Synthesis and Use of Bifunctional Chloromethylalkanedione Derivatives of Variable Chain Length for Cross-Linking Thiol Groups in Oligomeric Proteins

SPECIFIC CROSS-LINKING IN GLYCERALDEHYDE 3-PHOSPHATE DEHYDROGENASE

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Bischloromethylpentanedione, bischloromethylhexanedione, bischloromethyloctanedione and bischloromethyldecanedione were synthesized from their corresponding dicarboxylic acids via the bis-acyl chloride and the bisdiazomethylketone derivatives. These compounds proved to be highly specific cross-linking reagents for rabbit skeletal-muscle glyceraldehyde 3-phosphate dehydrogenase. Incubation of the enzyme with cross-linking reagents resulted in both a time- and concentration-dependent formation of covalently linked oligomeric structures. The major cross-linked product detected by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was the dimer (mol. wt. 72 000). Sepharose 6B chromatography of the cross-linked enzyme showed that it still existed as the tetramer. Cross-linking was dependent on the native structure of the enzyme, since it was abolished on denaturation of the enzyme. The actual covalently linked product depends on the conditions of modification and the chain length of the reagent. The maximum yield of dimer (70–80%) was obtained with bischloromethylhexanedione, and the yield decreased with either shorter- or longer-chain compounds. The calculated distance between the two reactive points in bischloromethylhexanedione is 1.21–1.45 nm. Bischloromethylhexanedione modified at least two thiol groups per monomer. Modification of the active-site thiol, cysteine-149, was not essential for cross-linking, since glyceraldehyde 3-phosphate dehydrogenase carboxymethylated on cysteine-149 still reacted to form the dimer. The rate of chemical cross-linking was markedly decreased by increasing the NAD⁺ occupancy of the enzyme active sites. These experiments are discussed in terms of the asymmetry of the enzyme structure in solution.

Cross-linking studies provide an important method for studying features in oligomeric proteins, including the distance between groups (D'Albis & Gratzer, 1976), the geometry of oligomer arrangement (Davies & Stark, 1970; Cohlberg *et al.*, 1972; Bickle *et al.*, 1972; Wang & Richards, 1974; Coggins *et al.*, 1976) and protomer interaction in regulatory proteins (Lad & Hammes, 1974). The most useful reagents developed so far are the imidates, such as dimethyl-suberimide, which modify lysine residues. The intention of the present project was to develop a similar type of reagent that would be specific for cysteine thiol groups. Although a number of reagents are available for this purpose, such as bifunctional alkyl halides and aryl halides (Wold, 1967), they have the disadvantage that synthesis of variable-chain-length compounds cannot be achieved with ease. My purpose in undertaking the synthesis of alternative compounds was to use the chloromethyl ketone group as a functional group because of its high specificity for thiol groups (Bloxham & Chalkley, 1976), and secondly to base the synthesis on dicarboxylic acids, which are readily available in different chain

lengths. Once these objectives are stated, a suitable synthesis suggests itself quite readily, and involves the sequential conversion of a dicarboxylic acid into the bis-acid chloride, the bisdiazomethylalkanedione and the bischloromethylalkanedione.

The present paper describes the synthesis of these compounds and shows that they can produce highly specific cross-linking in rabbit muscle glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12), the extent of which is dependent on the separation of the reactive groups in the cross-linking reagent.

Materials and Methods

Synthesis of bischloromethylhexanedione

The general route for the synthesis of the bischloromethylalkanediones is shown in Scheme 1. For the synthesis in question the starting dicarboxylic acid was adipic acid ($n = 4$; carboxypentanoic acid). Adipic acid (7.6 gm) was treated with 15 g of freshly distilled thionyl chloride for 16 h at room temperature (20°C) followed by refluxing for 1 h. Excess thionyl

Scheme 1. General route for the synthesis of bischloromethylalkanediones

chloride was removed under vacuum and adipyl chloride was purified by vacuum distillation (b.p. 95°C at 1.75 mmHg).

