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Self-association of α-chymotrypsin: Effect of amino acids

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Abstract. The concentration-dependent self-association of α-chymotrypsin is known to be influenced by various factors including the presence of small molecules and autolysis products. In this connection the effect of various amino acids on the self-association of α-chymotrypsin has been studied, as a point of interest, by measuring the sedimentation coefficient of α-chymotrypsin. The influence of an amino acid is seen to be governed by the nature of its side chain. Some amino acids do not affect the self-association of α-chymotrypsin at all while some affect it moderately and some others considerably. Functional groups such as the – OH group of Ser or the phenolic ring of Tyr do not seem to influence self-association behaviour. Based on these effects, amino acids could be categorized into 3 groups. Activity studies in the presence of amino acids indicate that the site of self-association and the active-site are probably mutually exclusive.

Keywords. Self-association; α-chymotrypsin; ultracentrifuge; sedimentation coefficient; amino acid effect.

Introduction

α-Chymotrypsin has been used by several workers as a model system for the study of the concentration-dependent self-association of proteins. The self-association of proteins is known to be dependent on various factors (Schwert, 1949; Smith and Brown, 1952; Rao and Kegeles, 1958; Ackers and Thompson, 1965; Sarfare *et al.,* 1966; Morimoto and Kegeles, 1967; Tellam and Winzor, 1977; Ikeda *et al.,* 1982c). Pandit and Rao (1974) showed that the self-association of α-chymotrypsin is influenced appreciably by autolysis products present in the solution. It is known that small molecules influence the self-association of proteins considerably (Ikeda *et al.,* 1982a). Certain specific amino acid derivatives have been shown to modify the association behaviour of α-chymotrypsin (Ikeda *et al.,* 1982b); however, the effect of various amino acids on the self-association behaviour of α-chymotrypsin has not been well studied. This report demonstrates that the presence of amino acids influences the self-association of α-chymotrypsin and that the extent of this effect depends on the nature of the side-chain group (s).

Materials and methods

Chemicals

Thrice crystallized α-chymotrypsin from Worthington (Batch CD17-JC) or from BDH Biochemicals (product No. 39009) was used without further purification. Amino acids were purchased from Calbiochem, USA, E. Merck AG, Germany, or BDH, England. All the chemicals used were of guaranteed reagent grade.

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Sedimentation velocity measurements

The sedimentation velocity measurements were made with a Spinco Model E ultracentrifuge at 260,000 *g*. Schlieren optics was used for recording sedimentation boundaries. All the measurements were made at 25 ± 1°C. From pictures taken at different times, the sedimentation coefficients of individual peaks were determined. The data were corrected for temperature and viscosity.

Sedimentation patterns were resolved into individual components from enlarged photographs and relative areas were measured by cutting out the peaks and weighing them. Johnston-Ogston correction was not applied as it is negligible for globular proteins.

Protein concentration was determined spectrophotometrically using a value of 20·6 for $E^{1\%}_{280\ \mathrm{nm}}$ (Rao and Kegeles, 1958).

Proteolytic activity

The proteolytic activity of α-chymotrypsin in the presence of amino acids was determined using casein as substrate. Tris-HCl buffer of pH 8·3 was prepared and the ionic strength made up to 0·05 by the addition of KCl. To 1 ml of the Tris buffer containing 5 μg of the enzyme and 1·4 μg of amino acid, 1 ml of 1% casein solution in the same buffer was added. The reaction mixture was incubated at 37°C for 20 min and the reaction stopped by the addition of 3 ml of 5% TCA solution. The precipitate was allowed to settle for 30 min at room temperature and then removed by centrifugation. Proteolytic activity was measured as optical density at 280 nm of the supernatant. The activity of the enzyme in the absence of any amino acid served as control. Change in the activity of the enzyme in the presence of amino acid was calculated with respect to the control.

