Disulphide cross-linked macromolecules formed by thiolated insulin and globin

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It has been considered by Mahbouba et al (1974) that thiolation (Benesch & Benesch 1958; 1962) of polypeptides and proteins of pharmacological interest, e.g. ACTH, asparaginase, followed by cross-linking through intermolecular disulphide bonds could give macromolecules in which the monomer units were afforded some protection from the action of tissue enzymes, provided that these units were closely packed together. Subsequent studies with insulin (Mahbouba & Smith 1977) and those reported here for globin have shown that the units in the macromolecules are more closely packed than expected from the earlier work with α-chymotrypsin macromolecules and have prompted an examination of the susceptibility of insulin macromolecules to attack by a protease.

Formation of macromolecules. Globin was thiolated using *N*-acetyl homocysteine thiolactone (AHTL) at pH 10–11·5 by the method described for α-chymotrypsin and subsequently oxidized to the corresponding disulphide cross-linked macromolecule using potassium ferricyanide in the usual manner (Mahbouba et al 1974). Thiolated globin was oxidized as soon as possible after its preparation and was not stored in the lyophilized state since freeze-drying was associated with loss in thiol content. The average molecular size of the oxidized thiolated globin macromolecule and the radius of gyration (R_Z) were calculated from light scattering measurements using the method of Mahbouba et al (1974). The aggregation number, n, of the macromolecules obtained was broadly related to the thiol content of the thiolated globin: n = 8 (4·1 –SH groups mol^{-1}), 8·6 (0·5), 11 (3·3), 13 (3·1), 37 (7·4) and 65 (12). The shapes of the aggregates were estimated by plotting their particle scattering factors (P_θ) against sin ($\theta/2$) and comparing the curve shape with those of various model shapes (Doty & Steiner 1950). The results show that the plots for the rod and coil models were both similar to that for the globin solution.

Macromolecules (n = 410–708 monomer units) containing native insulin carried by a modified insulin skeleton have been previously described (Mahbouba & Smith 1977) and were prepared by partially thiolating (0·5–0.7 –SH groups mol^{-1}) the zinc-insulin hexamer and cross-linking using copper (II) ion-catalysed oxygenation. The macromolecule contained 2 Cu (II) and 4–6 Cu(I) atoms as determined by atomic absorption spectroscopy and titration with 2,2¹-biquinolyl (Felsenfeld 1960). The ESR spectrum of the macromolecule was consistent with replacement of zinc in the hexamer by 2 Cu(II) atoms (Evans, J. C., Morgan, P. H., Smith, H. J., Mahbouba, M. in preparation). Oxidized thiolated α-chymotrypsin macromolecules (n = 13–40) have been previously described (Mahbouba et al 1974).

Susceptibility of native insulin, 2 Cu(II) - insulin and modified insulin macromolecule to attack by α-chymotrypsin. A solution of modified insulin macromolecule, native insulin or 2 Cu(II) - insulin (5–8 mg, 8 ml) in phosphate buffer (0·1 M) pH 8, was mixed with sodium chloride solution (1 M, 1·9 ml) and α-chymotrypsin solution (50–80 μg, 0·1 ml) added. The mixture was incubated at 37° C. Initially and at suitable intervals of time, aliquots (0·5 ml) were mixed with buffer (2·5 ml) and a solution of sodium trinitrobenzene sulphonate (TNBS) (1%, 25 μl) was added. The colour developed at λ420 nm (A) after a 2 h incubation and was a measure of the α-amino groups released in the reaction (Habeeb 1966; Snyder & Sobocinski 1975). The number of amino groups liberated mol^{-1} of insulin was calculated from $(A - A')/A'/3$ where A′ is the noted increase in absorption due to reaction of the three single amino groups in native insulin as determined in the absence of enzyme.

Appreciable enzymatic hydrolysis occurred during the long reaction period with TNBS and the 'initial' titres for released amino groups were 4·6 and 8·6 for modified and native insulin respectively. The values increased to 9·39 and 12·39 respectively after a 3½ h incubation. Under similar conditions Native 2 Zn-insulin and 2 Cu(II)-insulin gave 'initial' titres for released amino groups of 7·7 and 3·8 respectively and after 3½ h incubation these values increased to 10·1 and 6·0 respectively. The peptide linkages susceptible to α-chymotryptic hydrolysis in the insulin samples were phenylalanine (3) and tyrosine (4) as well as the less susceptible leucine (6) peptide linkages.