Diazomethane was derived from 0.15 mol of toluene-*p*-sulphonylmethylnitrosoamide (Bloxham & Chalkley, 1976) and dissolved in 300 ml of anhydrous diethyl ether. The mixture was then stirred on ice, and to this 0.03 mol of bis-acid chloride was added dropwise over a period of 10 min. During this period gas was evolved and the diazomethane decolorized. Reaction was continued for 1 h at 4°C, and during this time a heavy precipitate formed. An equal volume of ice-cold light petroleum (b.p. 60–80°C) was added and the product was obtained in 75% yield by filtration [bisdiazomethylhexanedione, m.p. 68–70°C, i.r. shows maxima at 2100 cm⁻¹ (N=N=CH) and 1620 cm⁻¹ (C=O); n.m.r.* shows: δ 4.88 p.p.m. (singlet, 2H, 2 × N=N=CH); δ 7.70 p.p.m. (triplet, 4H, 2 × -C-CH₂-); δ 8.34 p.p.m. (multiplet, 4H, -C-CH₂-CH₂-C-)].

The bisdiazomethylhexanedione (0.01 mol) was dissolved in 100 ml of dichloromethane and cooled to 4°C. Dry HCl gas was passed slowly through the mixture for 5–30 min. The end point of the reaction was obvious both from the disappearance of the yellow colour and the disappearance of the i.r. absorption of the diazomethyl group. When the reaction was complete, the mixture was concentrated under vacuum to the point of incipient crystallization, and

* Abbreviation: n.m.r., nuclear magnetic resonance.

then light petroleum (b.p. 60–80°C) was added. The product was obtained in 90% yield after filtration [bis(dichloromethyl)hexanedione; m.p. 80–81°C; i.r. 1720 cm⁻¹ (C=O); n.m.r., δ 6.04 p.p.m. (singlet, 4H, 2 × Cl-CH₂-C), δ 7.42 p.p.m. (triplet, 4H, 2 × C-CH₂-C), δ 8.40 p.p.m. (multiplet, 4H, C-CH₂-CH₂-C); mass spectrum showed *M* - Cl = 161 as the principal ion].

Synthesis of bischloromethylpentanedione (n = 3), bischloromethyloctanedione (n = 6) and bischloromethyl-decanedione (n = 8)

All of these compounds were readily synthesized by the general route, except that the glutaric acid (*n* = 3) series required a minor modification. Thus bisdiazomethylpentanedione does not crystallize directly from ethereal solution and it can only be crystallized after removing the ether *in vacuo*. The physical properties of these compounds are listed below.

Bisdiazomethylpentanedione; m.p. 54–57°C; n.m.r., δ 4.8 p.p.m. (singlet, 2H, 2 × N=N=CH), δ 7.60 p.p.m. (triplet, 4H, 2 × C-CH₂-), δ 8.0 p.p.m. (multiplet, 2H, C-CH₂-C).

Bischloromethylpentanedione; m.p. 76–77°C; n.m.r. δ 6.0 p.p.m. (singlet, 4H, 2 × Cl-CH₂-),

δ 7.35 p.p.m. (triplet, 4H, $2 \times \text{C}-\text{CH}_2$), δ 8.1 p.p.m.

(multiplet, 2H, $\text{C}-\text{CH}_2-\text{C}$).

Bisdiazomethyloctanedione: m.p. 78–80°C; n.m.r.,

δ 4.88 p.p.m. (singlet, 2H, $2 \times \text{N}=\text{N}^+-\text{CH}$), δ 7.70 p.p.m. (triplet, 4H, $2 \times \text{C}-\text{CH}_2$), δ 8.50 p.p.m. (broad

multiplet, 8H, $\text{C}-[\text{CH}_2]_4-\text{CH}_2$).

Bischloromethyloctanedione: m.p. 84–85°C; n.m.r., δ 6.04 p.p.m. (singlet, 2H, $2 \times \text{Cl}-\text{CH}_2$), δ 7.50 p.p.m. (triplet, 4H, $2 \times \text{C}-\text{CH}_2$), δ 8.50 p.p.m. (broad

multiplet, 8H, $\text{C}_2-[\text{CH}_2]_4-\text{C}$).

Bisdiazomethyldecanedione: m.p. 88–90°C;

n.m.r., δ 4.88 p.p.m. (singlet, 2H, $2 \times \text{N}=\text{N}^+-\text{CH}$), δ 7.70 p.p.m. (triplet, 4H, $2 \times \text{C}-\text{CH}_2$), δ 8.50 p.p.m.

(broad multiplet, 12H, $\text{C}-[\text{CH}_2]_6-\text{C}$).

Bischloromethyldecanedione: m.p. 90–91°C; n.m.r., δ 6.04 p.p.m. (singlet, 4H, $2 \times \text{Cl}-\text{CH}_2$), δ 7.50 p.p.m. (triplet, 4H, $2 \times \text{C}-\text{CH}_2$), δ 8.50 p.p.m. (broad

multiplet, 12H, $\text{C}-[\text{CH}_2]_6-\text{C}$).

