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SH GROUPS MASKED BY SUBUNIT INTERACTION IN GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE

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SUMMARY

The changes in reactivity of the less reactive SH groups of glyceraldehyde-3-phosphate dehydrogenase (D-glyceraldehyde-3-phosphate:NAD^+ oxidoreductase (phosphorylating) EC 1.2.1.12) apoenzyme towards thiol reagents were studied during the course of dissociation into dimers and inactivation of the enzyme induced by stoichiometric amounts of ATP. Neither the reactive SH group of Cys-149 nor that of Cys-153 (the latter being the second cysteinyl group in order of reactivity towards thiol reagents) is ozidized during dissociation and inactivation caused by ATP treatment. However, during this process one SH group per subunit which was previously masked in the native tetramer is oxidized.

This cysteinyl residue corresponds to Cys-281 as identified analytically. The molecular weight of the ATP-treated enzyme is 60 000 even in the presence of 6 M urea. Both enzymatic activity and the oxidized SH group can be fully recovered by treatment with 2-mercaptoethanol. We conclude that during dissociation into dimers by ATP, residues Cys-281 form interchain S–S bridges within the dimers.

INTRODUCTION

Muscle glyceraldehyde-3-phosphate dehydrogenase (D-glyceraldehyde 3-phosphate:NAD^+ oxidoreductase (phosphorylating) EC 1.2.1.12) consists of four sequentially identical subunits and contains four cysteinyl residues per subunit[1]. One of the SH groups, that located in the active center of the enzyme, Cys-149, is extremely reactive[2,3] (SH-I). Another cysteinyl residue (SH-II) reacts with *p*-chloromercuribenzoate (PCMB) and with other SH reagents after modification of Cys-149, faster than the remaining two. The kinetic behaviour of SH-II can be explained in terms of a fluctuating protein model and it has been proposed that SH-II corresponds to residue Cys-153 (ref. 4). In the presence of 2 M NaCl these 2 reactive SH groups

Abbreviations: DTNB, 5,5′-dithio-bis-(2-nitrobenzoate); MNB, 5-mercapto-2-nitrobenzoate; PCMB, *p*-chloromercuribenzoate; CM-Cys, carboxymethyl cysteinyl group.

(SH-I and SH-II) can be selectively titrated with 5,5'-dithio-bis-(2-nitrobenzoate) (DTNB)[5]. The other 2 SH groups of the enzyme, denoted as SH-III and SH-IV, are buried in the molecule and become unmasked in 6 M urea or following the structural change induced by the blocking of SH-I and SH-II[4–9].

In a previous communication we have reported that glyceraldehyde-3-phosphate dehydrogenase is dissociated into dimers and inactivated after binding of stoichiometric amounts of ATP[10]. During these processes the SH group of Cys-149 was not affected, as tested by the formation of the charge-transfer complex between NAD and the ATP-treated enzyme[11]. However, the degree of inactivation by ATP depended on whether 2-mercaptoethanol was present or not. Difference spectrophotometric studies suggested that dissociation into dimers caused by ATP was not accompanied by the unfolding of the molecule[10].

In the present paper we provide evidence for the formation of intersubunit disulfide bridges by SH groups which were masked in the native tetramer prior to the addition of stoichiometric amounts of ATP. Cys-281 may participate in the formation of the contact surface of the dimers within the tetrameric molecule.

MATERIALS AND METHODS

Four times recrystallized swine muscle glyceraldehyde-3-phosphate dehydrogenase[12] was used.

D-Glyceraldehyde 3-phosphate was prepared from fructose 1,6-diphosphate (Reanal) according to Szewczuk *et al.*[13]. ATP was either a Sigma or Reanal product. NAD was obtained from Reanal. DTNB was a Fluka preparation. PCMB was a preparation of British Drug Houses. Bromo[^{14}C]acetic acid was procured from Reanal (spec. act.: 1.22 Ci/mole). The other chemicals were commercial preparations of reagent grade.

