The Development of SS' -Polymethylenebis(methanethiosulphonates) as Reversible Cross-Linking Reagents for Thiol Groups and their Use to Form Stable Catalytically Active Cross-Linked Dimers within Glyceraldehyde 3-Phosphate Dehydrogenase

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The synthesis of a series of SS' -polymethylenebis(methanethiosulphonates) including the pentane, hexane, octane, decane and dodecane derivatives is described. These derivatives were synthesized by condensation between dibromoalkanes and potassium methanethiosulphonate in refluxing methanol and this seems an especially versatile reaction for the synthesis of asymmetric thiosulphonate derivatives. The synthesis of SS' -[1,8- $^3\text{H}_4$]-octamethylenebis(methanethiosulphonate) was also performed. Cross-linking was demonstrated in the four enzymes lactate dehydrogenase, phosphofructokinase, pyruvate kinase and glyceraldehyde 3-phosphate dehydrogenase. For all four enzymes cross-linking was efficiently reversed by reducing conditions in denaturing solvents. The reaction with glyceraldehyde 3-phosphate dehydrogenase was unique in that only the cross-linked dimer was produced in significant amounts (>90% of total products as dimer). This reaction was followed in detail with radioactive cross-linking reagent. Inhibition of enzyme activity was extremely fast and showed an asymmetric distribution of enzyme activity on subunits. Thus complete modification of only one subunit resulted in up to 75% inhibition of enzyme activity. Reaction of glyceraldehyde 3-phosphate dehydrogenase with 1.25 mol of SS' -octamethylenebis(methanethiosulphonate) per mol of enzyme subunit produced two species of protein. The first species was obtained in 20% yield and was only partially re-activated on mild reduction with 2-mercaptoethanol. The second species was isolated in 66% yield and was completely re-activated on mild reduction. Before reduction there was 4 mol of inhibitor per tetramer for the latter species, and more than 95% of the enzyme was present as a dimer on non-reducing electrophoresis. After mild reduction 2 mol of inhibitor was still bound per tetramer, the enzyme was now catalytically active and the dimer was still the major structure on non-reducing electrophoresis. Thus mild reduction of SS' -octamethylenebis(methanethiosulphonate)-treated glyceraldehyde 3-phosphate dehydrogenase enabled the production of active enzyme in which there is a stable cross-link across one of the molecular axes of the tetrameric enzyme. This cross-link was only reversed if reduction was performed when the enzyme was denatured. The molecular weight of cross-linked and re-activated cross-linked glyceraldehyde 3-phosphate dehydrogenase was established as 144000 (tetramer) by sucrose-density-gradient centrifugation. These observations are interpreted in terms of the molecular structure of glyceraldehyde 3-phosphate dehydrogenase.

Chemical cross-linking of proteins has found an increasing number of applications in the study of complex structure in multicomponent systems by the process of nearest-neighbour analysis (Peters & Richards, 1977). This area of analysis has particularly expanded since the introduction of reversible cross-linking reagents (Wang & Richards, 1974; Coggins *et al.*, 1976). However, in order to exploit these reagents to their maximum use there is great scope in reagent design. As an example, the reaction of a bis-imidate with ϵ -amino groups in lysine is frequently

used to examine nearest-neighbour relations in membrane-protein systems. (Wang & Richards, 1974; Louis & Shooter, 1972). Here is an example of a polar reagent reacting with a polar functional group trying to probe a potentially non-polar environment. Clearly some interactions in the non-polar environment or at alternative functional residues are not probed by the reagent. As part of a programme to develop alternative reagents we recently synthesized bischloromethylalkanediones as cross-linking reagents for thiol groups in non-polar

environments (Bloxham, 1977). The current work involves the synthesis and use of reversible reagents, the *SS'*-polymethylenebis(methanethiosulphonates). The usefulness of these reagents is particularly demonstrated in studies on molecular asymmetry in glyceraldehyde 3-phosphate dehydrogenase.

Materials and Methods

Synthesis of a series of SS'-polymethylenebis(methanethiosulphonates)

Potassium methanethiosulphonate was prepared as described by Bloxham *et al.* (1978). First 2.5 mmol of dibromoalkane was gently refluxed with potassium methanethiosulphonate (6.6 mmol) in 13 ml of dry methanol for 18 h. Solvent was then removed *in vacuo* and the solid was mixed with 100 ml of warm chloroform to dissolve the product. Excess KBr was removed by washing with water until the chloroform layer was clear. After drying over anhydrous Na_2SO_4 the solvent was removed *in vacuo*. The product was now crystallized from methanol/light petroleum (b.p. 60–80°C). The reaction usually gives 60–80% yields of product based on dibromoalkane. The melting points of the compounds were as follows: *SS'*-pentamethylenebis(methanethiosulphonate), 67–68°C; *SS'*-hexamethylenebis(methanethiosulphonate), 83–84°C; *SS'*-octamethylenebis(methanethiosulphonate), 88–89°C; *SS'*-decamethylenebis(methanethiosulphonate), 96.5–97.5°C; *SS'*-dodecamethylenebis(methanethiosulphonate), 103–104°C.

