chymotrypsin has been reported to be a symmetrical head-to-head process (Steitz *et al.*, 1969; Nichol *et al.*, 1972). Hence it seems reasonable to consider that the dimerization reaction is essentially the same under both sets of conditions (ionic strengths), and that higher polymers are formed from dimers by an entirely different mechanism that is largely electrostatic in nature.

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