Chemical cross-linking of a dimeric protein on a modified lectin matrix

A general probe for the chemical topology of oligomeric glycoproteins

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A dimeric glycoprotein, glucose oxidase, was allowed to react with lysine-specific cross-linkers, both when immobilized on a succinoylated lectin matrix at a critically low density and also at a high density in solution. Analysis of the cross-linked complexes thus obtained led to the following inferences with regard to the structure of this protein. (1) Of the 15 lysine residues on each glucose oxidase protomer, none is available on the non-interfacial surfaces. (2) Assuming that this protein possesses C_2 symmetry with isologous bonding between subunits, it may be inferred that on each protomer there are at least two lysine clusters along or close to the interprotomeric interface. (3) These 'interfacial' lysine residues on each protomer are so oriented that the ϵ -amino groups of lysine residues a and b on protomer 1 'face', and are very close to, the ϵ -amino groups of lysine residues b' and a' respectively on protomer 2. General inferences on the geometry of dimeric proteins derivable from an analysis of the cross-linked complexes obtained (as well as those not seen) by using this low-density matrix cross-linking approach were enumerated. Modified lectin matrices may prove useful in studying the three-dimensional structure of glycoproteins, particularly non-crystallizable oligomers.

Many glycoproteins of biological interest, including a large number of poorly soluble membrane-bound oligomers, may never prove to be crystallizable. Alternative probes, if available, would prove useful for obtaining structural information on such proteins, as well as for deriving preliminary topographical data for other glycoproteins that may be eventually crystallized and characterized.

Conventional 'in-solution' cross-linking methods have occasionally yielded valuable information on inter-residue distances in single-chain globular proteins (Hartman & Wold, 1967) and on the subunit stoichiometry of oligomeric proteins (Davies & Stark, 1970). The information derivable from these methods with regard to three-dimensional protein structure is, however, limited and often ambiguous. The immobilization of a protein at a critically low density on an affinity matrix (Pillai & Bachhawat, 1977, 1978; Pillai *et al.*, 1979) before treatment with a bifunctional reagent prevents the formation of intermolecular conjugates; by using such an approach, high concentrations of a bifunctional reagent may be added to a protein with impunity. The high intramolecular cross-linking efficiency thus attainable permits structural inferences to be confidently made on the basis of cross-linked complexes

not seen. This approach has been demonstrated to be capable of yielding useful information on the tertiary and quaternary structure of a tetrameric protein (Pillai & Bachhawat, 1979), which was confirmed by the available crystallographic data on this particular protein.

Glucose oxidase (from *Aspergillus niger*) is a glycoprotein made up of two identical 80000- M_r subunits united by two disulphide bridges (O'Malley & Weaver, 1972; Tsuge *et al.*, 1975). Each subunit contains 1 atom of iron and 1 molecule of FAD. No report is available on the crystallization of this protein or the study of its three-dimensional structure by any means other than X-ray diffraction.

Materials and methods

Concanavalin A-Sepharose (4 mg of protein/ml of packed gel) was prepared and succinoylated as described previously (Pillai & Bachhawat, 1977). Succinoylated concanavalin A-Sepharose was equilibrated at pH 8.3 in 50 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid]/ethanolamine buffer containing 150 mM-NaCl, 1 mM- CaCl_2 , 1 mM- MgCl_2 and 1 mM- MnCl_2 , and 10 ml portions of the equilibrated gel were distributed into

separate beakers each with 10 ml of the above pH 8.3 buffer. Various quantities of glucose oxidase (type V; Sigma Chemical Co., St. Louis, MO, U.S.A.) ranging from 300 μ g (30 μ g/ml of packed gel) to 20 mg (2 mg/ml of packed gel) were added dropwise to five separate stirred portions of succinoylated concanavalin A-Sepharose. After 2 h at 4°C the contents of each beaker were packed into a column that was washed with 2 bed volumes of the above Hepes buffer, pH 8.3. A 10 ml volume of 1.5% (v/v) glutaraldehyde (BDH Chemicals, Poole, Dorset, U.K.) in the above buffer was passed into each column, which was then closed. After 18 h at 4°C each column was washed with 7 bed volumes of 50 mM-Hepes/ethanolamine buffer, pH 8.3, containing 150 mM-NaCl, 1 mM-CaCl₂, 1 mM-MgCl₂ and 1 mM-MnCl₂. The columns were separately eluted at room temperature with 300 mM- α -methylmannoside (Sigma Chemical Co.) in the above buffer.