Characterization of the compounds was easily achieved by n.m.r. in C^2HCl_3 with tetramethylsilane as a reference, which showed δ 3.3 p.p.m. (s, 6H, 2- $\text{SO}_2\text{-CH}_3$) and δ 3.07–3.47 p.p.m. (m, 4H, 2- $\text{CH}_2\text{-S}$). In addition, the compounds contain variable resonances accounting for the internal methylene groups. The compounds were also characterized by g.l.c. and mass spectrometry. These data have been published separately (Corina *et al.*, 1979).

Synthesis of SS'-[1,8- $^3\text{H}_4$]octamethylenebis(methanethiosulphonate)

LiAlH_4 (8 mmol) and NaB^3H_4 (5 mg; 100 mCi) were mixed in a 100 ml round-bottomed flask containing 50 ml of sodium-dried diethyl ether. After 5 min, diethyl octanedioate (20 mmol) was added and the mixture was refluxed for 1 h. Then a further 8 mmol of LiAlH_4 was added and refluxed for another 1 h. The reaction was stopped by the sequential addition of 0.6 ml of water, 0.6 ml of 14% (w/v) NaOH and 0.6 ml of water. After standing, the precipitate was filtered off and the ethereal fraction was dried over anhydrous Na_2SO_4 . Then this was evaporated dry *in vacuo*. This yielded a liquid, which

solidified on standing. Crystallization from benzene/light petroleum (b.p. 60–80°C) yielded [1,8- $^3\text{H}_4$]-octane-1,8-diol (8 mmol; $2\mu\text{Ci}/\mu\text{mol}$; m.p. 57–59°C; literature m.p. 61°C).

[1,8- ^3H]Octane-1,8-diol (8 mmol) was dissolved in 40 ml of sodium-dried benzene. PBr_3 (8 mmol) in 12 ml of dry benzene was added dropwise under N_2 and refluxed for 1.5 h. The benzene was removed *in vacuo* and the products were dissolved in 100 ml of chloroform. The organic phase was washed 3 times with 100 ml of satd. NaHCO_3 , dried over anhydrous Na_2SO_4 and evaporated *in vacuo*. 1,8-Dibromo-[1,8- $^3\text{H}_4$]octane was obtained by vacuum distillation (4 mmol; b.p. 144°C at 2.13 kPa). The identity of the compound was confirmed by g.l.c. and mass spectroscopy by using authentic 1,8-dibromo-octane as a standard (M^+ 274, 272, 270, 1:2:1; $M^+ - \text{Br}$ 193, 191, 1:1; $M - \text{C}_8\text{H}_{15}$ 111). 1,8-Dibromo[1,8- $^3\text{H}_4$]octane was converted to the bis(methanethiosulphonate) by the procedure described above at an appropriate scale. The ^3H specific radioactivity of the product was $2\mu\text{Ci}/\mu\text{mol}$.

Enzymes, assays and modification reactions

Pig heart lactate dehydrogenase (EC 1.1.1.27; specific activity $6\mu\text{kat}/\text{mg}$ of protein at 25°C) and rabbit skeletal-muscle glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12; specific activity $1.2\mu\text{kat}/\text{mg}$ of protein at 25°C) were obtained from Boehringer Corp (London) Ltd., Lewes, Sussex, U.K. Rabbit skeletal-muscle pyruvate kinase (EC 2.7.1.40; specific activity $3.33\mu\text{kat}/\text{mg}$ of protein at 25°C) was purified by the method of Tietz & Ochoa (1962). Rabbit skeletal-muscle phosphofructokinase (EC 2.7.1.11; specific activity $2\mu\text{kat}/\text{mg}$ of protein at 25°C) was purified by the method of Ling *et al.* (1965). All enzymes were homogeneous by polyacrylamide-gel electrophoresis in sodium dodecyl sulphate, and were stored as suspensions in 60%-satd. $(\text{NH}_4)_2\text{SO}_4$. The protein was collected by centrifugation at 15000g for 30 min at 4°C. The pellet was then dissolved in 0.1 M-Tris/HCl, pH 8.0 at 20°C, containing 1 mM-EDTA to give a protein concentration of 1 mg/ml. Protein concentration was determined by using the molar absorption coefficient of each protein at 280 nm. Enzyme assays for lactate dehydrogenase and glyceraldehyde 3-phosphate dehydrogenase were performed as described by Bloxham & Wilton (1977) and Ferdinand (1964) respectively.

For modification studies, the *SS'*-polymethylenebis(methanethiosulphonates) were dissolved in warm methanol, and 50 μl of the solution was added to a 1 ml reaction volume at 18°C. With 5% (v/v) methanol the compounds remain in solution up to 0.5 mM concentrations. This concentration of methanol had no effect on any of the enzymes tested, all of which were stable for up to 18 h at 18°C.