Results and discussion

The sedimentation patterns of α-chymotrypsin in the presence of amino acids are given in figure 1. All the sedimentation velocity experiments were performed at a concentration of 1·6% of α-chymotrypsin and a concentration of 0·44% of the added amino acid (ratio of amino acid to protein $\simeq$ 1:4). The choice of this ratio was guided mainly by the earlier work of Pandit and Rao (1974) on α-chymotrypsin in which they showed the formation of about 20% autolysis product within a couple of hours. The influence of amino acids on the sedimentation behaviour is quite obvious from the sedimentation patterns. For example, in the presence of Arg, Arg·HCl or Lys, the sedimentation pattern shows only one peak similar to the slow-moving peak for monomers of α-chymotrypsin, indicating that these amino acids influence the self-association behaviour of α-chymotrypsin considerably. Table 1 summarizes the results of the sedimentation velocity experiments. The sedimentation pattern of α-chymotrypsin in the absence of any amino acid consists of an equilibrium reaction boundary with $S_{20,\ w}$ values of 2·94 and 6·06, The slow-moving peak consists of monomers while the fast peak reflects the aggregation reaction in equilibrium controlled by concentration (Pandit and Rao, 1974). Comparison of the $S_{20,\ w}$ values obtained (table 1) indicates that the presence of certain amino acids brings about a