Two 10 ml portions of succinoylated concanavalin A-Sepharose were each mixed with 300 μ g of glucose oxidase and packed in columns as described above. Portions (10 ml) of freshly prepared solutions (10 mg/ml in 50 mM-Hepes/ethanolamine buffer, pH 8.3, containing 150 mM-NaCl, 1 mM-MgCl₂, 1 mM-MnCl₂ and 1 mM-CaCl₂) of dimethyl suberimide (Sigma Chemical Co.) and of dimethyl adipimide (Pierce) were passed separately into the above two succinoylated concanavalin A-Sepharose columns, which were then closed. After 4 h at 4°C, these columns were washed and eluted as described above.

A 2 ml sample of a 2 mg/ml solution of glucose oxidase in 100 mM-potassium phosphate buffer, pH 8.3, was made 1% (v/v) with respect to glutaraldehyde, mixed at 4°C for 18 h and then made 100 mM with respect to ethanolamine. To separate 2 ml portions of the above glucose oxidase solution, dimethyl suberimide and dimethyl adipimide (20 mg of solid, each) were separately added in a stepwise fashion. After 4 h of stirring at room temperature the pH of each mixture was lowered to 6.5.

Sodium dodecyl sulphate/polyacrylamide gels (5%, w/v) were prepared by mixing the following in succession and pouring 2.4 ml amounts into capped inverted cylindrical glass tubes (internal diam. 6 mm): (a) 6.75 ml of a solution containing 22.2 g of acrylamide and 0.6 g of *NN'*-methylenebisacrylamide in 100 ml of water; (b) 6.75 ml of water; (c) 15 ml of a 750 mM-Tris/HCl buffer, pH 8.8, containing 0.2% w/v of sodium dodecyl sulphate; (d) 1.5 ml of a 15 mg/ml solution of ammonium persulphate (prepared fresh) in water; (e) 0.045 ml of *NNN'*-tetraethylmethylenediamine. All samples were dialysed against distilled water, and each sample was mixed with its own volume of a mixture

containing 20% (v/v) glycerol, 4% (w/v) sodium dodecyl sulphate, 10% (v/v) β -mercaptoethanol and 0.002% Bromophenol Blue in 125 mM-Tris/HCl buffer, pH 6.8. Samples were heated in boiling water for 2 min before being subjected to electrophoresis (8 mA per tube). The electrode buffer contained 25 mM-Tris, 192 mM-glycine and 0.1% sodium dodecyl sulphate at pH 8.3. All the glutaraldehyde-treated samples were also separately reacted with a similar mixture that did not contain β -mercaptoethanol and were incubated in boiling water and subjected to electrophoresis as described above.

All gels were stained with Coomassie Blue and densitometrically scanned after destaining.

Results and discussion

Reaction with glutaraldehyde at all tested densities on the matrix, as well as at a high density in solution, yielded a single 160 000-*M_r* band on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis; 'unreacted' glucose oxidase, as well as the imide-treated samples, all yielded a single 80 000-*M_r* band (see Fig. 1).

Table 1 lists various structural inferences with

Fig. 1. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of glucose oxidase after various treatments

Scanning densitometer patterns: trace I, glucose oxidase; trace II, glucose oxidase (30 μ g/ml of gel) reacted with glutaraldehyde on succinoylated concanavalin A-Sepharose; trace III, glucose oxidase (2 mg/ml) reacted with glutaraldehyde in solution; trace IV, glucose oxidase (2 mg/ml) reacted with dimethyl suberimide in solution; trace V, glucose oxidase (2 mg/ml) reacted with glutaraldehyde in solution, but electrophoresed without addition of β -mercaptoethanol.

Table 1. *Possible cross-linking patterns and corresponding structural inferences when a dimeric protein is reacted (both when immobilized at a critically low density on an affinity matrix and also at a high density in solution) with a high-efficiency lysine-specific flexible multiple-chain-length homobifunctional reagent*

Similar inferences may be arrived at when cross-linking is performed with suitable bifunctional reagents specific for other reactive amino acid residues.

	Low protein density on matrix	High protein density	Inferences
A	Monomers	Monomers	(1) No lysine residues are available on the non-interfacial surfaces of each protomer (2) No lysine residues are available close to or along the inter-protomeric interface; if any are so located, their ϵ -amino groups face away from the neighbouring protomer
B	Monomers	Dimers	(1) A single lysine cluster is available along the non-interfacial surfaces of each protomer (2) As in A (2)
C	Monomers	Higher polymers	(1) At least two lysine clusters are available along the non-interfacial surfaces of each protomer (2) As in A (2)
D	Dimers	Dimers	(1) As in A (1) (2) At least two lysine clusters are available along or close to the interprotomeric interface of each protomer (3) These lysine residues are so oriented that the ϵ -amino groups of lysine residues a and b on protomer 1 'face', and are very close to lysine residues b' and a' respectively on protomer 2 (the only theoretically possible exception is discussed in the text)
E	Dimers	Higher polymers	(1) At least one lysine cluster is available along the non-interfacial surfaces of each protomer (2) At least two lysine clusters are available either along or close to the interprotomeric interface, or even along the non-interfacial surfaces (3) As in D (3), but these lysine residues need not necessarily be close to the lysine residues that they 'face'

regard to the topological distribution of any specific reactive amino acid residue on a dimeric protein; these inferences may be made by studying the cross-linked products obtained by reacting such a protein with a specific high-efficiency multiple-chain-length flexible homobifunctional reagent, both when immobilized at a critically low density on an affinity matrix and at a high density in solution.