Gel electrophoresis of cross-linked products

The nature of the products depends upon whether the cross-linking reaction is terminated in a non-reducing or reducing medium. For non-reducing conditions an equal volume of 10mM-sodium phosphate, pH7.0, containing 1% (w/v) sodium dodecyl sulphate and 0.5% (w/v) *N*-ethylmaleimide was added and the mixture boiled for 2min. For reducing conditions an equal volume of 10mM-sodium phosphate and 1% (v/v) 2-mercaptoethanol was added and the mixture boiled for 2min. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was performed as described by Weber & Osborn (1969) by using either 5 or 7.5% (w/v) polyacrylamide gels.

NAD⁺ content of glyceraldehyde 3-phosphate dehydrogenase

The NAD⁺ concentration in glyceraldehyde 3-phosphate dehydrogenase was estimated by the A_{280}/A_{260} ratio (Fox & Dandliker, 1956). As prepared by centrifugation there were 2 NAD⁺ molecules per tetramer. Bound NAD⁺ was removed by charcoal treatment.

Sucrose-density-gradient centrifugation

Linear sucrose gradients (approx. 15ml) were between 5 and 25% (w/v) sucrose in 25mM-Tris/HCl, pH8.0, containing 1mM-EDTA. Centrifugation was performed for 45h at 30000rev./min in a 6×16.5ml MSE swing-out rotor. At the end of the

run, 25-drop samples were collected and analysed for refractive index, radioactivity, A_{280} and enzyme activity. The gradients were calibrated with 1mg of lactate dehydrogenase, 1mg of pyruvate kinase and 2mg of bovine serum albumin.

Results and Discussion*Synthesis and reaction of SS'-polymethylenebis(methanethiosulphonates)*

SS'-Polymethylenebis(methanethiosulphonates) were synthesized by condensation, in refluxing methanol, between a dibromoalkane and an excess of potassium methanethiosulphonate (Scheme 1). Reactions of this type are generally performed in *NN*-dimethylformamide (Smith *et al.*, 1975; Bloxham *et al.*, 1978; Corina *et al.*, 1979), but methanol was preferred in these syntheses because, although the reaction rate is considerably lower, the isolation of product is considerably easier. The potential for synthesis of asymmetrically substituted thiosulphonate derivatives by this procedure seems virtually unlimited. So far we have synthesized bis(methanethiosulphonates) in the pentane, hexane, octane, decane and dodecane series. As expected all these compounds are very non-polar and have only limited solubility in water. Radioactively labelled compounds can be synthesized as illustrated in Scheme 1 and we have achieved the synthesis of [1,8-³H₄]octamethylenebis(methanethiosulphonate) by this method.

SS'-Polymethylenebis(methanethiosulphonates) are very stable compounds but they react readily with thiols to eliminate methanesulphinate and form a

Scheme 1. *Synthesis and reactions of SS'-polymethylenebis(methanethiosulphonates)*

disulphide (Scheme 1). The methanesulphonate is probably oxidized subsequently to methane-sulphonic acid. This makes the generation of disulphides a virtually irreversible process and explains why some of the cross-linked products were obtained in greater than 90% yield. On reduction the mixed disulphide generates an alkanedithiol (Scheme 1). Alkanedithiols were identified as the products of direct reduction of *SS'*-polymethylenebis(methanethiosulphonates); however, these compounds are unstable and readily form the alkane disulphide ring structure (Corina *et al.*, 1979).

Cross-linking and inhibition studies using pig heart lactate dehydrogenase

Pig heart lactate dehydrogenase contains four cysteine residues per subunit of mol.wt. 36000 (Holbrook *et al.*, 1975). Of these residues cysteine-165 was once considered essential for catalysis (Fondy *et al.*, 1965; Holbrook & Pfeleiderer, 1965; Holbrook, 1966), but it is now appreciated that its role is principally in substrate binding and maintenance of the correct enzyme conformation (Bloxham & Wilton, 1977; Bloxham *et al.*, 1979). Whatever the exact role of the thiol it is buried in the protein structure and is not readily accessible to externally added reagents unless they are reasonably non-polar (Holbrook & Pfeleiderer, 1965; Kolkenbrock *et al.*, 1978) or alternatively if the protein is denatured (Bloxham *et al.*, 1979). When lactate dehydrogenase was allowed to react with *SS'*-pentamethylenebis(methanethiosulphonate) the enzyme was progressively inactivated (Fig. 1). With high concentrations of inhibitor the enzyme was completely inhibited. Time-dependent inhibition did not follow pseudo-first-order kinetics (Kitz & Wilson, 1962), indicating that the reaction proceeds as a simple bimolecular reaction. Generally, these observations confirm our suggestion that *SS'*-polymethylenebis(methanethiosulphonates) will prove excellent reagents for thiol groups in non-polar regions of protein structure.