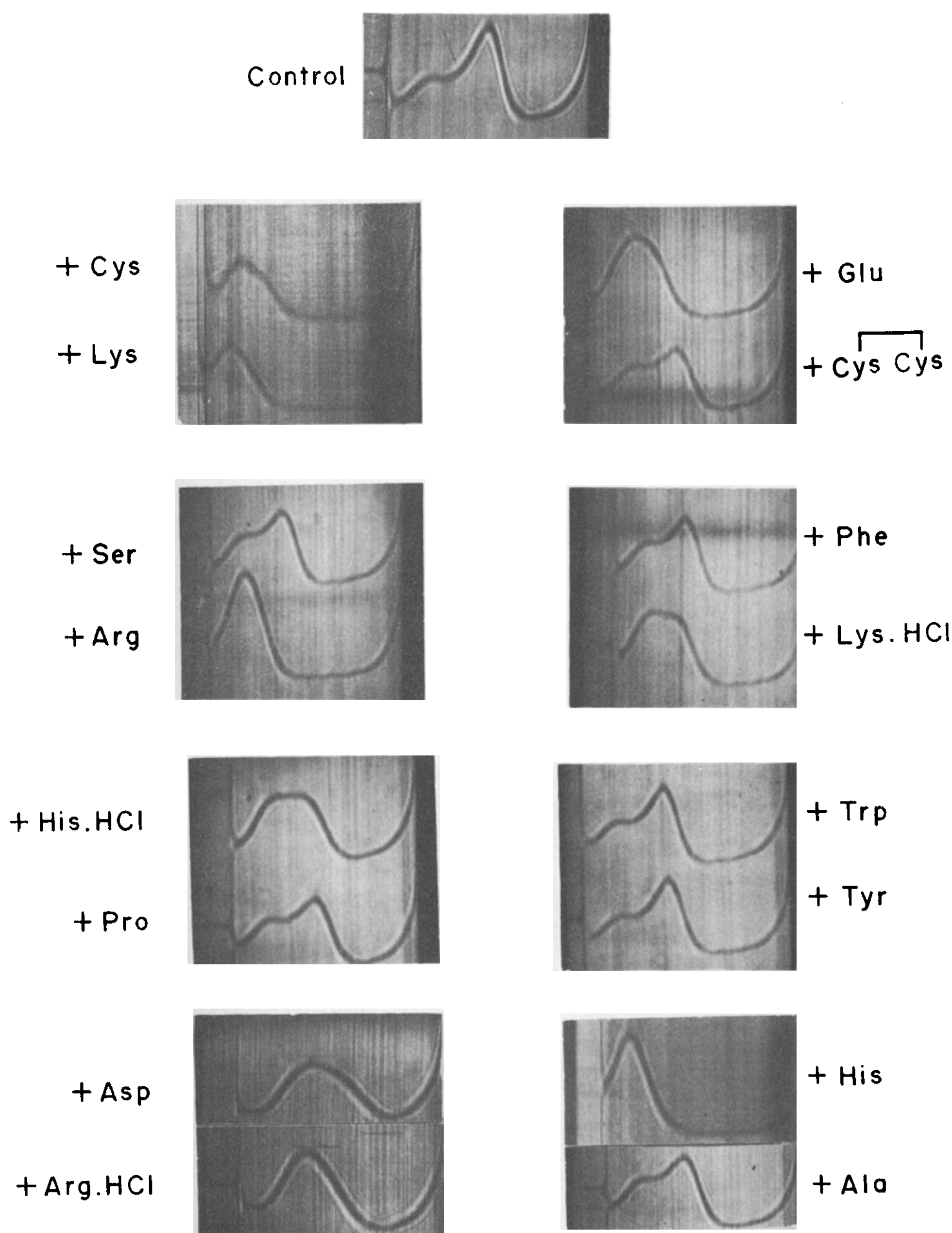

Figure 1. Sedimentation velocity patterns of α-chymotrypsin (control) and α-chymotrypsin in the presence of amino acids. The concentration of α-chymotrypsin was 16 mg/ml in Tris-HCl buffer (pH 8·3, ionic strength 0·05), and the ratio of protein to amino acid was 4:1 (w/w) in all the cases. Sedimentation is from left to right.

drastic change in the sedimentation pattern, sometimes even reducing it to a single slow-moving peak. The influence of the presence of amino acids is reflected not only in the $S_{20,w}$ values at the peak positions but also in the relative proportion of

Table 1. Effect of amino acids on the sedimentation behaviour of α-chymotrypsin.

Amino acid added to α-chymotrypsin	S_s(area %)	S_f(area %)	$\bar{S}_{20,w}$	ΔS(%)	Category, based on effect on self-association
Ser	3·27 (22·8)	5·91 (77·2)	5·31	− 2·2	A
Tyr	3·17 (20·6)	5·80 (79·4)	5·26	− 3·1	–"–
Pro	3·13 (26·5)	5·76 (73·5)	5·06	− 6·8	–"–
Ala	3·03 (16·1)	5·51 (83·9)	5·11	− 5·9	–"–
Cys Cys	3·19 (22·4)	5·64 (77·6)	5·09	− 6·3	–"–
Trp	3·18 (22·7)	5·63 (77·3)	5·08	− 6·4	–"–
Phe	2·91 (26·2)	5·67 (73·8)	4·95	− 8·8	–"–
His·HCl	3·16 (48·7)	4·51 (51·3)	3·85	−29·1	B
Lys·HCl	3·08 (36·0)	4·55 (64·0)	4·02	−26·0	–"–
Cys	2·77 (35·5)	4·44 (64·5)	3·85	−29·1	–"–
Glu	3·16 (100)	—	3·16	−41·8	C
His	2·95 (100)	—	2·95	−45·7	–"–
Asp	2·98 (100)	—	2·98	−45·1	–"–
Lys	2·74 (100)	—	2·74	−49·5	–"–
Arg	2·87 (100)	—	2·87	−47·1	–"–
Arg·HCl	2·80 (100)	—	2·80	−48·4	–"–
α-chymotrypsin alone (control)	2·94 (20·3)	6·06 (79·7)	5·43	0	

monomers in the reaction boundary. It is possible to quantify both these influences by computing $\bar{S}_{20,w}$ (the weight-average $S_{20,w}$) from the $S_{20,w}$ values for the individual components and their relative proportions using the formula

$$\bar{S}_{20,w} = (S_s A_s + S_f A_f)/A_t,$$

where S_s and S_f are the sedimentation coefficients ($S_{20,w}$) of the slow-moving and fast-moving components respectively, A_s and A_f are the areas under the respective peaks, A_t is the total area, and $\bar{S}_{20,w}$ is the weight-average sedimentation coefficient for all the species present.

The extent of effect on the self-association could be estimated to a first approximation by computing the per cent difference, ΔS, between the $\bar{S}_{20,w}$ of α-chymotrypsin alone and that in the presence of amino acid. Thus,

$$\Delta S = 100[(\bar{S}_{20,w})_o - (\bar{S}_{20,w})_{+aa}]/(\bar{S}_{20,w})_o,$$

where $(\bar{S}_{20,w})_0$ and $(\bar{S}_{20,w})_{+aa}$ are the weight-average sedimentation coefficients of α-chymotrypsin alone and in the presence of amino acid respectively. The $\bar{S}_{20,w}$ values obtained for α-chymotrypsin in the presence of amino acids are given in table 1. The changes in $S_{20,w}$ of individual peaks and their relative proportions obviously influence $\bar{S}_{20,w}$ values in terms of ΔS, as shown in table 1.

