Formation of a Polymethylene Bis(disulfide) Intersubunit Cross-Link between Cysteine-281 Residues in Rabbit Muscle Glyceraldehyde-3-phosphate Dehydrogenase Using Octamethylene Bis(methane[^{35}S]thiosulfonate)†

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ABSTRACT: The synthesis of a radioactive cross-linking agent, *S,S'*-octamethylene bis(methane[^{35}S]thiosulfonate) (OBMTS), is described. The route of synthesis can be generally used in the synthesis of ^{35}S-labeled thiosulfonates for the selective modification of thiols in proteins. Glyceraldehyde-3-phosphate dehydrogenase (G3PD) reacts asymmetrically with the bifunctional inhibitor. Initially two molecules of OBMTS react rapidly with the active-site thiol, Cys-149, on two of the four subunits to inhibit the enzyme completely without cross-linking. This is followed by the modification of four Cys-281 residues to incorporate two cross-links into the tetramer. Reduction of modified G3PD with 5 mM dithioerythritol under nondenaturing conditions released the inhibitor blocking the active-site thiol and completely restored enzyme activity while leaving the cross-link intact. Sodium dodecyl sulfate (NaDodSO$_4$) gel electrophoresis of the cross-linked enzyme under nonreducing conditions showed a dimer (M_r 72 000) as the major species which was only cleaved by reduction in NaDodSO$_4$ containing β-mercaptoethanol. The monomer formed was still radioactive, showing that the first disulfide in the cross-link was reduced at a much faster rate than the second disulfide. The latter was only reduced by using vigorous conditions. The location of the intersubunit cross-linked residues was established by isolation of the cyanogen bromide and tryptic subdigest peptides containing modified Cys-281. These were identified by molecular weight, amino terminal sequence, and amino acid composition.

Cross-linking reagents have found important applications in protein chemistry which have been reviewed (Peters & Richards, 1977). Most applications attempt to measure distances between pairs of reactive residues by nearest neighbor analysis. Currently the most widely used reagents are bifunctional methylimidates containing a reduction cleavable disulfide bond (Traut et al., 1973; Sun et al., 1974; Peretz & Elson, 1976). The identification of cross-links frequently depends upon an off-diagonal analytical process (Wang & Richards, 1974). Although this method can be very powerful in identifying proteins having close contacts, it suffers from the drawback that the method of identification depends upon cleavage of the cross-links, and this complicates analysis of specific residues involved in the cross-link. Examination of the literature reveals that whereas cross-linking patterns of polypeptides are frequently very sophisticated [for examples, see Peretz et al. (1976); Huang & Richards, 1977; Henriques & Park, 1978; Pilch & Czech, 1979; Witzemann et al., 1979; Dombrâdi et al., 1980] the information on residues involved in the cross-link is usually quite limited.

Thiosulfonate reagents provide a specific electrophilic reaction center for thiols, and their use in the study of thiols in proteins has expanded recently (Smith et al., 1975; Nishimura et al., 1975; Maggio et al., 1977; Bloxham et al., 1979; Botts et al., 1979; Collier & Nishimura, 1979). Recently the synthesis of bifunctional thiosulfate reagents which cross-link thiol groups in a number of proteins including lactate dehydrogenase, pyruvate kinase, phosphofructokinase, and G3PD[1] was developed (Bloxham & Sharma, 1979). The reaction with G3PD was especially interesting because preliminary evidence indicated that it was possible to form a single cross-link across one crystallographic axis to form a catalytically active dimer within the tetrameric enzyme. G3PD is a classic example of the apparent asymmetric distribution of enzyme activity in that although the enzyme is a tetramer (Harris & Perham, 1965) the functional unit of enzyme activity may be a dimer (Osborne & Hollaway, 1974, 1975; Cherednikova et al., 1980). Because of the potential interest of an active G3PD species which is chemically fixed across a crystallographic axis, we felt that it was essential to determine the exact nature of the cross-link. To achieve this we synthesized a ^{35}S-labeled cross-linking agent which could be used readily in the autoradiographic identification of cross-links.

Materials and Methods

Synthesis of [1,8-$^{35}S_2$]OBMTS. The route for the synthesis of the ^{35}S compound and its expected reaction with protein thiols is shown in Scheme I. Sulfur-35-labeled sodium methanethiosulfonate was prepared by addition of methanesulfonyl chloride (1 mmol) to a stirred aqueous solution (3 mL) of $Na_2{}^{35}S$ (1 mmol; 10 mCi). The mixture was heated in a sealed vessel at 100 °C for 24 h. Water was then removed by lyophilization and the residue filter extracted with warm ethanol (10 mL). The filtrate was concentrated in vacuo to give $Na^{35}SSO_2CH_3$.