When lactate dehydrogenase was allowed to react with *SS'*-pentamethylenebis(methanethiosulphonate) for periods of up to 18 h then cross-linked products were formed when the gels were analysed by non-reducing electrophoresis (Fig. 2). The largest molecular species was the octamer, although this represents only a very small fraction of the total protein applied to the gel. The fact that there was not a sharp cut-off when cross-linked products equivalent to the tetramer were formed must mean that inter-oligomer cross-linking occurs quite readily. If the electrophoresis was repeated under reducing conditions, then only the monomer (mol.wt. 36000) was observed. This shows that the cross-linking reactions must proceed by the formation of disulphide bridges as suggested in Scheme 1. The newly

Fig. 1. Time-dependent inhibition of lactate dehydrogenase. Lactate dehydrogenase ($6.4 \mu\text{M}$ in subunits) in 0.1 M -Tris/HCl, pH 8.0, containing 1 mM -EDTA was allowed to react at 18°C with various concentrations of *SS'*-pentamethylenebis(methanethiosulphonate) as follows: $\bullet$, 5 mM ; $\circ$, 2.5 mM ; $\blacktriangle$, 1 mM ; $\triangle$, 0.25 mM ; $\blacksquare$, 0.1 mM ; $\square$, none.

formed disulphide bridges are readily broken, provided that reduction is performed under denaturing conditions.

Insertion of pentane disulphide cross-links into lactate dehydrogenase does not produce any unusual properties in the electrophoretic migration of the polymeric products. This is shown by a plot of $\log_{10}$ (predicted molecular weight for cross-linked products) against relative mobility, which is reasonably linear (Fig. 3). Indeed the migration of cross-linked products of lactate dehydrogenase under non-reducing conditions provides a good calibration curve for proteins of unknown molecular weight.

The cross-linking reaction of lactate dehydrogenase by 5 mM -*SS'*-pentamethylenebis(methanethiosulphonate) was considerably slower than inactivation. Thus at least one thiol group associated with enzyme activity is rapidly attacked, and subsequent reaction with other thiol groups is much slower. It is well known that cysteine-165 is the most reactive thiol group in pig heart lactate dehydrogenase (Fondy *et al.*, 1965). A detailed time course for the

disappearance of the monomer and the appearance of the dimer, trimer and tetramer is shown in Fig. 4. The pattern of results indicates that the dimer is

Fig. 2. Cross-linked products of lactate dehydrogenase analysed by non-reducing and reducing SDS/polyacrylamide-gel electrophoresis

Lactate dehydrogenase was allowed to react for 18 h with 5 mM-SS'-pentamethylenebis(methanethiosulphonate) and 100 μ g of protein was analysed by SDS/polyacrylamide-gel electrophoresis on 7.5% gels. Traces (a) and (b) show scans of Coomassie Brilliant Blue-stained gels run under non-reducing and reducing conditions respectively.

Fig. 3. Mobility of cross-linked products of lactate dehydrogenase

The molecular weights of cross-linked products as in Fig. 2 were calculated by assuming that they were formed by the sequential addition of 36000 mol.wt. subunits.

formed first and this is followed by polymerization to trimers and tetramers. This type of result is similar to that predicted for a tetramer with equal numbers of contact along each of the three crystallographic axes (Hajdu *et al.*, 1976).

The current experiments do not throw light upon the exact nature of the groups that react to give cross-linked products, but it was possible to show a high degree of specificity for reagent size in cross-linking. When lactate dehydrogenase was allowed to react with a series of SS'-polymethylenebis(methanesulphonates) the pentane, hexane and octane reagents gave cross-linked products (Table 1), whereas the decane and dodecane derivatives were unreactive in the cross-linking reaction. However, the decane and dodecane reagents still inactivate the enzyme, showing that they react with cysteine-165. Conceivably if this residue is located in a hydrophobic region of the protein (Holbrook & Pfeleiderer, 1965; Kolkenbrock *et al.*, 1978), then the unchanged end of the molecule may be adsorbed out of solution as a result of non-polar interactions between the protein and the polymethylene chain of the reagent.

Cross-linking in rabbit muscle pyruvate kinase

Rabbit muscle pyruvate kinase contains at least four thiol groups per protomer (mol.wt. 56000; Cottam *et al.*, 1969), which react with thiosulphonate reagents (Bloxxham *et al.*, 1978). Of these thiol groups, at least three are involved in maintenance of enzyme conformation and one participates in catalysis. When pyruvate kinase was allowed to react with SS'-pentamethylenebis(methanethiosul-

Fig. 4. Time course for cross-linking in lactate dehydrogenase. Lactate dehydrogenase was allowed to react with 5 mM-SS'-pentamethylenebis(methanethiosulphonate) for various times. The proportions of enzyme present as monomers ($\circ$), dimers ($\bullet$), trimers (Δ) and tetramers ($\blacktriangle$) were analysed by SDS/polyacrylamide-gel electrophoresis.

Table 1. *Extent of cross-linking in lactate dehydrogenase with SS'-polymethylenebis(methanethiosulphonates) of various chain lengths*

Lactate dehydrogenase was allowed to react for 6 h with 5 mM-SS'-polymethylenebis(methanethiosulphonate) of various chain lengths and the extent of reaction was measured by SDS/polyacrylamide-gel electrophoresis. Only products up to the pentamer were analysed for convenience.