By closely examining the values for $\bar{S}_{20,w}$ and ΔS, the amino acids studied could be categorized into 3 groups with respect to their influence on the association behaviour of α-chymotrypsin. The first group of amino acids (category A) affect the sedimentation behaviour of α-chymotrypsin to a negligible extent ($\Delta S \leqslant 9\%$) and the sedimentation pattern always consists of two peaks with $S_{20,w}$ values similar to those of α-chymotrypsin alone. Category B consists of 3 amino acids which affect the

self-association in a way that is reflected in the reduction of the $S_{20,w}$ value of the fast-moving peak indicating that there is a reduction in the size of aggregates. However, there still exist two components of the reaction boundary, though these are not as well resolved as in the case of category A. The change (ΔS) in the $\bar{S}_{20,w}$ in this category is in the range 26–29%. The third group of amino acids (category C) shows appreciably strong influence on the self-association. The sedimentation patterns in this case consist of a single peak corresponding to the monomer peak of α-chymotrypsin; ΔS values are in the range 41–50%. It is interesting to see that amino acids such as His and Lys which greatly affect the self-association of α-chymotrypsin are not as effective in the hydrochloride form as they are in the non-hydrochloride form. It is known that ionic strength of the medium plays an important role in controlling the extent of aggregation: an increase in the ionic strength normally reduces the extent of aggregation (Pandit and Rao, 1974, 1975). The change in ionic strength due to the hydrochloride form is expected to be of the order of 0·03 leading to an effective ionic strength of 0·08 of the medium. Pandit and Rao (1974) have studied the self-association of α-chymotrypsin under conditions of isoelectric pH and 0·5 and 0·1 ionic strength. Comparison of $\bar{S}_{20,w}$ values in table 1 with their results indicates that Lys·HCl and His·HCl may influence the self-association of α-chymotrypsin by increasing the ionic strength. If this contention were true, then Lys and His should show much lower effect on the self-association behaviour than their respective hydrochlorides. The change(%) in $\bar{S}_{20,w}$ values brought about by the presence of hydrochloride form of amino acid over its non-hydrochloride form can be calculated by using the formula

$$\text{Per cent change} = 100\,[(\bar{S}_{20,w})_{+aa-\mathrm{HCl}} - (\bar{S}_{20,w})_{+aa}]/(\bar{S}_{20,w})_{+aa}.$$

The positive values of the change obtained for Lys (+ 46·3%) and His (+ 30·5%) indicate that both Lys and His influence the self-association of α-chymotrypsin to a greater extent than their hydrochloride forms. Hence, the effect seen in the case of Lys·HCl and His·HCl cannot be attributed to the increase in ionic strength alone. α-Chymotrypsin at ionic strength 0·08 aggregates ($\bar{S}_{20,w}$ = 3·9; Pandit and Rao, 1975) while in the presence of Arg-HCl it exists in the monomeric form ($\bar{S}_{20,w}$ = 2·8; table 1). Therefore, the reduction in the association brought about by Arg-HCl cannot be explained merely on the basis of increase in ionic strength.

The addition of amino acids can influence the self-association of α-chymotrypsin through a change in pH. In the present experiments, under the conditions used the change in pH due to the addition of amino acid was of the order of 0·2 unit in most of the cases and was 0·4 unit in the case of Lys, Arg and Cys. In the case of Glu and Asp pH of the final solution was 4·8 and 4, respectively (change in pH 3·5 and 4·3 units, respectively). The reduction in the self-association of α-chymotrypsin in the presence of Glu and Asp could thus be attributed to the drastic change in pH. This cannot be true in the case of a majority of the amino acids in category C. These results show that one cannot generalize the effect of amino acids on the self-association behaviour of α-chymotrypsin. However, whenever there is such an effect its extent appears to depend upon the nature of the side-chain of the amino acid.

It can be seen from the shapes of the patterns and the reduction in the $\bar{S}_{20,w}$ values that amino acids having one carboxyl group and one amino (imino in the case of Pro) group (category A) do not affect the self-association of α-chymotrypsin significantly. The results also indicate that side-chains (the – OH group of Ser or the phenolic ring

of Tyr) do not influence the self-association behaviour. All the amino acids in category C, which influence the self-association considerably, contain extra carboxyl, amino or imino (imidazole and guanidino) groups in the side-chain. It appears, therefore, that whenever there is a change in the balance of amino and carboxyl groups in added amino acid, the extent of aggregation of α-chymotrypsin is reduced. Cys falls in category B, indicating that the – SH group has an influence on the self-association behaviour of α-chymotrypsin.