1,8-Dibromooctane (0.5 mmol) in dry methanol (5 mL) was added to the $Na^{35}SSO_2CH_3$ and reacted for 12 h at 60 °C. Analysis of the products by TLC in chloroform/diethyl ether (1:1) showed that after this initial reaction the bulk of the radioactive compound was located in the monothiol ester product [R_f(8-bromooctyl methanethiosulfonate) 0.65; R_f(dibromooctane) 0.73; R_f(OBMTS) 0.44]. The reaction was forced to completion by the addition of 1.1 mmol of $NaSSO_2CH_3$ followed by heating at 60 °C for a further 24 h. Methanol was removed under vacuum, water (20 mL) was

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[1] Abbreviations: G3PD, glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12); OBMTS, *S,S'*-octamethylene bis(methanethiosulfonate); DTNB, 5,5'-dithiobis(nitrobenzoate); NaDodSO$_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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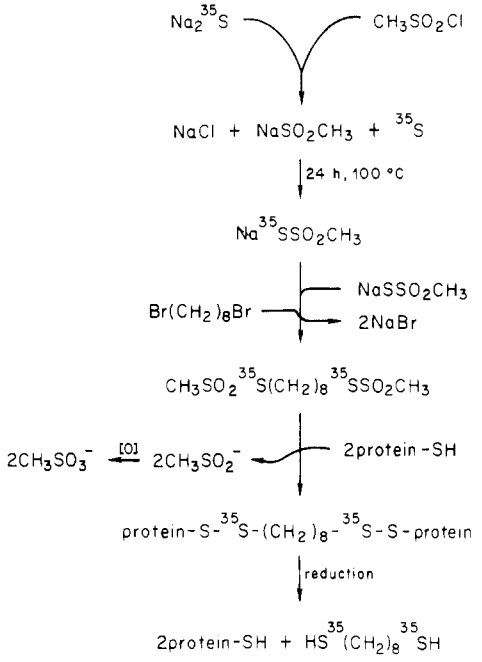
Scheme I: Synthesis and Reactions of [^{35}S]OBMTS



added, and the product was extracted 3 times with ethyl acetate (10 mL). The organic extracts were combined, dried over anhydrous Na_2SO_4, and concentrated under vacuum. Crystallization from ethyl acetate/petroleum ether (bp 40–60 °C) yielded [^{35}S]OBMTS which gave a single radioactive spot on TLC. All other physical characteristics of the compound were identical with values reported previously (Bloxham & Sharma, 1979).

In addition to the current synthesis, $Na^{35}SSO_2CH_3$ may be generally used for the synthesis of thiosulfonates according to the general reaction

$$R\text{-}Br + Na^{35}SSO_2CH_3 \rightarrow NaBr + R\text{-}^{35}SSO_2CH_3$$

Assay for Thiosulfonate Concentration. Thionitrobenzoate anion was prepared by reducing a 5,5′-dithiobis(nitrobenzoate) (DTNB) solution in methanol (5 mg/mL) with $NaBH_4$. After 30 min at 4 °C, excess borohydride was destroyed by acidification to pH 2 with hydrochloric acid (1 M). The solution was then neutralized with $KHCO_3$ (1 M) and added to 0.1 M Tris-HCl, pH 8, to give a concentration of 75 μM. Thiosulfonates react rapidly and stoichiometrically with thionitrobenzoate to form a mixed disulfide. Therefore following the addition of a known volume of thiosulfonate in methanol, its concentration was estimated by the decrease in absorption at 412 nm.

Formation of Cross-Linked G3PD. Rabbit skeletal muscle G3PD was obtained as a suspension in 60% saturated $(NH_4)_2SO_4$ from Boehringer Corp. (London). The protein was collected by centrifugation at 15000*g* for 30 min at 4 °C and the pellet dissolved in 0.1 M Tris-HCl, pH 8.0, at 20 °C, containing 1 mM EDTA. These enzyme solutions at 1 mg/mL (28 μM in subunits) were incubated for 2 h at 18 °C with 30 μM OBMTS (1.1 mol of inhibitor/mol of subunit) to obtain complete inactivation and cross-linking. The inhibitor was added from a stock solution (4 mM) in methanol.

The enzyme was reactivated with 5 mM dithioerythritol at 18 °C for 15 min. This completely restores enzyme activity but leaves the subunit cross-link intact (Bloxham & Sharma, 1979).

$NaDodSO_4$–Polyacrylamide Gel Electrophoresis. All gel electrophoresis was performed by using the system described by Laemmli (1970). When cross-linked proteins were run under nonreducing conditions, they were denatured at 80 °C for 2 min with an equal volume of 1% (w/v) $NaDodSO_4$ containing 30 mM *N*-ethylmaleimide. Under these conditions the polymethylene disulfide cross-links were stable. Alternatively, the proteins were prepared under denaturing reducing conditions when samples were treated at 80 °C for 2 min with 1% (w/v) $NaDodSO_4$ containing 1% (v/v) β-mercaptoethanol. As will be shown later this treatment cleaves only one of the two disulfide bonds in the cross-link. So that both disulfide bonds were completely cleaved, very strong reduction conditions were required. In this case protein samples were treated under denaturing reducing conditions at 110 °C and 15 psi for 60 min.

Following gel electrophoresis samples were stained with Coomassie brilliant blue, and gels were vacuum dried for autoradiography using Kodak X-Omat film.

