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Disulphide cross-linking of thiolated α-chymotrypsin to form macromolecules

M. MAHBOUBA, W. J. PUGH AND H. J. SMITH

Welsh School of Pharmacy, University of Wales Institute of Science and Technology, Cathays Park, Cardiff, U.K.

Thiolation of α-chymotrypsin with *N*-acetyl homocysteine thiolactone (AHTL) occurs in the presence of Ag^+ and imidazole with the introduction of 4–5·5 SH groups per mole. Oxidation of the thiolated enzyme with ferricyanide gave a macromolecule consisting of thiolated enzyme residues cross-linked through intermolecular disulphide bonds. The number of residues linked in this manner varied with each batch of thiolated enzyme but was within the range 16–44 residues. The light scattering data indicated that the macromolecule was rod-shaped.

In recent years thiol groups have been introduced into proteins before the addition of heavy metal labels for electron microscopy studies (Benesch & Benesch, 1958, 1962; Kendall, 1965, 1972; Kendall & Barnard, 1969). Benesch & Benesch (1958) showed that formation of inter- or intramolecular disulphide bonds between the thiol groups was possible by oxidation with ferricyanide. We considered that intermolecular bonding in this manner between polypeptides of pharmacological interest, such as ACTH and asparaginase, might yield macromolecules with potentially useful sustained-release properties, provided, (a) that thiolation was not associated with excessive loss in pharmacological activity, and (b) that the aggregate had a reasonable area/volume ratio, so that attack by plasma enzymes would be confined to the exposed molecules on its periphery. Gradual release of the active molecules would occur by reduction of the disulphide bridges by plasma thiols.

We report here a preliminary study on the properties of such macromolecules formed using α-chymotrypsin as a model protein.

METHODS AND RESULTS

Materials

α-Chymotrypsin from bovine pancreas which had been recrystallized three times (Koch-Light) was further purified by passage down a column (45 cm × 2·5 cm i.d.) of Sephadex G-25 (41 g) with collection of the main protein fraction detected by ultraviolet absorption measurement at λ 282 nm.

Thiolation of α-chymotrypsin by N-*acetyl homocysteine thiolactone* (*AHTL*)

A solution of silver nitrate (2 ml, 0·3M) was mixed with a solution of imidazole (2 ml, 2·4M) and the suspension was adjusted to pH 8 with dilute sulphuric acid (0·5M). A solution of α-chymotrypsin (5 ml, 100 mg) was added and the resulting mixture was re-adjusted to pH 8 with dilute sodium hydroxide (M) and a solution of AHTL (2 ml, 0·3M) added. The suspension was stirred for 0·5 h with continuous re-adjustment to pH 8 when a yellow opalescent solution was formed. A cold neutral solution of potassium cyanide (10%) was then added until a clear colourless

solution was obtained. The solution was immediately transferred to a column of Sephadex G-25 equilibrated with phosphate buffer pH 8 (0·1M). The column was developed with the same buffer and fractions (4 ml) collected after rejection of the first 60 ml. The fractions were examined spectrophotometrically for protein absorption at λ 282 nm and the fractions (2–8) containing the thiolated protein were combined and freeze dried.

The determination of –SH groups in thiolated α-chymotrypsin

The –SH groups in the thiolated α-chymotrypsin were determined by Ellman's method (Ellman, 1959) using a value of 13 600 (Ellman, 1958) for the reported extinction coefficient for the coloured reaction product.

The initial titre in the thiolated protein varied with the batch and was within the range 4–5·5 SH groups per mole.

Assay for enzyme activity. The activity of the thiolated enzyme was determined in a pH-Stat (Radiometer, Copenhagen) by a continuous titration method using *N*-acetyl tyrosine ethyl ester (ATEE) at pH 7·4 and 25° (Al Shabibi & Smith, 1974).

The activity per mole of the thiolated enzyme towards ATEE was compared with that of the native enzyme using Lowry's method (Lowry, Rosebrough, & others, 1951) for determination of protein nitrogen. A freshly prepared solution of the thiolated enzyme (5·5 SH) had 60% of the activity shown by the native enzyme.

Disulphide coupling with potassium ferricyanide

An aliquot (1 ml) of a solution (100 ml) containing potassium ferricyanide (1·82 g), ammonium chloride (4·86 g) and ammonia (0·880, 7·1 ml) was added to a solution of thiolated enzyme (9 ml, 85 mg) and the mixture stored at room temperature (20°) for 1 h. The mixture was then passed down a column of Sephadex G-25 to remove excess reagent. The protein fraction was collected in the usual manner and the thiol titre was found to be zero which confirmed that all the thiol groups had been coupled in disulphide linkages.

A freshly prepared solution of the ferricyanide cross-linked thiolated enzyme (0·2%) in phosphate buffer pH 8·0 had 54% of the activity (ATEE) possessed by the native enzyme under similar conditions.

Light scattering studies

Measurements were made using a Brice-Phoenix instrument at λ 546 nm. Refractive index differences were measured with a differential refractometer (Polymer Consultants Ltd.). Solutions of cross-linked thiolated enzyme in phosphate buffer pH 8 (0·1M) were clarified by filtration through Millipore filters of pore size 0·22 μm in a Millipore filtration unit and the protein concentration determined using Lowry's method.