Number of methylene groups (<i>n</i>)	Percentage of cross-linked species				
	Monomer	Dimer	Trimer	Tetramer	Pentamer
5	45.7	25.9	13.8	8.6	5.1
6	41.7	25	15	10	8.3
8	45	28.5	12.5	8.1	6.1
10	100	—	—	—	—
12	100	—	—	—	—

phonate), cross-linked products were formed. Initially dimers, trimers and tetramers were formed, but the reaction proceeded rapidly beyond this to produce high-molecular-weight products. After 18 h most of the products had molecular weights of between 330 000 (hexamer) and 440 000 (octamer). The cross-linking reactions were completely reversed when the products were analysed under reducing conditions. Clearly for pyruvate kinase, inter-oligomer cross-links form much more easily than for lactate dehydrogenase. This, of course, may reflect the fact that more thiol groups are surface-exposed in pyruvate kinase and react readily, whereas the thiols are buried in lactate dehydrogenase.

Cross-linking in rabbit muscle phosphofructokinase

Rabbit muscle phosphofructokinase is a tetramer composed of subunits of mol.wt. approx. 80 000 (Coffee *et al.*, 1973; Simpson *et al.*, 1977). It contains several classes of thiol groups based on their reaction with thiol reagents and these thiol groups are involved in catalysis and maintenance of enzyme structure (Bloxham & Lardy, 1973; Bloxham *et al.*, 1973). However, their exact function is not yet established. Reaction of phosphofructokinase with SS'-polymethylenebis(methanethiosulphonates) resulted in the formation of cross-linked products that were stable only in non-reducing electrophoresis. Cross-linked products with phosphofructokinase never exceeded the molecular weight of the tetramer, showing that inter-oligomer cross-links were virtually absent.

Use of cross-linking to study molecular asymmetry in rabbit muscle glyceraldehyde 3-phosphate dehydrogenase

Inhibition of enzyme activity. Rabbit muscle glyceraldehyde 3-phosphate dehydrogenase is a tetramer composed of subunits of mol.wt. 36 000 (Harris & Perham, 1965). There is a catalytically essential cysteine thiol, cysteine-149 (Harris *et al.*, 1963), which is readily alkylated. There are three

other cysteine residues per subunit; however, these are not readily accessible to polar reagents, so presumably they are in the hydrophobic interior of the protein.

Molecular asymmetry of the chemical properties of rabbit muscle glyceraldehyde 3-phosphate dehydrogenase has been shown by NAD⁺ binding (Conway & Koshland, 1968; De Vijlder & Slater, 1968), by hybridization studies (Osborne & Holloway, 1974, 1975) and by cross-linking with bischloromethylalkanediones (Bloxham, 1977). However, if rabbit muscle glyceraldehyde 3-phosphate dehydrogenase is allowed to react with iodoacetic acid, then all four cysteine-149 residues react in an equivalent manner and inhibition is directly proportional to the number of molecules of inhibitor bound (MacQuarrie & Bernhard, 1971). This was not the case with the SS'-polymethylenebis(methanethiosulphonate) reagents. All of these reagents were powerful covalent inhibitors of glyceraldehyde 3-phosphate dehydrogenase. The covalent reaction was so fast that it has proved impossible so far to demonstrate a time-dependence for the reaction. For experimental convenience we allow a reaction period of 5 min. When glyceraldehyde 3-phosphate dehydrogenase was titrated with SS'-octamethylenebis(methanethiosulphonate) (Fig. 5), the loss of activity was completely asymmetric when compared with the result for iodoacetic acid. Thus the addition of one molecule of inhibitor per tetramer causes up to 75% inhibition of activity. For iodoacetic acid the equivalent result is 25% inhibition. The asymmetric inhibition of enzyme activity was given by all SS'-polymethylenebis(methanethiosulphonates) synthesized in the present project. Of course, the bi-functional reagent contains twice the concentration of thiol-reactive groups per molecule. However, the observation that four molecules of inhibitor must be added to the tetramer to give complete inhibition suggests that there must be one molecule of inhibitor bound on each subunit.

The asymmetric nature of enzyme inhibition is not greatly influenced by the content of bound

Fig. 5. Inhibition of glyceraldehyde 3-phosphate dehydrogenase by SS'-octamethylenebis(methanethiosulphonate). Glyceraldehyde 3-phosphate dehydrogenase ($1.2 \mu\text{M}$ based on tetramer mol.wt.) in 0.1 M -Tris/HCl, pH 8.0, containing 1 mM -EDTA was allowed to react for 5 min at 20°C with various concentrations of SS'-octamethylenebis(methanethiosulphonate). The enzyme activity was then measured. The experiment was performed with NAD^+ at three concentrations as follows: ○, none; ●, 2 mM /tetramer; △, $300 \mu\text{M}$. The dashed line (a) shows the experimental result for modification by iodoacetamide (MacQuarrie & Bernhard, 1971).

NAD^+ (Fig. 5). However, increasing NAD^+ content does decrease the inhibition by SS'-octamethylenebis(methanethiosulphonate), especially at the lowest inhibitor concentrations. Protection of the active-site thiol by NAD^+ can be expected from other experimental results (Conway & Koshland, 1968; Bloxham, 1977).