Protein Cleavage Reactions. Native G3PD was reduced and carboxymethylated prior to any cleavage reaction (Crestfield et al., 1963). Active cross-linked G3PD in 0.1 M Tris-HCl, pH 8, containing 1 mM EDTA (1 mg of protein/mL) was treated for 30 min with 12 mM *N*-ethylmaleimide to modify all free thiol groups. The protein was dialyzed exhaustively vs. distilled water and then lyophilized. The product was dissolved in 70% (v/v) formic acid and cleaved with cyanogen bromide (10 mg/mL) for 18 h at room temperature (Gross & Witkop, 1962). Then the sample was diluted with 5 volumes of water, stirred for 3 h, and lyophilized. The cleaved products were dissolved in 9% (v/v) formic acid and analyzed by chromatography on a Sephadex G-50 superfine column (100 × 2.5 cm) equilibrated in 9% formic acid at room temperature.

Tryptic digestions were performed in 50 mM NH_4HCO_3 with 0.1 mg/mL diphenylcarbamoyl chloride treated trypsin (Wooton et al., 1975) for 6.5 h at 25 °C. The reaction was terminated by the addition of 1 mM phenylmethanesulfonyl fluoride (Fahrney & Gold, 1963) in ethanol, and the products were analyzed after removal of the NH_4HCO_3 by lyophilization.

General Methods. Amino acid analysis was performed with a Durrum D-500 amino acid analyzer. The decomposition of amino acid residues was corrected for by analyzing samples hydrolyzed for 24, 48, and 96 h. Amino-terminal sequences were confirmed by using a Beckman sequencer (model 890B) according to the method of Edman & Begg (1967) with modifications as described by Koide et al. (1978). We are indebted to R. D. Wade (University of Washington, Seattle, WA) for his help in these experiments. Phenylthiohydantoin-amino acids were identified by high-performance liquid chromatography (Hermann et al., 1978).

The activity of G3PD was routinely assayed in the direction of nicotinamide adenine dinucleotide reduction by the method of Ferdinand (1964). Only lots of commercial enzyme with specific activities between 140 and 160 units/mg at 25 °C were used. Results were confirmed on six enzyme preparations. The specific activities for both the commercially obtained enzyme and the active cross-linked species were the same. The protein concentration of G3PD was calculated on the basis of an extinction coefficient at 280 nm of 1000 $cm^2\ g^{-1}$ (Dandliker & Fox, 1955).

The synthesis of [^{3}H]OBMTS has already been described (Bloxham & Sharma, 1979).

Results

Inhibition and Cross-Linking of G3PD. OBMTS reacts very rapidly with G3PD, and complete inhibition was obtained

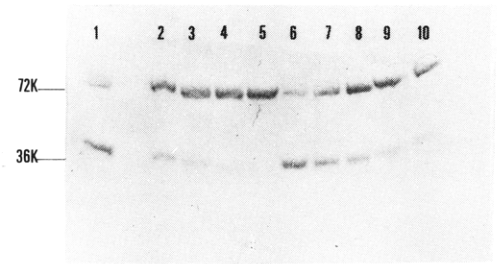


FIGURE 1: $NaDodSO_4$–polyacrylamide gel electrophoresis of OBMTS-modified G3PD. The modified enzyme (25 μg) was analyzed under nonreducing electrophoretic conditions on 7.5% gels and stained. For tracks 6–10 the enzyme was reactivated by reduction prior to electrophoresis. The molar ratio of OBMTS to subunit concentration for each track was as follows: tracks 1 and 6, 0.3; tracks 2 and 7, 0.6; tracks 3 and 8, 0.9; tracks 4 and 9, 1.2; tracks 5 and 10, 2.0.

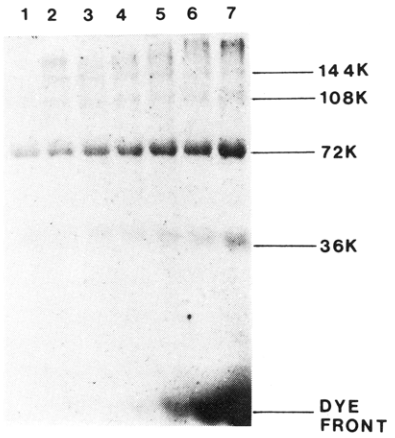


FIGURE 2: Autoradiograph of [^{35}S]OBMTS cross-linked G3PD. The enzyme was cross-linked with increasing [^{35}S]OBMTS concentration and electrophoresed under nonreducing conditions followed by autoradiography. The molar ratio of OBMTS per subunit was as follows: track 1, 1.0; track 2, 1.5; track 3, 2.0; track 4, 3.0; track 5, 4.0; track 6, 8.0; track 7, 16.

within the time required to manually mix the enzyme with excess inhibitor. The extent of the inhibition observed was 15%, 27.1%, 53%, 75%, 96%, and 100% following the addition of 0.032, 0.068, 0.13, 0.25, 0.5, and 1.0 mol of inhibitor/mol of subunit, respectively. The extent of inhibition was identical for both the nonradioactive and ^{35}S-labeled inhibitors. This pattern of inhibition has been reported earlier (Bloxham & Sharma, 1979; see their Figure 5).