Enzyme solutions were freshly prepared. The crystals were dissolved in Tris–HCl buffer, pH 8.5. All experiments described in this paper were carried out with apoenzyme. Firmly bound NAD was removed by charcoal treatment, which increased the absorbance ratio $A_{280\text{ nm}}/A_{260\text{ nm}}$ from 1.05 to 1.95.

10 μM apoenzyme in Tris–HCl buffer, pH 8.5, was treated with 40 μM of ATP adjusted to the same pH. The solution was kept at 0 °C in an ice bath. The apoenzyme treated in a similar manner in the absence of ATP served as control.

Protein concentration was determined spectrophotometrically at 280 nm, with $E^{0,1\%}_{1\text{ cm}} = 0.9$ for the apoenzyme.

Enzymatic activity with D-glyceraldehyde 3-phosphate as substrate was determined by the optical test as described previously[14]. The specific activity of the preparation was 15–20 kmoles NADH produced·min^{-1}·mole enzyme^{-1}. The molecular weight of the tetramer was taken to be 145 000 (ref. 15).

The number of SH groups was determined with Ellman's reagent[16] in the presence of 6 M urea or 2 M NaCl atp H 8.0. Calculations were based on a molar extinction coefficient for mercaptonitrobenzoate of 13 600 (ref. 16).

The kinetics of mercaptide formation were measured in 0.1 M Tris–HCl buffer, pH 8.5, by adding 2, 3 and 4 moles of PCMB per mole of subunit to 1.25 μM enzyme. The number of mercaptide bonds formed was calculated by Boyer's method[17] ($A_{255\text{ nm}} = 6000$).

The [^{14}C]carboxymethylation was carried out as follows: 10 μM enzyme solutions were treated with ATP for 10 min as described above. Then $4 \cdot 10^{-3}$, $8 \cdot 10^{-3}$, $1 \cdot 10^{-2}$ and $1.6 \cdot 10^{-1}$ M bromo-[^{14}C]acetate was added to the samples and incubation further proceeded. At appropriate times 2-ml aliquots were gel filtered on a Sephadex G-50 column (2 cm $\times$ 30 cm). Fractions of 1.5 ml were collected. Radioactivity was measured in Packard Tri-carb 2420 spectrometer.

Identification of [^{14}C]carboxymethylated peptides: Apoenzyme, 10 μM, was treated with 40 μM ATP and without ATP for 10 min. Residue Cys-149 was blocked with unlabelled bromoacetate. The radioactive bromoacetate was added (final concentration 0.01 M) and the samples were kept at 0 °C for 5 h. Under these conditions carboxymethylation of only cysteinyl residues could be detected. Carboxymethylation was stopped by addition of 0.5 M 2-mercaptoethanol. The [^{14}C]CM-Cys ([^{14}C]carboxymethylcysteinyl group) enzyme samples were dialysed against 1 mM HCl and were digested at pH 8.0 with 1% trypsin (Calbiochem) at 37 °C for 2 h and the digest was freeze-dried. Peptide maps were prepared by two-dimensional electrophoresis at pH 6.5 and 1.9, and the pattern of radioactive peptide was revealed by radioautography.

The spectrophotometric measurements were carried out in an Opton PMQ II spectrophotometer.

RESULTS AND DISCUSSION

Effect of ATP treatment on the SH groups

During ATP treatment some of the SH groups of glyceraldehyde-3-phosphate dehydrogenase become non-titratable with DTNB or PCMB. Apoenzyme was incubated in the presence and absence of 1 mole of ATP per mole of subunit in Tris–HCl buffer, pH 8.5, at 0 °C. Under these conditions ATP is bound to the enzyme within 10 min[10] and dissociation into dimers and inactivation take place during incubation. At different times of incubation the number of SH groups was determined with DTNB in the presence of 6 M urea.

As seen in Fig. 1 during ATP treatment the number of SH groups decreases more rapidly than in the control experiment, probably due to the oxidation of some SH groups. The loss in the number of SH groups caused by ATP treatment, the spontaneous oxidation taken into account, follows simple first-order kinetics with a rate constant of $3.8 \cdot 10^{-1}$ h^{-1}.