All n.m.r. spectra were recorded with a Perkin-Elmer RB12 spectrometer with CHCl_3 as solvent and tetramethylsilane as a reference.

Cross-linking of glyceraldehyde 3-phosphate dehydrogenase

Rabbit muscle glyceraldehyde 3-phosphate dehydrogenase was dialysed at 4°C against 0.1 M-Tris/HCl, pH 8.0, containing 1 mM-EDTA. Protein concentration was then adjusted to 1 mg/ml and the enzyme was treated at 20°C with the appropriate concentration of cross-linking reagent. The usual reaction volume was 0.25 ml. The reaction was terminated by the addition of an equal volume of 10 mM-sodium phosphate buffer, pH 7.0, containing 1% (w/v) sodium dodecyl sulphate and 1% (v/v) β -mercaptoethanol. As shown below, the mixture could be stored in this form for a considerable period of time before analysis. Detection of various oligomeric forms of the enzymes was performed by gel electrophoresis in 7.5% polyacrylamide gels by the method of Weber & Osborn (1969). Just before electrophoresis, complete denaturation of protein samples was ensured by heating at 90°C for 2 min. At the end of electrophoresis, samples were stained with Coomassie Brilliant Blue dye and the gels were scanned with a Joyce-Loebl u.v. scanner. The protein content of each component was then assessed by integrating the area be-

neath each curve. For each electrophoretic run the correct mobility of the glyceraldehyde 3-phosphate dehydrogenase dimer was ensured by calibration against the monomer of bovine serum albumin (mol.wt. 68000). The catalytic activity of the enzyme was determined by the method of Velick (1955).

Sources of chemicals and reagents

All the materials in this work were obtained from BDH, Poole, Dorset, U.K., Aldrich Chemical Co., Gillingham, Dorset, U.K., Koch-Light Laboratories, Colnbrook, Bucks, U.K., or Boehringer Corp. (London) Ltd., Lewes, Sussex, U.K.

Results

Synthesis of bischloromethylalkanediones for cross-linking purposes

The basic route for the synthesis of these compounds is described in the Materials and Methods section and is illustrated in Scheme 1. The method uses the readily available dicarboxylic acids as starting compounds. Fortunately the intermediate compounds are readily isolated and the conversions are high at each step. The syntheses are particularly convenient, which means that these compounds are readily available. The longest dicarboxylic acid used in the present work is sebacic acid (carboxynonanoic acid); however, much longer compounds are also commercially available, such as hexadecanedioic acid ($\text{HO}_2\text{C}-[\text{CH}_2]_{16}-\text{CO}_2\text{H}$). These can be converted into the bischloromethylalkanediones by an identical route and should lead to reagents that are capable of linking groups up to 2 nm apart. Currently, the synthesis of the bifunctional compounds of short dicarboxylic acids has not succeeded below glutaric acid (carboxybutyric acid). Although we have tried a large variety of routes to convert succinic acid into its bifunctional derivative, these have all failed, owing to the formation of cyclic compounds.

For the present work, chloromethyl ketone derivatives were used, because of the high reaction specificity of this group in covalent modification. However, for some purposes it may be advantageous to have more reactive compounds, which are obtained by converting the chloromethyl ketone derivative into the iodomethyl ketone. This is obtained by treating the bis(chloromethyl ketone) with an equimolar amount of NaI in acetone at 0°C with stirring. After 1 h, all the precipitated NaCl is removed by filtration and the solvent removed *in vacuo* to yield the product. Iodomethyl ketones are up to 10^4 times as reactive as their chloromethyl ketone analogues. One feature that has become prominent in the use of bifunctional reagents is the introduction of a cleavable reagent linked

by a disulphide bridge (Wang & Richards, 1974; Peretz & Elson, 1976). It seems unlikely that reagents of this type can be synthesized, owing to the inherent reactivity of the chloromethyl ketone group with a thiol group. An alternative may be to incorporate an olefinic bond in the reagent by using an unsaturated dicarboxylic acid in the synthesis. This bond can then be cleaved under fairly mild conditions by using a mixture of KMnO_4 and periodate (Pelletier *et al.*, 1970). The use of periodate to cleave a glycol-based cross-linking reagent has been introduced by Coggins *et al.* (1976).

An additional difference between the chloromethyl ketone reagents and the imidates is that the former reagents are non-polar and relatively insoluble in water. This means that they could be useful in linking enzymes in hydrophobic environments, such as membranes that may not be easily available to the polar imidate reagents.