The inhibited enzyme was analyzed by gel electrophoresis in the presence of $NaDodSO_4$ under nonreducing conditions (Figure 1). Part of the enzyme was reactivated by reduction prior to the analysis (Figure 1, tracks 6–10). A dimer, M_r 72 000, was the major product of cross-linking, and maximum conversion of monomer to dimer (95% yield of dimers) required at least 1 mol of inhibitor/mol of subunit. From the results on inhibition of enzyme activity and Figure 1, we can deduce that the chemical modification leading to inhibition, presumably by thioalkylation of the essential Cys-149 (Harris et al., 1963), preceded modification leading to cross-linking.

The availability of the ^{35}S-labeled inhibitor means that enzyme cross-linking can also be monitored by autoradiography. Modifying the enzyme with increasing molar excesses of OBMTS up to a large excess of inhibitor yielded the dimer as the major radioactive product, although traces of higher molecular weight products were also identified (Figure 2). The extent of the radioactive labeling of the dimer, as judged by the intensity of the labeled band, increased as the molar ratio of inhibitor/subunit was raised above 1. This must result

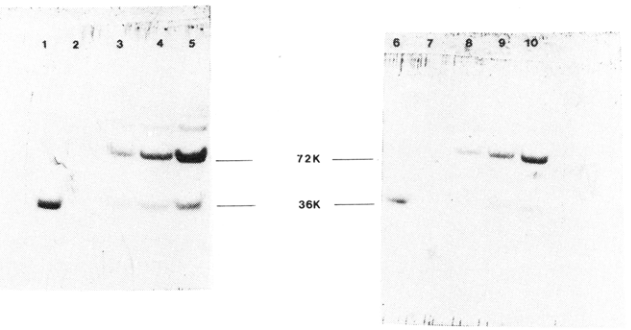


FIGURE 3: Reduction of polymethylene bis(disulfide) links in G3PD. The enzyme was modified with [^{35}S]OBMTS (tracks 1–5) and part was reactivated by reduction under nondenaturing conditions (tracks 6–10). The following reduction conditions were used when samples were denatured for $NaDodSO_4$ gel electrophoresis: tracks 3–5 and 8–10, nonreducing; tracks 1 and 6, 1% (v/v) β-mercaptoethanol at 80 °C for 2 min; tracks 2 and 7, 1% (v/v) β-mercaptoethanol at 110 °C and 15 psi for 60 min. The results show the autoradiograph of 7.5% gels. The results in tracks 3–5 and 8–10 represent increased protein loading of 5, 10, and 25 μg.

from the slower reaction of the remaining thiols in G3PD. Presumably these thiols are spaced at such a distance that further cross-linking reactions are prohibited. Consistent with the modification of further thiols, unreacted inhibitor identified at the dye front of gels (Figure 2) was not detected in significant amounts until the molar ratio of inhibitor/subunit exceeded 3. This is close to the number of four cysteine residues per subunit (Harris & Perham, 1968). Less than 4 mol of inhibitor/mol of subunit is expected at complete modification since the bifunctional compound forms at least two cross-links.

Reduction of [^{35}S]OBMTS Cross-Linked G3PD. The known chemistry of the reaction of thiols with thiosulfonates (Scheme I) predicts that the formation of a cross-link should involve the formation of two apparently equivalent disulfide bonds. We would expect these to be equally susceptible to reductive cleavage. Cross-linked G3PD was reduced in three separate ways to investigate this. First, the enzyme was reduced with 5 mM dithioerythritol under nondenaturing conditions. This led to the complete recovery of catalytic activity (Bloxham & Sharma, 1979). Second, both the inhibited cross-linked and active cross-linked enzymes were reduced in the presence of 1% $NaDodSO_4$ and 1% β-mercaptoethanol at 80 °C for 2 min. Third, the enzyme was reduced under vigorous denaturing conditions at 110 °C and 15 psi. The autoradiography of the resultant gels is shown in Figure 3. Nondenaturing mild reduction, which results in enzyme reactivation, leaves the cross-link intact (Figure 3, tracks 8–10). However the intensity of the radioactive dimer is halved (shown quantitatively in Figure 4), consistent with the removal of the group blocking the active-site thiol, cysteine-149. This shows that reduction preferentially removes the disulfide bond which involves the proteins most reactive thiol group (Harris et al., 1963).

When the protein was reduced under strongly denaturing conditions, then the dimer was completely reversed, and all traces of radioactivity were removed from the M_r 36 000 region (Figure 3, tracks 2 and 7). In contrast, if the reduction was performed under milder denaturing conditions (80 °C for 2 min), then the dimer was completely reversed; however, radioactivity was still present in the monomer (Figure 3, tracks 1 and 6).