When glyceraldehyde 3-phosphate dehydrogenase cross-linked by excess concentrations of SS'-octamethylenebis(methanethiosulphonate) was studied, a dimer was formed as the major product (mol.wt. 72000) and the reaction was too fast for time-dependence to be established (Fig. 6). Thus cross-linking and enzyme inhibition occur on the same time scale. In order to observe any time-dependence for cross-linking it was necessary to use the poorest cross-linking reagent observed in the present studies, namely SS'-pentamethylenebis(methanethiosulphonate) (see Fig. 7). However, even with this reagent the reaction proceeded to its maximum extent within 1 h, and the major product was the cross-linked dimer. Cross-linking was reversed completely provided that reduction with 2-mercaptoethanol was carried out under denaturing

Fig. 6. Formation of the cross-linked dimer of glyceraldehyde 3-phosphate dehydrogenase

Glyceraldehyde 3-phosphate dehydrogenase ($25 \mu\text{M}$ in subunits) was allowed to react for 30 min with either 1 mM - (a) or $25 \mu\text{M}$ - (b) SS'-octamethylenebis(methanethiosulphonate). The product was analysed by electrophoresis on SDS/5% polyacrylamide gels under non-reducing conditions. Gel (c) shows the result when (a) was analysed by electrophoresis under reducing conditions. The molecular weights are indicated.

conditions (Fig. 6). This result may be contrasted with later results on reduction under non-denaturing conditions.

An optimum distance between reactive centres on the bifunctional molecule was sought by measuring the extent of cross-linking after 30 min reaction time with reagents of various chain length (Fig. 7). All reagents gave the cross-linked dimer, but the reaction was optimal with SS'-octamethylenebis(methanethiosulphonate). Assuming that the cross-linked product contains the structure enzyme-S-S-[CH₂]₈-S-S-enzyme, this corresponds to an optimal separation of about 1.5 nm. This distance assumes that the methylene groups orient in a linear staggered configuration, and also that C-S and S-S bond lengths are about 0.18 and 0.189 nm respectively. A distance of 1.5 nm corresponds closely to the separation of 1.77 nm estimated for the Cys₍₂₈₁₎-Cys₍₂₈₁₎ pair located on the P-axis of rabbit muscle glyceraldehyde 3-phosphate dehydrogenase (R. M. Garavito & M. G. Rossmann, personal communication). A distance of 1.21–1.45 nm was estimated from studies with bischloromethylalkanediones, where the optimal cross-linked structure was enzyme-S-CH₂-CO-[CH₂]₄₋₆-CO-CH₂-S-enzyme (Bloxham, 1977).

Cross-linking and enzyme inhibition with SS'-[1,8-³H₄]-octamethylenebis(methanethiosulphonate)

The reaction of glyceraldehyde 3-phosphate dehydrogenase with SS'-[1,8-³H₄]octamethylenebis(methanethiosulphonate) appeared identical with

Fig. 7. Influence of reagent chain length on cross-linking of glyceraldehyde 3-phosphate dehydrogenase

Glyceraldehyde 3-phosphate dehydrogenase (25 μ M in subunits) was allowed to react for 30 min with 1 mM-SS'-polymethylenebis(methanethiosulphonate). The formation of dimer (●) and disappearance of monomer (○) were estimated by electrophoresis on SDS/5% polyacrylamide gels under non-reducing conditions.

respect to enzyme inhibition and extent of cross-linking when compared with the unlabelled reagent. To analyse the extent of reaction, cross-linking was performed on a much larger scale. Glyceraldehyde 3-phosphate dehydrogenase (15 ml; 75 nM in subunits) was allowed to react with 1.25 mol of SS'-[1,8-³H₄]-octamethylenebis(methanethiosulphonate)/mol of enzyme subunit for 3 h at 18°C. This long reaction period was chosen to ensure completion of all possible chemical reactions. At the end of this reaction the enzyme was completely inhibited (Table 2).

To isolate the labelled protein, (NH₄)₂SO₄ fractionation was used, and this revealed that the cross-linked glyceraldehyde 3-phosphate dehydrogenase was not a homogeneous product. Thus 0–65% saturation with (NH₄)₂SO₄ precipitated 6 mg of protein (20% of total) and this fraction was designated E₁ (Table 2). Raising the (NH₄)₂SO₄ to 95% saturation produced a second precipitate (20 mg of protein; 66% of total) and this was designated fraction E₂. Gel electrophoresis of both samples (Fig. 8) showed that fraction E₂ was exclusively the dimer (>95% of total protein) whereas fraction E₁ was a mixture of monomer (25%) and dimer (75%). In the case of fraction E₂ the bound radioactivity corresponded to approx. 1 mol of inhibitor (0.88 $\pm$ 0.13)/mol of subunit. This coincided with the disappearance of 8 thiol groups per tetramer (Table 2), showing that each bifunctional group modified two enzyme thiol groups. Clearly this is the perfect result. In contrast, fraction E₁ had incorporated approx. 6 mol of inhibitor/tetramer, but there were no detectable thiol groups present as measured by 5,5'-dithiobis-(2-nitrobenzoate) titration. The exact reason for this discrepancy is unknown, but it is conceivable that free thiosulphonate groups interfere with the result. We have shown that methyl methane-