The following inferences with regard to the tertiary and quaternary structure of glucose oxidase could be drawn from the present study.

(1) Of the 15 lysine residues in each protomer none is available along the non-interfacial surfaces of this protein. The contributions, if any, of the sugar side chains, the iron atom or the dinucleotide molecule bound to each protomer towards the steric non-availability of any lysine residues that might actually be located along these surfaces remain unknown.

(2) A dimeric protein in which the protomers are sterically equivalent is expected to possess cyclic (C_2) symmetry (with a single twofold axis of symmetry) and isologous bonding between subunits (Monod *et al.*, 1965; Hanson, 1966; Klotz *et al.*, 1975). It may be inferred that on each glucose

oxidase protomer there are at least two lysine clusters along or close to the interprotomeric interface.

(3) These 'interfacial' lysine residues in each glucose oxidase protomer are sterically so oriented that the ϵ -amino groups of lysine residues a and b in protomer 1 'face', and are very close to, the ϵ -amino groups of lysine residues b' and a' respectively on protomer 2.

(The theoretical possibility nonetheless exists that a single lysine cluster is present in each protomer, located close to and directly opposite the identical residue in the other protomer; an imaginary line joining these lysine residues would intersect and be roughly perpendicular to the single dyad axis about which the two protomers are related. The existence of two disulphide bridges between the protomers, however, suggests the presence of two specialized binding subset regions in each protomer, and hence the probable existence of two lysine clusters as described above.)

A single lysine cluster at the non-interfacial surfaces of each protomer combined with the absence of any lysine clusters close to the inter-protomeric interfaces could also lead to the obser-

vation (on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis) of dimers at a high density (but monomers on a low-density matrix). In such a situation, however, electrophoretic analysis in the absence of a disulphide-cleaving reagent would have resulted in the detection of higher polymers. Such an analysis (performed without β -mercaptoethanol) of all glutaraldehyde-treated glucose oxidase samples consistently revealed a single dimer band.

The inability of dimethyl suberimidate and dimethyl adipimidate to generate either dimers or higher polymers of the glucose oxidase protomer further indicates that the lysine residues bridged by glutaraldehyde must be close to each other [the former reagents cannot generate cross-links over distances less than 0.8 nm (8 Å) (Peters & Richards, 1977)]. Such an inference must, however, be made with caution, since the chemical reactivities of the amino groups concerned towards imidates may well differ from their reactivities towards glutaraldehyde. The structural heterogeneity and flexibility of glutaraldehyde in solution (Monsan *et al.*, 1975), coupled with the high efficiency with which specific and stable inter-lysine cross-links may be generated with this cross-linker (Richards & Knowles, 1968), make it a particularly useful reagent for obtaining conclusive structural inferences with regard to the topology of lysine residues on the basis of cross-linked complexes not seen.

The distance between any two cysteine residues involved in disulphide-bridge formation is known to be approx. 0.74 nm (7.4 Å) (Kuntz *et al.*, 1979). Assuming, according to the foregoing argument, that the cross-linked lysine residues are close to one another [0.8 nm (8 Å) separating them], it is probable that the distance between complementary binding subset areas is approx. 0.7–0.8 nm (7–8 Å) and that lysine residues a, b, a' and b' are each close to a cysteine residue involved in interprotomeric disulphide-bridge formation.

Even when highly efficient cross-linkers are employed, the results obtained by such an approach may be influenced, not only by the spatial locations of the side chains concerned, but also by their local microenvironments. It must be stressed that, even though the experimental findings are readily reproducible, the validity of the above interpretations further depends on the assumption that a dimeric protein must possess C_2 symmetry (with both subunits equivalently located in three-dimensional space). The above interpretations must there-

fore be categorized only as 'likely topological relations' in the three-dimensional structure of this protein. These inferences may provide base-line information for crystallographers who might eventually study this dimeric protein.

The ability of lectins to bind glycoproteins in the presence of many detergents (Lotan *et al.*, 1977) facilitates their use for the immobilisation of poorly soluble glycoproteins. The use of a battery of suitable cross-linkers in combination with chemically modified lectin matrices may prove useful as a general method for studying the three-dimensional structures of glycoproteins, particularly non-crystallizable oligomers.

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