A solution (21 ml) containing Cu(II) ions and α-chymotrypsin was placed in the titration cell of the pH stat (Radiometer, Copenhagen) and assayed in the usual manner (Al Shabibi & Smith 1974) with ATEE (2 ml, 0·02 M) at pH 7·4 and 25 °C. The final concentration of Cu(II) ions and α-chymotrypsin in the cell was the same as was present in the incubation mixture containing the copper-insulin preparations. A control was conducted in the absence of Cu(II) ions. The activity of the enzyme was lowered 25% in the presence of the Cu(II) ions.

Compactness of macromolecules. The ratio M/R_Z increases with M (molecular weight) for each of the three protein macromolecules. We suggest that the

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noted curvature of the plot is due to the macromolecules becoming more compact with increasing molecular weight, a feature probably due to inflexion of any chains of molecules, on the basis of the following arguments. Consider the two extreme degrees of compactness possible in a model macromolecule, namely a stiff rod and a sphere. The relationship between the radius of gyration of the rod and its length (Stacey 1956) is $L = R_z\sqrt{12}$ and that between R_z and diameter for a sphere is $D = R_z\sqrt{20/3}$. The molecular weight of these models is proportional to the length and (diameter)^3 respectively, so that for the rod-shaped model, the ratio M/R_z would be independent of M whereas for the sphere-shaped model the ratio would be expected to increase with M. Change in an aggregate to a more compact form as M increases would lead to a resultant change in M/R_z which is the sum of two terms; a term due to the increase in the rod-like characteristic which would tend to keep M/R_z constant, and a term due to an increase in the spherical characteristic which would tend to increase this ratio. The ratio M/R_z may therefore be regarded as a measure of the compactness of the aggregate. The results shown in Fig. 1 for the modified insulin, globin and α-chymotrypsin macromolecules indicate increasing compactness of the macromolecule with increasing molecular weight. The greatest rod→coil transition is implied for insulin which is in accord with the results of shape determination by the traditional light scattering methods which showed a rod→coil transition at high molecular weight (Mahbouba & Smith 1977). The results for α-chymotrypsin are included from earlier work in which the probability that the aggregates were more compact than a stiff rod model was argued from a consideration of the known dimensions of the molecules.

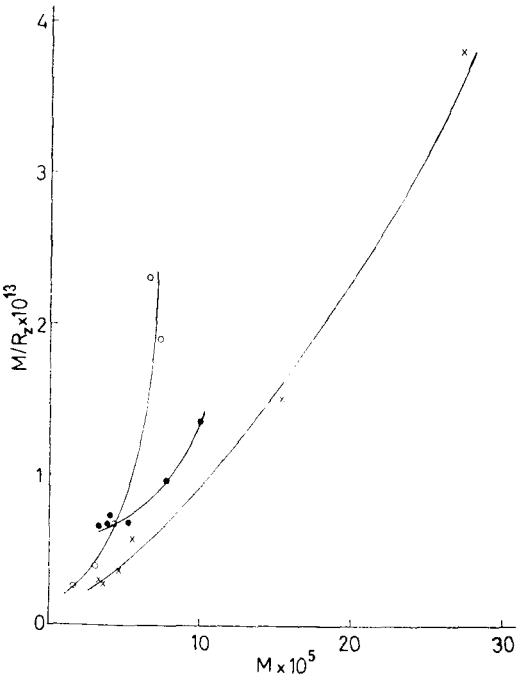

FIG. 1. Change in the ratio M/R_z with increasing M for disulphide cross-linked macromolecules formed from α-chymotrypsin (●), insulin (○) and globin (×).

The results reported here would suggest that the macromolecules formed in this work, may have a greater compactness than was originally suspected from the previous studies with α-chymotrypsin macromolecules. The steep rise in M/R_z with M, which is particularly marked for insulin, suggested that the monomer units were sufficiently closely packed to afford protection to each other from attack by tissue enzymes.

The susceptibility of specific peptide linkages in native insulin to α-chymotrypsin action was apparently greater than in modified insulin since native insulin was degraded at approximately twice the rate of the macromolecule. However, a similar rate difference was noted in the α-chymotryptic digestion of native 2 Zn-insulin and 2 Cu(II)-insulin. It would seem that the apparent greater stability of the macromolecule to enzyme attack is not due to the beneficial effects of the closer packing of the units noted in the macromolecule but mainly to the noted inhibition of the enzyme by Cu(II) ions which are released once the integrity of the hexamer is violated. The results from the enzyme studies show that the intermolecular spaces between the units of the macromolecule are sufficiently large to allow unhampered penetration by the α-chymotrypsin molecule ($4{\cdot}93 \times 10^{-9} \times 6{\cdot}7 \times 10^{-9} \times 6{\cdot}59 \times 10^{-9}$ M; Sigler et al 1968).

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