Table 2. Properties of glyceraldehyde 3-phosphate dehydrogenase cross-linked with SS'-[1,8-³H₄]octamethylenebis(methanethiosulphonate)

Glyceraldehyde 3-phosphate dehydrogenase was modified with SS'-[1,8-³H₄]octamethylenebis(methanethiosulphonate) as described in the Results and Discussion section. The cross-linked enzyme was resolved into two distinct fractions (E₁ and E₂) by (NH₄)₂SO₄ precipitation. Gel scans of labelled products are shown in Fig. 8. Thiol-group content was determined by titration with 5,5'-dithiobis-(2-nitrobenzoate) (Ellman, 1959). The concentration of bound radioactivity was determined after exhaustive dialysis of the labelled proteins. The mol. wt. of protein samples was determined by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis under non-reducing conditions. Inhibitor binding is presented as means $\pm$ S.E.M. (n = 5).

Glyceraldehyde 3-phosphate dehydrogenase species	(NH ₄) ₂ SO ₄ fraction (% satn.)	Mol. wt.	Specific activity at 18°C (μ kat/mg of protein)	Thiol groups/tetramer	Molecules of inhibitor/tetramer
Native	—	36000	0.66	16	0
E ₁	0–65	36000 (25%) 72000 (75%)	0	0	5.6 $\pm$ 0.3
E ₁ mild reduction	—	36000 (25%) 72000 (75%)	0.17	—	2.7 $\pm$ 0.1
E ₂	65–95	72000	0	8	3.5 $\pm$ 0.5
E ₂ mild reduction	—	72000	0.66	—	2.0 $\pm$ 0.1

Fig. 8. SDS/polyacrylamide-gel electrophoresis of glyceraldehyde 3-phosphate dehydrogenase cross-linked with SS'-[1,8- $^3\text{H}_4$]octamethylenebis(methanethiosulphonate).

The products of cross-linking experiments as described in Table 2 were analysed by electrophoresis on SDS/5% polyacrylamide gels. The traces show scans of Coomassie Brilliant Blue-stained gels as follows: (a) E_2 ; (b) E_1 ; (c) E_2 , mild reduced; (d) E_1 , mild reduced; (e) control glyceraldehyde 3-phosphate dehydrogenase; (f) E_2 , mild reduced; (g) E_1 , mild reduced. Gels (a)–(e) were analysed under non-reducing conditions whereas (f) and (g) were analysed under strongly reducing conditions; 25 μg of protein was applied to each gel.

thiosulphonate groups react rapidly with the thio-nitrobenzoate anion.

Both samples of cross-linked enzyme are re-activated by mild reduction with either dithiothreitol or 2-mercaptoethanol. In the case of fraction E_2 , mild reduction, giving complete re-activation, was achieved by exposure to 1 mM-2-mercaptoethanol for 10 min at 18°C. No time-dependence for regain of enzyme activity on reduction has been demonstrated so far. The partial recovery of activity in fraction E_1 was not enhanced by using 50 mM-dithiothreitol or 50 mM-2-mercaptoethanol. After mild reduction both re-activated samples were analysed by gel electrophoresis and in both cases the major structure had remained as a dimer of mol.wt. 72 000 (Table 2; Fig. 8). Provided that non-denaturing conditions were used, the stable dimer was always observed

Fig. 9. Sucrose-density-gradient centrifugation of cross-linked glyceraldehyde 3-phosphate dehydrogenase.

(a) Profile for SS'-[1,8- $^3\text{H}_4$]octamethylenebis(methanethiosulphonate) cross-linked E_2 glyceraldehyde 3-phosphate dehydrogenase (1 mg of protein) and (b) profile for the same enzyme that had been re-activated by mild reduction (300 μg of protein). The graph shows the distribution of radioactivity ($\circ$) and the sucrose concentration on the gradient ($\bullet$).

and this occurred even when high dithiothreitol concentrations (50 mM) and long reaction times (3 h) were chosen. The inclusion of sodium dodecyl sulphate (1 %, w/v) during reduction led to the formation of the monomer (Fig. 8).

Analysis of the bound radioactivity after mild reduction, showed that for fraction E₂, 2 mol of inhibitor/tetramer was retained. There are two cross-links within every tetramer, and presumably these are buried in a hydrophobic region of the protein and not easily removed.

The technique of modification of glyceraldehyde 3-phosphate dehydrogenase with *SS'*-octamethylene-bis(methanethiosulphonate) followed by mild reduction has established that it is possible to isolate a fully active enzyme (fraction E₂, mild reduced), which has a cross-link across one crystallographic axis to give a chemically stable dimer. This observation supports the concept of the highly interacting dimer as the functional unit of enzyme activity for glyceraldehyde 3-phosphate dehydrogenase. The active cross-linked dimer of glyceralde-

Scheme 2. Model for cross-linking in glyceraldehyde 3-phosphate dehydrogenase

There are four thiol groups per subunit of glyceraldehyde 3-phosphate dehydrogenase, but only three are represented for simplicity. The solid bar (■) represents potential cross-links. It is proposed that linkages involving cysteine-149 are more susceptible to reduction. Cysteine-149–cysteine-149 cross-links were not considered, since the separation is approx. 3 nm, which is greater than the distance spanned by the reagent (Harris & Waters, 1976). The cysteine residues are represented by S₁₄₉, S₁₅₃ and S₂₈₁.

hyde 3-phosphate dehydrogenase should be of considerable interest in studying aspects of the molecular asymmetry of the enzyme and its influence on the properties of enzyme hybrids.