The results for autoradiography in this section were also checked quantitatively by analyzing the distribution between monomer and dimer following slicing and treatment of the gel

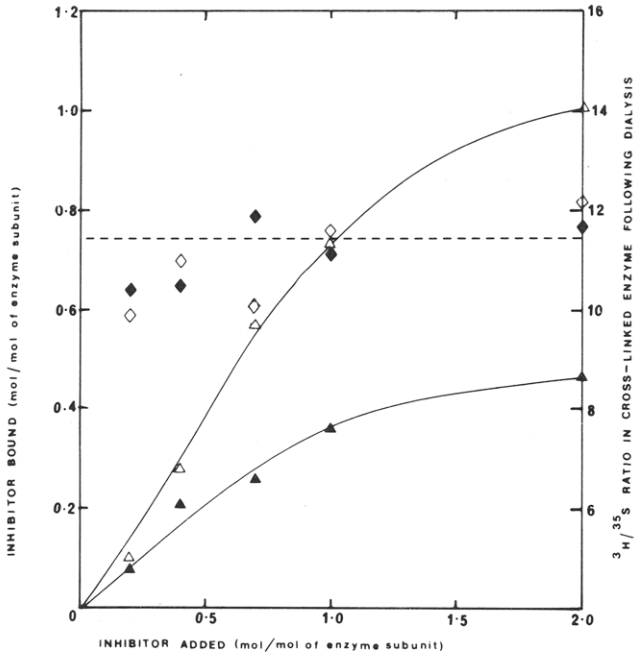


FIGURE 4: Covalent modification of G3PD by [^{3}H,^{35}S]OBMTS ($^3H/^{35}S$ = 11.4). Open and closed symbols are for inhibited and reactivated G3PD, respectively. (△, ▲) Inhibitor bound after exhaustive dialysis; (◇, ◆) $^3H/^{35}S$ ratio of bound inhibitor. The dashed line shows the $^3H/^{35}S$ ratio of free inhibitor.

with NCS tissue solubilizer. Under mild denaturing reducing conditions then at least 50% of the radioactivity remained in the monomer following reductive cleavage of the dimer. The result was observed with either the inactive cross-linked enzyme or the reduced active cross-linked enzyme.

The preceding results raise the possibility that the two disulfide bonds involved in the cross-link are reduced at very different rates or alternatively that cross-linking might involve a more complex chemical reaction than that predicted in Scheme I. G3PD was reacted with various molar amounts of [^{3}H,^{35}S]OBMTS ($^3H/^{35}S$ ratio = 11.4) to varify that cross-linking involves the incorporation of both ^{35}S atoms and all the methylene groups of OBMTS. Both the amount of inhibitor bound and the $^3H/^{35}S$ ratio were measured (Figure 4). Irrespective of the amount of inhibitor bound, the $^3H/^{35}S$ ratio was reasonably constant and virtually identical with the value of the free inhibitor. This result confirms that cross-linking must proceed by the formation of two disulfide bonds (Scheme I). In addition Figure 4 shows that reactivation of the enzyme by reduction under nondenaturing conditions does not alter the $^3H/^{35}S$ ratio although it does decrease the amount of inhibitor bound. In the fully cross-linked active enzyme, there was ~0.8 mol of inhibitor bound per 2 mol of subunit. The retention of radioactivity on reduction is a clear illustration of the difference in susceptibility of disulfide bonds to reduction.

Demonstration That Cys-281 Participates in Subunit Cross-Linking. The complete sequence of G3PD from pig muscle has been determined, and tryptic peptide mapping showed the sequence to be strictly homologous with the rabbit muscle enzyme (Harris & Perham, 1968). On the basis of this sequence, cyanogen bromide cleavage of rabbit muscle G3PD would yield 10 peptides, the largest of which (residues 229–325, M_r 10700) would contain residue 281. The next largest contains residues 44–102 (M_r 6400) and is readily distinguishable on electrophoresis. Any peptides containing a homo-cross-link (i.e., between two identical cysteine residues) would have twice the expected molecular weight and give a

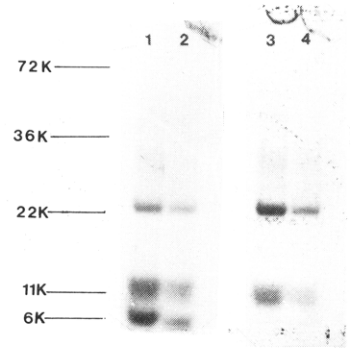


FIGURE 5: Cyanogen bromide digest of active ^{35}S-labeled cross-linked G3PD. The enzyme was cross-linked with 1.1 mol of [^{35}S]-OBMTS/mol of subunit and reactivated with dithioerythritol. After CNBr digestion, the products were electrophoresed under nonreducing conditions using 15% polyacrylamide gels. Tracks 1 (50 μg) and 2 (20 μg) show the protein staining bands from duplicate digests, and tracks 3 and 4 show the corresponding autoradiogram.