In order to test whether the reactive SH groups or some of the buried SH groups were oxidized, the SH-I and SH-II content was determined separately in the presence of 2 M NaCl. As shown in Fig. 1, on incubation of the enzyme either in the presence or in the absence of ATP there is only a slight decrease in the number of reactive SH groups. This decrease practically equals the loss in the total number of SH groups of the untreated enzyme. These data suggest that during ATP treatment, due to the dissociation into dimers, half of the previously buried SH groups of the enzyme become unmasked and oxidized.

As Fig. 2 shows, in the control experiment the enzymatic activity and the number of reactive SH groups decrease with the same rate which is to be expected since SH-I is carried on residue Cys-149 located in the active center of the enzyme[18].

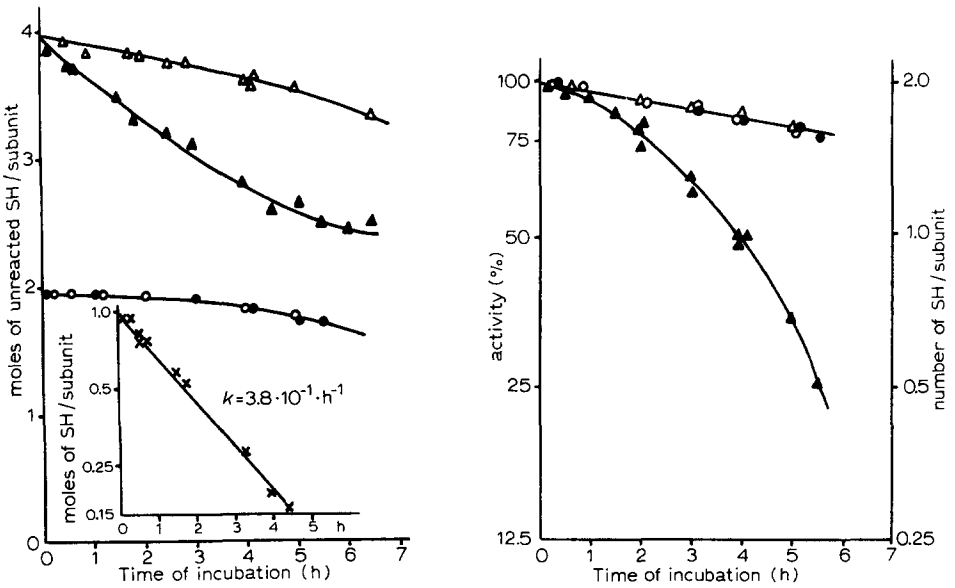

Fig. 1. Effect of ATP treatment on the loss of SH groups of glyceraldehyde-3-phosphate dehydrogenase. Apoenzyme (10 μM) was incubated in the presence and absence of 1 mole of ATP per mole of subunit in 0.1 M Tris–HCl buffer, pH 8.5, at 0 °C. The SH groups were titrated with DTNB at the times indicated on the abscissa. Titration in 6 M urea: incubation in the absence (△) and presence (▲) of ATP. Titration in the presence of 2 M NaCl: incubation in the absence (○) and presence (●) of ATP. Insert: Semilogarithmic plot of the time course of SH loss due to ATP treatment (control values substracted) as measured in 6 M urea.

Fig. 2. Kinetics of ATP-induced inactivation and oxidative loss of SH-I and SH-II. Conditions of ATP treatment as in legends to Fig. 1. The sum of SH-I and SH-II was determined by titration with DTNB in the presence of 2 M NaCl. Enzymatic activity during incubation in the absence (△) and presence (▲) of ATP. Sum of SH-I and SH-II during incubation in the absence (○) and presence (●) of ATP.

However, inactivation during ATP treatment is a more rapid process than is the loss in the reactive SH groups.

On the basis of these data we assume that inactivation caused by ATP treatment may be related to the oxidation of the SH groups which are buried in the tetrameric form.

To obtain more evidence whether SH-I or SH-II is involved in the ATP-induced inactivation process we tested the accessibility of the SH groups also by PCMB. Glyceraldehyde-3-phosphate dehydrogenase was incubated with ATP for 5 h and the kinetics of mercaptide bond formation were determined after the addition of 2, 3 and 4 moles of PCMB per mole of subunit, respectively (Fig. 3).