Molecular weight of native cross-linked glyceraldehyde 3-phosphate dehydrogenase

To establish the native molecular weight of cross-linked glyceraldehyde 3-phosphate dehydrogenase, sucrose-density-gradient centrifugation was used. Centrifugation conditions were established that would resolve proteins of mol.wt. 224 000 (pyruvate kinase), 144 000 (lactate dehydrogenase) and 68 000 (bovine serum albumin) on linear 5–20% (w/v) sucrose gradients. ^3H -labelled samples of cross-linked and cross-linked re-activated glyceraldehyde 3-phosphate dehydrogenase (E_2) were then analysed under identical conditions (Fig. 9). In both cases, the ^3H -labelled protein exhibited distribution profiles across the gradient that were characteristic of 144 000-mol.wt. proteins rather than of 72 000-mol.wt. proteins. We conclude that formation of the cross-link does not interfere with the quaternary structure of the native enzyme and the cross-linked enzyme remains as a tetramer. Presumably dissociation to the dimer occurs primarily under denaturing conditions.

Models for cross-linking and inhibition of glyceraldehyde 3-phosphate dehydrogenase

Clearly, a very detailed analysis of the implications of the enzyme-inhibition and cross-linking studies awaits the determination of the sequences around labelled cysteine residues. However, since we have accurate details of inhibitor binding and there is substantial knowledge of the crystal structures of

glyceraldehyde 3-phosphate dehydrogenase (Moras *et al.*, 1975), we have presented a pictorial representation of the cross-linking reaction (Scheme 2).

The simplest structure to visualize is the fully active mildly reduced form of E_2 (model C, Scheme 2). This would contain two cross-links per tetramer presumably between two cysteine-281 residues, since these are separated by about the correct reaction distance (1.4–1.77 nm). Since all cysteine-149 residues are unmodified, the enzyme should be catalytically active as observed. Assuming that model C is correct, then it could be formed by the reduction of two precursors. Model A and model B (Scheme 2) should represent forms E_2 and E_1 respectively. Our scheme visualizes that form E_1 is formed by the slow reaction of form E_2 with excess reagent. It is well known that, in the asymmetric chemical modification of glyceraldehyde 3-phosphate dehydrogenase, two cysteine-149 residues per tetramer react very rapidly. The last two cysteine-149 residues, although seriously hindered, will eventually react (MacQuarrie & Bernhard, 1971).

The presence of cysteine 149–cysteine-153 bridges is perfectly plausible in forms E_1 and E_2 (Parker & Allison, 1969; Wassarman *et al.*, 1969). However, it must be remembered that a single modification will give identical results (no available thiol groups) on titration with 5,5'-dithiobis-(2-nitrobenzoate) as shown in Scheme 3.

Model A seems to fit all the experimental data for form E_2 . The principal interest of this model is that only two additional cysteine-149–cysteine-153 cross-links would be required to fit the inhibitor-binding and thiol-titration data. Complete enzyme inhibition would occur while two active-site cysteine-149 residues were unmodified. Clearly this would only occur if enzyme activity was distributed asymmetrically which is in accord with the enzyme-

Scheme 3. Reaction of 5,5'-dithiobis-(2-nitrobenzoate) with enzyme after half-reaction with SS'-polymethylenebis(methanethiosulphonate)

Abbreviations: DTNB, 5,5'-dithiobis-(2-nitrobenzoate); TNB, 5-thio-(2-nitrobenzoate).

inhibition experiments. This result is also found in modification of muscle glyceraldehyde 3-phosphate dehydrogenase with the substrate analogue, furacroyl phosphate (MacQuarrie & Bernhard, 1971). Furthermore when the rabbit muscle enzyme was allowed to react with a structural analogue of NAD⁺, [3-(3-bromoacetylpyridine)propyl]adenosine pyrophosphate, complete inhibition of enzyme activity coincided with the modification of only two subunits in the tetrameric enzyme (results of Woenckhaus discussed in Hollaway *et al.*, 1977). Presumably modification in two subunits would induce a conformational change in the last two subunits, making cysteine-149 an inferior nucleophile (equivalent to an increase in pK_a) and less suitable for catalysis. Interestingly, studies on the crystal structure of the holoenzyme of lobster muscle glyceraldehyde 3-phosphate dehydrogenase (Moras *et al.*, 1975) have demonstrated that the orientation of cysteine-149 with respect to histidine-176 (an invariant residue) depends upon the subunit analysed. If histidine-176 functions by proton abstraction from cysteine-149 (Harrigan & Trentham, 1973), then moving histidine-176 further away from cysteine-149 could raise the pK_a of the thiol group and explain the decrease in nucleophilic reaction.

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