Taken together these effects appear to relate to the constellation of charges on α-chymotrypsin involving at least 3 centres. The amino acids may bind to the site of association, if there is any, of α-chymotrypsin, and reduce the self-association by causing complete or partial blocking of the site. This contention is further supported by the fact that the hydrochloride forms of Lys and His had a much smaller effect on the self-association than the non-hydrochloride forms, the effect of the hydrochloride possibly being the neutralization of the influence of one of the charge centres.

It is obvious from these results that at least some of the amino acids, probably through their binding to a specific site on α-chymotrypsin, reduce the extent of self-association. As α-chymotrypsin is a proteolytic enzyme and the process of self-association can be looked upon as a mimicking of substrate–enzyme complex formation (Egan *et al.,* 1957; Kezdy and Bender, 1965), it is likely that the site involved in the self-association is also the active site. Earlier attempts in this direction indicated that the situation is quite complex (Schwert and Kaufman, 1951; Smith and Brown, 1952; Neurath and Dreyer, 1955). Martin and Niemann (1958) investigated the effect of dimerization of α-chymotrypsin on its kinetics and found that the dimer of the enzyme could bind the substrate without hydrolysing it. The studies of Sarfare *et al.* (1966) on the relationship between the active site and the polymerization site in α-chymotrypsin in the presence of *β*-phenylpropionate—a competitive inhibitor of the enzyme—indicate that the sites for the polymerization of protein and the binding of the inhibitor are mutually exclusive.

If the active site and the site of association are the same, or if they overlap, either completely or partially, then one would expect that the amino acids that strongly influence the self-association would also reduce the proteolytic activity. In order to find out if this is the case the enzyme was assayed for activity in the presence of various amino acids. The results of these experiments are summarized in table 2. It is

Table 2. Effect of various amino acids on the proteolytic activity of α-chymotrypsin.

Amino acid	Change (%) in proteolytic activity
Ser	+12·9
Tyr	+16·6
Pro	+18·6
Ala	+ 1·0
Cys Cys	+ 5·5
Trp	+19·5
Phe	+10·0
Lys · HCl	+17·4
Glu	+ 2·0
His	+ 6·0
Arg	+10·6

interesting to note that none of the amino acids reduced the activity of the enzyme. On the other hand, in most cases the presence of amino acid led to a slight increase (1–20%) in the activity over that in the control. This clearly indicates that the active site of the enzyme is completely free even in the presence of amino acid. It is known that α-chymotrypsin when dissolved in Tris-HCI buffer (pH 8·3 μ = 0·05) undergoes a time-dependent autolysis. Pandit and Rao (1974) showed that most of the autolysis takes place within the first 30 min and the process reaches a steady value of about 20%. They observed further that the extent of autolysis was decreased to the level of 10% upon prior addition of autolysis products to the incubation mixture. The increase in the activity of α-chymotrypsin which we observed in the presence of amino acids may well be the effect of a decrease in autolysis of the enzyme. It is interesting to note that the increase (av. 11 %) in the activity of the enzyme in the presence of amino acids coincides very well with the decrease in autolysis reported by Pandit and Rao (1974). Therefore, it is most likely that the increase in activity in the presence of amino acids is a reflection of reduced autolysis. A number of monomeric enzymes have been found to exhibit either positive or negative co-operativity (Meunier *et al.,* 1974; Niemeyer *et al.,* 1975; Ainslie and Neet, 1979) when bound by effector molecules. Therefore, a second possibility, that the binding of amino acid at some other site may influence the activity through a conformational change at the active site, cannot be ruled out.

In conclusion, the presence of amino acids influences the self-association behaviour of α-chymotrypsin. This influence appears to be governed by the nature of the side-chain of the amino acids and is, therefore, related to the charge distribution which determines their binding to certain functional groups on the protein. However, side-chains such as the – OH group of Ser or the phenolic ring of Tyr do not influence the self-association behaviour. The presence of amino acids has a strong influence on the self-association but does not cause any reduction in the activity, indicating that the active site is not involved in the self-association of α-chymotrypsin under the conditions studied.

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