NAD⁺ content of glyceraldehyde 3-phosphate dehydrogenase

The rate of reaction of thiol groups in rabbit muscle glyceraldehyde 3-phosphate dehydrogenase is influenced by the number of NAD^+ molecules bound per tetramer. Thus Conway & Koshland (1968) showed that the addition of NAD^+ to the apoenzyme accelerated the rate of reaction of cysteine-149 with either bromoacetamido-4-nitrophenol or iodoacetic acid. This process was saturated when one NAD^+ molecule was bound per enzyme tetramer. For the present work rabbit muscle glyceraldehyde 3-phosphate dehydrogenase was obtained from Boehringer and the enzyme was routinely prepared by dialysis against 0.1M-Tris/HCl, pH 8.0, containing 1 mM-EDTA at 4°C for 18h. After this period of time the A_{280}/A_{260} ratio was 1.31 ± 0.03 (S.E.M. for three preparations), which corresponds to approx. 1.6 NAD^+ molecules bound per tetramer from the data of Fox & Dandliker (1956). This value is similar to that obtained by Conway & Koshland (1968), who dialysed the rabbit muscle enzyme against 1 mM-EDTA, pH 7.0, and found 2 NAD^+ molecules bound per tetramer. The value of 1.6 NAD^+ molecules per tetramer was also confirmed after heat denaturation of the enzyme and assay of the liberated NAD^+ (Fox & Dandliker, 1956). In conclusion, the enzyme routinely used in the present work has sufficient NAD^+ bound to exceed the concentration required for maximum thiol reaction in the protein.

Inhibition of rabbit muscle glyceraldehyde 3-phosphate dehydrogenase

To ensure that the enzyme reacted with the bi-functional reagents, glyceraldehyde 3-phosphate dehydrogenase was incubated with a variety of con-

Fig. 1. Inactivation of glyceraldehyde 3-phosphate dehydrogenase by bischloromethylhexanedione

The enzyme was modified by the procedure described in the Materials and Methods section and the progressive fall in enzyme activity was followed. The control incubation mixture contained 10% (v/v) methanol, since this was the concentration used for dissolving the inhibitor. The concentrations of inhibitor were (○) 1 mM, (●) 0.5 mM, (△) 0.2 mM, (▲) 0.1 mM, (□) 0.05 mM, (■) none. The apparent rate constants for inactivation were determined from the slopes of the lines.

centrations of bischloromethylhexanedione, and the loss of activity was followed. Fig. 1 shows that inactivation followed pseudo-first-order kinetics and the rate of inactivation was concentration-dependent. The inclusion of 10% (v/v) methanol had no effect on enzyme activity in these short-term incubations.

Cross-linking of glyceraldehyde 3-phosphate dehydrogenase by bischloromethylhexanedione

The extent of cross-linking was assessed by incubating the enzyme with 5 mM-bischloromethylhexanedione under conditions identical with those used in the experiment in Fig. 1. However, for the purpose of this experiment the reaction period was extended to 18 h and cross-linking was measured by gel electrophoresis under denaturing conditions (Weber & Osborn, 1969). An example of the time course for the alteration in the monomer and the cross-linked dimer content of glyceraldehyde 3-phosphate dehydrogenase is shown in Fig. 2. The formation of cross-linked dimer (mol.wt. 72000) is time-dependent, and is compensated by the decline in free monomer concentration. The maximum yield of cross-linked dimer in this experiment corresponds to around 80% of the total protein on the basis of the intensity of Coomassie Brilliant Blue staining. The actual extent of cross-linking is slightly variable: 80% of cross-linked dimer

appears to be the maximum obtainable value, but on a number of occasions this value has fallen to around 60%. There is little evidence for the formation of very-high-molecular-weight aggregates during cross-linking, since the total amount of protein entering the gel remains reasonably constant during the cross-linking reaction.

One important feature of the cross-linking reaction is that there is a substantial difference between the rate of enzyme inhibition and the rate of the cross-linking reaction. If the fall in enzyme activity was measured in the presence of 5 mM-bischloromethylhexanedione, the enzyme was completely inactive within 3 min; however, only 6% of the enzyme was present as the dimer at this time and the maximum extent of the cross-linking reaction (80% present as the dimer) was only achieved after 18 h. Clearly, the chemical reactions for enzyme inhibition and cross-linking are different.