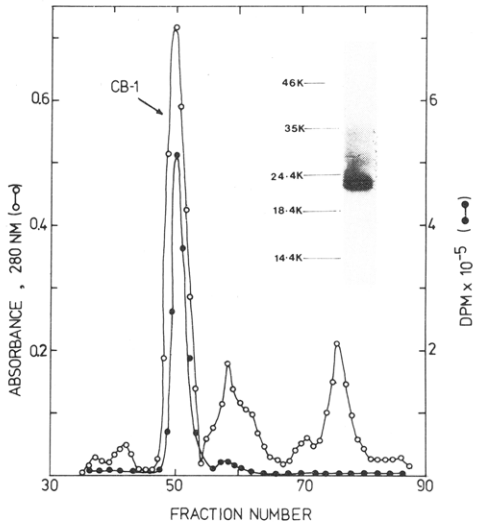


FIGURE 6: Isolation of [^{35}S]OBMTS cross-linked peptide. The CNBr digest of cross-linked reactivated G3PD (30 mg) was chromatographed on Sephadex G-50 in 9% formic acid. The insert shows a 15% gel of fraction CB-1. Both stain and autoradiography were identical.

single peptide on reduction. Cyanogen bromide digestion of cross-linked G3PD showed three protein staining bands on gel electrophoresis with M_r 22000, 11000, and 6000 (Figure 5). Autoradiography revealed that the two largest polypeptides contained radioactivity. The M_r 22000 peptide was absent in digests of native G3PD.

Furthermore reduction of the digestion products from the cross-linked protein prior to electrophoresis resulted in the disappearance of the M_r 22000 polypeptide, and radioactivity was only associated with the M_r 11000 polypeptide. As pointed out earlier, mild reduction would still leave ^{35}S in the peptide if only one of the two disulfide bonds in the cross-link was reduced.

The M_r 22000 polypeptide (CB-1) was isolated by gel filtration on Sephadex G-50 superfine in 9% formic acid (Figure 6). Most (95%) of the radioactivity was associated with a single peptide fraction. NaDodSO$_4$–polyacrylamide gel electrophoresis of CB-1 confirmed the molecular weight as 22000, which upon reduction with β-mercaptoethanol was converted to a radioactive M_r 11000 polypeptide. For removal of all the radioactivity from CB-1, reduction with β-mercaptoethanol in an autoclave for 1 h at 15 psi was necessary. Following carboxymethylation of CB-1, its amino-terminal sequence was identified as Ala-Phe-Arg-Val-Pro-Thr-Pro-

Table I: Amino Acid Analysis[a] of Cyanogen Bromide Fragment 229-325[b] (CB-1) from Rabbit Muscle G3PD

amino acid	required from pig muscle sequence	found
Cys	2	1.03[c]
Asx	15	14.75
Thr	5	5.4
Ser	8	7.62
Glx	6	6
Pro	4	3.49
Gly	6	7.8
Ala	6	7.8
Val	11	10.7
Met	1	0.98[d]
Ile	4	4.5
Leu	7	7.05
Tyr	4	3.52
Phe	5	5.1
His	2	2.5
Lys	7	7.1
Arg	3	2.95

[a] Based on hydrolysis times of 24, 48, and 96 h. [b] The ^{35}S cross-linked peptide from CNBr digestion (Figure 8; CB-1) was reduced and carboxymethylated prior to analysis. [c] Cysteine was determined as *S*-(carboxymethyl)cysteine. [d] Methionine was determined as homoserine + homoserine lactone.

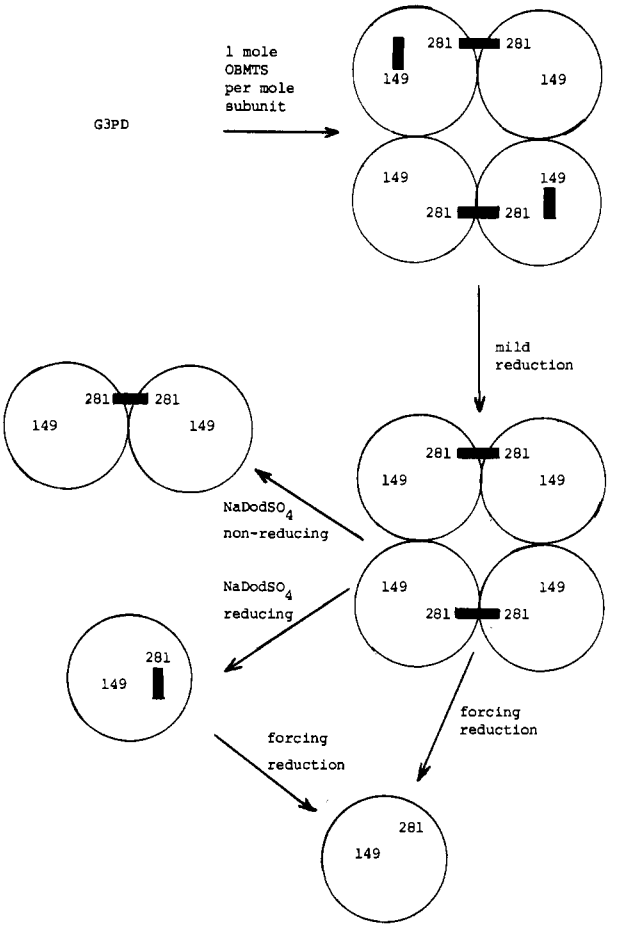
Asn-Val- which corresponds to the sequence of residues 229–237 in pig muscle G3PD. Therefore the isolated cross-linked peptide corresponds to the cyanogen bromide peptide from residues 229–237. This was confirmed by comparison of the theoretical amino acid composition of this region with the amino acid analysis of reduced carboxymethyl-CB-1 (Table I). The analysis shows only half the cysteine was present as carboxymethylcysteine. This agrees with the fact that thiol groups not involved in the cross-link, namely, cysteine-244, were blocked with *N*-ethylmaleimide. Therefore the carboxymethylcysteine analysis confirms that there is only one intersubunit cross-link.