Measurements were made in a cylindrical cell with flat entry and exit faces over angles ranging from 45° to 135°. After applying the appropriate Fresnel corrections (Stacey, 1956) the results were analysed by plotting Kc/R_θ against $\sin^2(\theta/2)$ as described by Zimm (1948). The intercept at zero angle gives the value 1/M.

Results were obtained for several different batches of ferricyanide cross-linked thiolated enzyme and the aggregation numbers (n) were 16–44, the more frequently observed values being in the narrower range 16–32.

Shape of the macromolecule

The shape of the aggregates was deduced from the light scattering results by comparison of the reciprocal particle scattering factors at 90°, P^{-1} (90°), with those for model shape having the same dissymmetry values. Tables of P^{-1} (90°) and dissymmetry for spheres, rods, random coils and discs are given by Beattie & Booth (1960).

The results are presented in Table 1 and it can be seen that the best fits for all samples, irrespective of the molecular size, are given by the rod and coil models. Attempts to obtain data corresponding to an ellipsoid of revolution were unsuccessful because the limited range of values available in the literature (Koch, 1961; Beidl, Bischof & others 1957) lay outside the range of radii of gyration of the aggregates.

Table. 1. *Comparison of experimental P^{-1} (90) values for cross-linked thiolated α-chymotrypsin with theoretical P^{-1} (90) values for models having the same dissymmetry as the protein solutions.*

Aggregation number	P^{-1} (90)	Dissymmetry	P^{-1} (90) − [P^{-1} (90) for model with same dissymmetry] Sphere	Rod	Disc	Coil
31	2·04	2·12	0·36	0·16	0·37	0·14
40	1·85	1·91	0·29	0·06	0·26	0·14
16	1·58	1·71	0·12	0·02	0·11	0·05
13	1·42	1·48	0·10	0·07	0·10	0·07
21	1·99	2·11	0·32	0·18	0·27	0·10
16	1·47	1·59	0·21	0·15	0·19	0·16

The choice between the rod and coil models was made by computing x and $x^{\frac{1}{2}}$ values respectively (Doty & Steiner, 1950), interpolating P (θ) values and plotting these over a wide range of angles. In most cases the experimental values corresponded to the rod model.

DISCUSSION

AHTL reacted with α-chymotrypsin in the presence of Ag^+ and imidazole with the introduction of several residues containing thiol groups into the enzyme molecule. The number of thiol groups introduced varied with each batch of product and was within the range 4–5·5 SH groups per mole. The noted variation was possibly due in part to exposure of the thiolated enzyme to non-uniform oxidizing conditions during the isolation procedure (Benesch & Benesch, 1958).

A heterogeneous mixture of thiolated derivatives, difficult to separate, is usually obtained on thiolation of proteins with AHTL (Benesch & Benesch, 1958), e.g. ribonuclease (White & Sandoval, 1962), α-chymotrypsinogen (Abadi & Wilcox, 1960), and further purification of the mixture was not pursued.

Both the oxidized and non-oxidized thiolated enzymes had about 60% of the activity of α-chymotrypsin. The native structure of the enzyme had apparently not been altered which is in accord with thiolation occurring on the ε-amino groups of lysine residues on the outer surface of the molecule (Benesch & Benesch, 1962) away from the active site.

Oxidative coupling of the thiol groups in thiolated gelatine has been reported to occur with the formation of either intramolecular or intermolecular disulphide bonds (Benesch & Benesch, 1958), although quantitative studies of the extent of the cross-

linking in the latter instance were not made. The cross-linking obtained in the oxidized thiolated enzyme was quantitatively studied in this work by light scattering measurements. The aggregation number, n, found for the number of enzyme residues in the cross-linked macromolecule was not a constant value and varied with each batch of thiolated enzyme used in the oxidation. Values of n ranged from 16–44 the more frequently observed values being in a narrower range 16–32, although in all instances the systems were homogeneous.

The light scattering data for the macromolecule suggested that its shape was closer to that of a rod than a coil, other shapes considered such as a sphere or disc being much less likely. Although the innate flexibility of a linear chain of protein molecules would favour a coil structure the bulkiness of the enzyme residues relative to the length of the bonds joining them would be expected to reduce such coiling to a considerable extent. The thin rod model represented the nearest approximation to the shape of the macromolecule but it is probably not an exact description for the following reason. The calculated length of the rod from light scattering studies is given by, length $= 3{\cdot}46 \times$ radius of gyration, (Fowle, 1934), which where for example $n = 31$, is $2{\cdot}8 \times 10^{-7}$ m. The unit cell dimensions for α-chymotrypsin are $a = 4{\cdot}93 \times 10^{-9}$ m, $b = 6{\cdot}73 \times 10^{-9}$ m, $c = 6{\cdot}59 \times 10^{-9}$ m from crystallographic data (Sigler, Blow & others, 1968) so that the maximum length obtainable by aggregation of 31 molecules ($2{\cdot}086 \times 10^{-7}$ m) through disulphide residues is $2{\cdot}64 \times 10^{-7}$ m. We consider that this discrepancy is due to the chain forming the backbone of the macromolecule bearing short side chains along its length, this branching being possibly because of the presence of the many thiol groups per enzyme residue available for coupling. The redistribution of mass in this manner towards the centre of the macromolecule would have a relatively greater effect on the length of the molecule than on the radius of gyration, and it is the latter quantity that is given by the light scattering measurements.

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