Concentration-dependence of the cross-linking reaction

The rate of cross-linking was determined at a variety of concentrations of bischloromethylhexanedione. This process showed a normal hyperbolic saturation process (Fig. 3) and enabled the apparent K_1 to be estimated as 0.60 (s.d. ± 0.08) mM.

Influence of aqueous methanol on the molecular structure of glyceraldehyde 3-phosphate dehydrogenase

The bischloromethylalkanediones are not soluble in water. Therefore the inhibitors were dissolved in methanol and mixed with the enzyme so that the final methanol concentration was 10% (v/v) (3M). Under these conditions the inhibitors were soluble; however, it meant that the enzyme was continuously exposed to high methanol concentrations, which might influence the oligomeric structure of the enzyme. To examine any possible action of methanol, glyceraldehyde 3-phosphate dehydrogenase (1 mg/ml) was incubated at 20°C in 0.1 M-Tris/HCl, pH 8.0, containing 1 mM-EDTA and 3 M-methanol. The activity

Fig. 2. Time-dependent formation of the cross-linked glyceraldehyde 3-phosphate dehydrogenase dimer

Traces (a-g) show the gel scans of glyceraldehyde 3-phosphate dehydrogenase which had been treated with 5 mM-bischloromethylhexanedione. The reaction periods were (a) 0 min, (b) 15 min, (c) 30 min, (d) 60 min, (e) 120 min, (f) 18 h, (g) 18 h. For curve (g) the enzyme was denatured with sodium dodecyl sulphate before the alkylation reaction. The arrow marks the mobility of the bovine serum albumin monomer (mol.wt. 68 000). Conditions for enzyme incubation and gel electrophoresis are described in the Materials and Methods section.

Fig. 3. Concentration-dependence of the cross-linking reaction

The rate of formation of the cross-linked dimer of glyceraldehyde 3-phosphate dehydrogenase was measured at various concentrations of bischloromethylhexanedione. The data were analysed by a least-squares regression analysis (Wilkinson, 1961). K_1 was estimated as 0.60 (s.d. ± 0.08) mM and the maximum rate of dimer formation was 51.7 (s.d. ± 2.2) %/h.

was followed for 18 h, the maximum duration of the cross-linking experiments, and during this period there was no detectable loss of enzyme activity, indicating that the enzyme was quite stable. After 18 h, the enzyme was applied to a column (70 cm $\times$ 2.5 cm) of Sepharose-6B equilibrated with 0.1 M-Tris/HCl, pH 8.0, containing 1 mM-EDTA. The enzyme was eluted at the calibrated position for a tetrameric protein of mol. wt. 140 000. These results are taken to indicate that the presence of methanol does not induce the formation of the stable dimer before cross-linking.

Molecular weight of the enzyme under non-denaturing conditions

To estimate the molecular weight of glyceraldehyde 3-phosphate dehydrogenase once it was covalently cross-linked, two incubations were set up. In the first control incubation, glyceraldehyde 3-phosphate dehydrogenase (1 mg) was incubated in 1 ml of 0.1 M-Tris/HCl, pH 8.0, containing 1 mM-EDTA and 10% (v/v) methanol. In the second incubation 5 mM-bischloromethylhexanedione was added. After 18 h a small insoluble residue was removed by centrifugation at 1000g for 5 min, and both samples were applied to the same Sepharose 6B column (Fig. 4) with a separation of ten fractions between the control and cross-linked samples. Both samples were then eluted from the column with exactly the same separation between them. The enzyme samples were easily identi-

fied, since the first was active and the second was inactive, corresponding to the control and cross-linked enzymes respectively. Clearly this result indicates that the cross-linked enzyme exists predominantly as the tetrameric form in aqueous solution, with covalent bonds stabilizing the dimer.

Specificity of the cross-linking reaction with bischloromethylhexanedione

The results so far have only been concerned with the formation of the cross-linked dimer. The formation of this species is very specific, since the amounts of either the trimeric or tetrameric species formed is relatively small. When the glyceraldehyde 3-phosphate dehydrogenase was cross-linked with bischloromethylhexanedione at 20°C, tetrameric product was never observed; however, the trimer was formed in very small yield and accounted for up to 5% of the total protein after 24 h reaction. To see if this yield might be increased by using conditions that might make the rigid tetramer less stable, cross-linking was carried out at the higher temperatures of 25, 30, 36 and 43°C. With these conditions 0.17, 0.20, 0.27 and 0.43% cross-linked trimer formation/h respectively were observed, showing that the yield of trimer increased progressively with increased temperature. Most of this increase was due to an increase in the further reaction between a dimer and monomer, since increasing reaction temperature caused a fall in the yield of cross-linked dimer, which corresponded reasonably to the increase in the trimer species. Although it proved difficult to produce trimeric species with bischloromethylhexanedione, cross-linking reagents of other chain lengths did not lead to such exclusive formation of the dimer.