The sequence 229–325 contains two cysteine residues at positions 244 and 281 so that either of these might participate in the cross-link. CB-1 was subdigested with trypsin to distinguish between these two possibilities. Tryptic digestion of the 229–325 peptide at arginine and lysine residues would yield 11 peptides, with the largest (residues 269–306) of M_r 4000 (cross-linked M_r 8000) containing cysteine residue 281. When the products of tryptic digestion of CB-1 were resolved by gel filtration using the conditions in Figure 6, then a radioactive peptide was isolated with an approximate molecular weight of 8000 by $NaDodSO_4$ gel electrophoresis. This radioactive peptide disappeared on reduction and carboxymethylation. Its amino-terminal sequence was identified as Gly-Ile-Leu-Gly-Tyr- which corresponds to the sequence of residues 269–273. This result demonstrates unambiguously that the cross-link must form between two cysteine-281 residues.

Discussion

The current work shows that bifunctional thiosulfonates provide an important additional class of compounds for studying spatial relationships between pairs of thiol groups (Wold, 1972). Although compounds such as the cupric bis-(1,10-phenanthroline) complex (Kobashi & Horecker, 1967) and bifunctional maleimides (Burke & Reisler, 1977; Weiss & McCarty, 1977; Pilch & Czech, 1979) can also be used for this purpose, the bifunctional thiosulfonates have a number of useful features. The reagents are readily synthesized with varying polymethylene chain lengths which are vital for probing intersulfhydryl distances. Most importantly since

Scheme II



OBMTS spans two Cys-281 residues in G3PD corresponding to a crystallographic distance of 1.5–1.77 nm (Moras et al., 1975), this should serve as a reasonable guide for the spanning distance of the reagent with other systems not amenable to crystallography.

The attack of sulfide on dicoordinate sulfur is generally very facile, so it is anticipated that modification of protein thiols by thiosulfonates will proceed very rapidly. We estimate that the thiosulfonate OBMTS is 10^3–10^4 times more reactive than the related chloromethyl ketone bis(chloromethyl)hexanedione (Bloxham, 1977).

The bifunctional reagents have now been synthesized with ^{35}S located in the thiosulfonate so that all of the radioactivity is retained in the cross-link. This allows identification of cross-linked peptides by autoradiography. Furthermore since reduction in $NaDodSO_4$/β-mercaptoethanol at 80 °C for 2 min completely reduces one of the disulfide bonds in the cross-link but leaves a substantial proportion of the second disulfide intact, this means that reacted proteins can be identified following reduction. This could be especially useful in diagonal mapping since cross-linked peptides could be identified in their off-diagonal position by autoradiography. Finally the polymethylene disulfide cross-link is stable to both cyanogen bromide and tryptic cleavage, and this allows the residue modified to be identified with reasonable precision. This is an important though frequently neglected part of the cross-linking experiment.

The analysis of the reacted thiol groups in G3PD and their susceptibility to reduction can now be described in detail (Scheme II). Addition of 1 mol of OBMTS/mol of subunit completely inhibits the enzyme and cross-links the enzyme to the dimer. Inhibition must result from modification of two

Cys-149 residues per tetramer since it is this thiol which is most reactive (Harris et al., 1963) and forms the acyl thioester during catalysis (Krimsky & Racker, 1955; Seydoux et al., 1973). Cross-linking results from the modification of all four Cys-281 residues per tetramer. Removal of the disulfide bond involving the active-site Cys-149 residue and the inhibitor was easily achieved by mild reduction under nondenaturing conditions. This led to reactivation of the enzyme while the intersubunit cross-link remained intact. The intersubunit cross-link between the Cys-281 pair is virtually completely stable to reduction under nondenaturing conditions, suggesting steric shielding of the new disulfides. Once the enzyme was denatured with $NaDodSO_4$, the disulfide bonds in the intersubunit cross-link were reduced. However, ^{35}S-labeled inhibitor was still attached to the monomer, showing that the two disulfide bonds in the cross-link were reduced at quite different rates. To account for this unusual chemical stability in the denatured protein–inhibitor complex, we propose that the polymethylene chain of the inhibitor molecule once released from its taut (linking) configuration enfolds the final disulfide bond to form a hydrophobic cell affording protection from attacking nucleophiles. Forcing reducing conditions must be employed to overcome this effect.