An additional feature for the specific formation of the cross-linked dimer is that it is dependent on the native configuration of the enzyme. If the oligomeric structure of glyceraldehyde 3-phosphate dehydrogenase was destroyed by incubating the enzyme in 50 mM-Tris/HCl, pH 8.0, containing 1 mM-EDTA and 1% (w/v) sodium dodecyl sulphate, then the subsequent addition of 5 mM-bischloromethylhexanedione did not result in any appreciable cross-linking (Fig. 2, curve g).

Influence of the length of cross-linking reagent on the extent of dimer formation

If the cross-linking reaction to form the dimeric species occurs fairly specifically between two cysteine thiol groups, then it would be expected that there would be a fairly strict requirement for the distance between the two reactive groups on the cross-linking molecule. To evaluate this possibility, four cross-linking reagents of different chain length were tested. The results in Fig. 5 clearly show that the cross-

Fig. 4. Molecular weight of cross-linked glyceraldehyde 3-phosphate dehydrogenase by Sepharose 6B chromatography. The Figure shows the elution profile of control (A) and bischloromethylhexanedione-cross-linked (B) enzyme. The first two arrows show sample application. Chromatography was performed on a Sepharose 6B column (70cm×2.5cm) equilibrated at 4°C with 0.1 M-Tris/HCl, pH 8.0, containing 1 mM-EDTA. The fraction volume was 1.8 ml. The conditions for enzyme preparation are described in the text.

Fig. 5. Dependence of cross-linking on an optimal distance between the reactive groups in the bifunctional reagent. The extent of cross-linking was measured after reaction with a fixed concentration (5 mM) of the different-chain-length bifunctional reagents: n represents the number of bridge methylene groups separating the carbonyl group in the cross-linking reagent. Reaction times were 1 h (○), 2 h (●) and 18 h (△).

of reaction decreases with either shorter- or longer-chain compounds. Compounds that did not produce the optimal formation of dimer resulted in slightly increased formation of both the trimeric and tetrameric species, up to a maximum yield of 10% for the trimer and 3% for the tetramer.

Thiol modification by cross-linking reagents

I have not synthesized radioactively labelled cross-linking reagents to evaluate the incorporation of the inhibitor into the protein. This meant that to assess the extent of reaction it was necessary to estimate the loss of thiol groups during modification. Amino acid analysis has shown that there are four cysteine groups per protomer (mol.wt. 36 000) in rabbit muscle glyceraldehyde 3-phosphate dehydrogenase (Harris & Perham, 1965). With enzyme prepared for the cross-linking studies, titration with 5,5'-dithiobis-(2-nitrobenzoate) (Ellman, 1959) in 60 mM-Tris/HCl, pH 8.0, containing 6 M-urea, then only three thiol groups were identified per protomer. Once the enzyme was modified with bischloromethylhexanedione, this declined to one free thiol group per protomer, corresponding to the modification of at least two cysteine residues in each protomer. In the earlier section on cross-linking of glyceraldehyde 3-phosphate dehydrogenase by bischloromethylhexanedione it was pointed out that the rate of inhibition of enzyme activity and cross-linking were markedly different. The basis of this divergence was easily observed when 5,5'-dithiobis-(2-nitrobenzoate) was used to modify cysteine thiol groups, since when native glyceraldehyde 3-phosphate dehydrogenase was treated with 5,5'-dithiobis-(2-nitrobenzoate) in 50 mM-potassium

linking reaction is dependent on the chain length, indicating that optimal cross-linking occurs with four bridge methylene groups in the reagent, and the extent

phosphate, pH 8.0, then 1–1.5 groups per protomer reacted very rapidly (within minutes), whereas the remaining groups reacted extremely slowly.