References

Bloxham, D. P. (1977) *Biochem. J. 167*, 201–210.

Bloxham, D. P., & Sharma, R. P. (1979) *Biochem. J. 181*, 355–366.

Bloxham, D. P., Sharma, R. P., & Wilton, D. C. (1979) *Biochem. J. 177*, 769–780.

Botts, J., Ile, K., Hozumi, T., & Samet, J. (1979) *Biochemistry 18*, 5157–5163.

Burke, M., & Reisler, E. (1977) *Biochemistry 16*, 5559–5563.

Cherednikova, T. V., Muronetz, V. I., & Nagradova, N. K. (1980) *Biochim. Biophys. Acta 613*, 292–308.

Collier, G. E., & Nishimura, J. S. (1979) *J. Biol. Chem. 254*, 10925–10930.

Crestfield, A. M., Moore, S., & Stein, W. H. (1963) *J. Biol. Chem. 238*, 622–627.

Dandliker, W. B., & Fox, J. R. (1955) *J. Biol. Chem. 214*, 275–283.

Dombrâdi, V., Hajdu, J., Bot, G., & Freiderich, P. (1980) *Biochemistry 19*, 2295–2299.

Edman, P., & Begg, G. (1967) *Eur. J. Biochem. 1*, 80–91.

Fahrney, D., & Gold, A. M. (1963) *J. Am. Chem. Soc. 85*, 997–1000.

Ferdinand, W. (1964) *Biochem. J. 92*, 578–585.

Gross, E., & Witkop, B. (1962) *J. Biol. Chem. 237*, 1856–1860.

Harris, J. I., & Perham, R. N. (1965) *J. Mol. Biol. 13*, 876–884.

Harris, J. I., & Perham, R. N. (1968) *Nature (London) 219*, 1025–1028.

Harris, J. I., Merriwether, B. P., & Park, J. H. (1963) *Nature (London) 198*, 154–157.

Henriques, F., & Park, R. B. (1978) *Arch. Biochem. Biophys. 189*, 44–50.

Hermann, J., Titani, K., Ericsson, L. H., Wade, R. D., Neurath, H., & Walsh, K. A. (1978) *Biochemistry 17*, 5672–5679.

Huang, C.-K., & Richards, F. M. (1977) *J. Biol. Chem. 252*, 5514–5521.

Kobashi, K., & Horecker, B. L. (1967) *Arch. Biochem. Biophys. 121*, 178–186.

Koide, A., Titani, K., Ericsson, L. H., Kumar, S., Neurath, H., & Walsh, K. A. (1978) *Biochemistry 17*, 5657–5672.

Krimsky, I., & Racker, E. (1955) *Science (Washington, D.C.) 122*, 319–321.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Maggio, E. T., Kenyon, G. L., Markham, G. D., & Reed, G. H. (1977) *J. Biol. Chem. 252*, 1202–1207.

Moras, D., Olsen, K. W., Sabesan, M. N., Buehner, M., Ford, G. C., & Rossmann, M. G. (1975) *J. Biol. Chem. 250*, 9137–9162.

Nishimura, J. S., Kenyon, G. L., & Smith, D. J. (1975) *Arch. Biochem. Biophys. 170*, 461–467.

Osborne, H. H., & Hollaway, M. R. (1974) *Biochem. J. 143*, 651–662.

Osborne, H. H., & Hollaway, M. R. (1975) *Biochem. J. 151*, 37–45.

Peretz, H., & Elson, D. (1976) *Eur. J. Biochem. 63*, 77–82.

Peretz, H., Towbin, H., & Elson, D. (1976) *Eur. J. Biochem. 63*, 83–92.

Peters, K., & Richards, F. M. (1977) *Annu. Rev. Biochem. 46*, 523–551.

Pilch, P. F., & Czech, M. P. (1979) *J. Biol. Chem. 254*, 3375–3381.

Seydoux, F., Bernhard, S., Pfennig, O., Payne, M., & Malhotra, P. (1973) *Biochemistry 12*, 4290–4300.

Smith, D. J., Maggio, E. T., & Kenyon, G. L. (1975) *Biochemistry 14*, 766–771.

Sun, T. T., Bollen, A., Kahan, L., & Traut, R. R. (1974) *Biochemistry 13*, 2334–2340.

Traut, R. R., Bollen, A., Sun, T. T., Hershey, J. W. B., Sundberg, J., & Pierce, L. R. (1973) *Biochemistry 12*, 3266–3273.

Wang, K., & Richards, F. M. (1974) *J. Biol. Chem. 249*, 8005–8018.

Weiss, M. A., & McCarty, R. E. (1977) *J. Biol. Chem. 252*, 8007–8012.

Witzemann, V., Muchmore, D., & Rafferty, M. A. (1979) *Biochemistry 18*, 5511–5518.

Wold, F. (1972) *Methods Enzymol. 25*, 623–651.

Wooton, J. C., Taylor, J. G., Jackson, A. A., Chambers, G. K., & Fincham, G. S. (1975) *Biochem. J. 149*, 739–748.