Cross-linking in glyceraldehyde 3-phosphate dehydrogenase carboxymethylated on cysteine-149

The role of cysteine-149 in the cross-linking reaction was evaluated by examining cross-linking when cysteine-149 was protected as the carboxymethyl group. Carboxymethylated glyceraldehyde 3-phosphate dehydrogenase was prepared by treating 30 mg of enzyme in 3 ml of 0.1 M-Tris/HCl, pH 7.0, containing 1 mM-EDTA, with 0.5 mg of recrystallized iodoacetic acid (Harris *et al.*, 1963). After 1 h at 0°C, 2.0 g of enzyme-grade $(\text{NH}_4)_2\text{SO}_4$ was added and 30 min later the enzyme was collected by centrifugation at 15 000g for 15 min. The enzyme was dissolved in 0.1 M-Tris/HCl, pH 8.0, containing 1 mM-EDTA. Titration with 5,5'-dithiobis-(2-nitrobenzoate) in 50 mM-potassium phosphate, pH 8.0, containing 6 M-urea showed that, whereas the control enzyme contained 3.2 thiol groups per protomer, the modified enzyme contained 2.4 thiol groups per protomer, corresponding to the modification of cysteine-149. When carboxymethylated glyceraldehyde 3-phosphate dehydrogenase was treated with 5 mM-bischloromethylhexanedione under conditions described in the Materials and Methods section, the dimer was formed virtually exclusively. The rate of formation of the dimer was indistinguishable from the data presented in Figs. 2 and 6 for cross-linking in the control enzyme with 1.6 NAD^+ molecules per tetramer. This result demonstrates that cysteine-149 does not participate in the cross-linking reaction and reinforces the idea presented above that the chemical reactions for enzyme inhibition and cross-linking were different.

Influence of NAD^+ content of the tetramer on the rate of cross-linking by bischloromethylhexanedione

The most difficult question associated with this work is whether the cross-linking reaction takes place within the tetramer or within a free dimer in solution. This may be approached by studying the effect of NAD^+ on the cross-linking reaction. The hybridization studies by Osborne & Hollaway (1975) have shown that NAD^+ appears to decrease the rate at which the rabbit muscle enzyme dissociates from the tetramer to the dimer. Therefore it would be of interest if NAD^+ slowed the rate of cross-linking. For this experiment three samples of enzyme were used containing 0, 2 and 4 NAD^+ molecules per tetramer and the rate of dimer formation with 5 mM-bischloromethylhexanedione was followed. Fig. 6 shows that increasing the NAD^+ occupancy of the enzyme active sites produced a substantial inhibition of the rate of dimer formation. By 4 NAD^+ molecules bound per

Fig. 6. Inhibition of cross-linking by NAD^+ occupancy of the enzyme active site

NAD^+ -free glyceraldehyde 3-phosphate dehydrogenase was prepared by treatment with acid-washed charcoal. The A_{280}/A_{260} ratio was 1.93, which is the same as the value obtained by Fox & Dandliker (1956) for the apoenzyme. Cross-linking was then performed with 7 μM -apoenzyme (based on mol.wt. 140 000) supplemented with no NAD^+ ($\circ$), 14 μM - NAD^+ (Δ ; 2 NAD^+ molecules per tetramer) and 1 mM- NAD^+ ($\bullet$; 4 NAD^+ molecules per tetramer). The analysis of dimer formation was performed by the general procedure described in the Materials and Methods section.

tetramer, the initial rate of dimer formation was virtually negligible. This result appears to be consistent with cross-linking occurring with a dimer in free solution, on the basis of the interpretation of the hybridization experiments of Osborne & Hollaway (1975).

Discussion

Although glyceraldehyde 3-phosphate dehydrogenase is composed of four chemically identical protomers, in the oligomeric form of the enzyme these subunits are not equivalent. This has been revealed by X-ray crystallography (Buehner *et al.*, 1974; Moras *et al.*, 1975), chemical modification (Malhotra & Bernhard, 1973; Stallcup & Koshland, 1974; Levitzki, 1974), hybridization studies (Osborne & Hollaway, 1974, 1975) and negative co-operativity in binding NAD^+ to the rabbit muscle enzyme (Conway & Koshland, 1968; DeVijlder & Slater, 1968). The dimer (mol.wt. 72 000) may in fact be regarded as the functional subunit within the enzyme. This is consistent with the majority of bonding contacts occurring between either the 'red' and 'blue' or 'green' and 'yellow' subunits [Moras *et al.* (1975) provides a detailed explanation of the 'colour coding' in the

tetramer], the formation of a dimer in hybridization and the apparent half-site reactivity of several chemical modification reagents. Additional information could be obtained on the molecular geometry of glyceraldehyde 3-phosphate dehydrogenase by examining cross-linking reactions in the enzyme in free solution. At least two previous cross-linking experiments have been performed on glyceraldehyde 3-phosphate dehydrogenase by using bis(imidates) (Davies & Stark, 1970; Hucho *et al.*, 1975). In neither case was there any evidence for preferential formation of dimers, since trimers and tetramers were produced. In fact the specific study by Hucho *et al.* (1975) provides poor evidence for the existence of a dimer-of-dimers configuration in glyceraldehyde 3-phosphate dehydrogenase and suggests that this may result from complete absence of preference for reactions involving lysine residues. Clearly an alternative reagent reacting with different amino acid residues would be convenient in this case. For this purpose the synthesis of a new group of reagents, bischloromethylalkanediones, was developed. These reagents should prove potentially useful in cross-linking thiol groups in oligomeric proteins.

The principal observations for the reaction between glyceraldehyde 3-phosphate dehydrogenase and the cross-linking reagents are that (a) the inhibitor causes a rapid loss of enzyme activity, (b) the inhibitor produces a much slower cross-linking reaction, which appears highly specific for formation of the dimeric species, (c) dimer formation is optimal with the distance between the reactive groups found in bischloromethylhexanedione, (d) cross-linking is associated with the loss of at least eight titratable thiol groups per tetramer.

The most important question from the present work is why should the cross-linked dimer be produced with such a high degree of specificity. A model that appears to explain observed results is the following:

This model is based primarily on the observation that NAD^+ slows both the rate of hybridization and the rate of cross-linked-dimer formation. It assumes that hybridization obligatorily requires the participation of a free dimer in solution (Osborne & Hollaway, 1975). The re-formation of the 'tetramer containing cross-linked dimers' is based on the observation that the molecular weight of the cross-linked enzyme is the same as that of the native enzyme (Fig. 4). An additional contribution to the specificity is that the cross-linking reaction may occur across a preferred

axis. This preference could clearly be accounted for by the asymmetry of the tetrameric structure of glyceraldehyde 3-phosphate dehydrogenase, which has been observed to form a dimer-of-dimers configuration, with three 2-fold symmetry axes perpendicular to one another. In this form the largest number of contacts is along the *P*-axis (Moras *et al.*, 1975), and it is tempting to propose that this is the axis along which cross-linking can occur. Hajdu *et al.* (1976) have developed a general theory to explain the pattern of cross-linking within tetramers. They propose that for an isologous tetrameric system the rate constants for reaction along the three crystallographic axes are different and described by k_p, k_q, k_r . For the system producing exclusively dimers $k_r = 0$ and $k_p > 50k_q$.

Finally we must consider the possible groups that could have reacted to produce cross-linking. There are at least two reactive groups per subunit, and it may be questioned why the functional group that reacts with chloromethyl ketone has produced this non-specific reaction when iodoacetate reacts quite specifically (Harris *et al.*, 1963). However, cross-linking only occurs under relatively extreme conditions, and normally when modifying cysteine-149 with a reagent such as iodoacetate only a small molar excess of inhibitor over active sites is used.

A plausible mode is that cross-linking occurs provided that a pair of cysteine residues are separated by a similar distance to that between functional groups on the bifunctional reagent. The specificity of the reagent length for cross-linking suggests that the optimal distance between these sites would be given by the structure subunit-S- $\text{CH}_2\text{-CO-[CH}_2\text{]}_{4-6}\text{-CO-CH}_2\text{-S}$ -subunit. Assuming the methylene groups occupied a staggered configuration, then this distance would correspond to 1.21–1.45 nm. From the X-ray crystal structure of the lobster enzyme and assuming that the pig and lobster enzyme sequences are essentially homologous, then it appears that the only pair

of cysteine residues separated by a suitable distance is the Cys-281–Cys-281 pair located on the *P*-axis subunit interface (R. M. Garavito & M. G. Rossmann, personal communication). In the lobster enzyme the equivalent residue is serine, and the separation is 1.77 nm, which could decrease to 1.4 nm on the basis of freedom of rotation of the residue. In this model, the loss of enzyme activity would not be related to cross-linking. It seems unlikely that cross-linking could occur between a pair of cysteine-149 residues, since these are separated by at least 3.5 nm.

Furthermore the experiments with glyceraldehyde 3-phosphate dehydrogenase carboxymethylated on cysteine-149 eliminate this possibility.

In conclusion, the present work describes the synthesis of a new class of cross-linking reagents for thiol groups, the bischloromethylalkanediones. These compounds were screened by analysing their reaction with glyceraldehyde 3-phosphate dehydrogenase. This enzyme was found to be specifically cross-linked, resulting predominantly in the formation of the covalently linked dimer within the tetramer.

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