On addition of 2 moles of PCMB per mole of subunit the availability of SH-I and SH-II is the same in the control and in the ATP-treated enzyme. In both cases the kinetics follow the expected pattern[4]. The same was observed if titration was carried out with 3 moles of PCMB per subunit. If, however, 4 moles of PCMB per mole of subunit were added to the enzyme, after preincubation for 5 h with ATP only 3 mercaptide bonds per subunit were formed. On the other hand, in the control experiment all the 4 SH groups of the subunits could be titrated, in agreement with

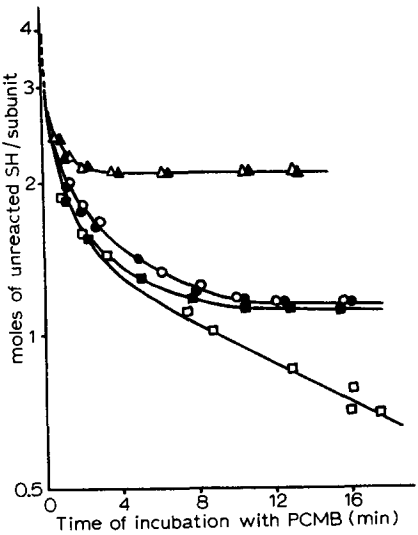

Fig. 3. Accessibility of the SH groups of ATP-treated glyceraldehyde-3-phosphate dehydrogenase to PCMB. Apoenzyme was incubated with and without ATP for 5 h under the conditions described in the legends to Fig. 1. The kinetics of mercaptide bond formation were followed with a 1.25 μM enzyme solution in 0.1 M Tris–HCl buffer, pH 8.5, at 0 °C, after the addition of 2 (△, ▲), 3 (○, ●) and 4 (□, ■) moles of PCMB per mole subunit. Open symbols: control; closed symbols: ATP-treated enzyme.

the data in the literature[6,9]. This further suggests that during ATP treatment there is practically no loss in either SH-I or SH-II. Treatment with ATP should effect the oxidation of SH-III and/or SH-IV, the sulfhydryl groups that are buried in the native tetramer and become exposed on dissociation.

What is the oxidation product of the SH groups thus exposed? If the vanished SH groups and the enzyme activity can be recovered after addition of 2-mercaptoethanol, we have evidence for the formation of S–S bridges. Therefore an excess of 2-mercaptoethanol was added to the enzyme which was previously practically fully inactivated and lost about 1.3 moles of cysteinyl residue per subunit. After about 2 h of incubation the sample was gel filtered on a Sephadex G-50 column and the enzymatic activity as well as the amount of titratable SH groups in 6 M urea were determined. We found that the reactivation was complete (100%) and about 3.7 moles of SH groups per subunit could be recovered. It should be mentioned that ATP remained bound to the enzyme. On the basis of these results, we assume that oxidation resulted in S–S bridge formation and, furthermore, that on the action of 2-mercaptoethanol the inactive dimeric form was transformed into an active form. A slow loss of the SH content similar to that measured in the basic experiment (*cf.* Fig. 1) could be detected in the reactivated enzyme after separation from 2-mercaptoethanol which further suggests the reassociation of dimers into tetramers.

Effect of ATP on the accessibility of SH groups as measured by [^{14}C]carboxymethylation

The transformation of the previously buried SH groups into S–S bridges seems to be a very rapid reaction and the oxidation of the SH groups should be limited by

their exposure. Since we assumed that the unmasking of these SH groups was connected with the ATP-induced dissociation into dimers, we studied the time course of the exposure of SH groups. To prevent the oxidation of free SH groups, and of those which become unmasked, the ATP-induced dissociation was carried out in the presence of such a great excess of bromo[^{14}C]acetate that alkylation be a more rapid reaction than oxidation.

Samples of apoenzyme in 10 μM concentration were incubated in the presence of 1 mole of ATP per mole of subunit and without ATP for 10 min. During this period ATP is already bound to the enzyme, but dissociation is a much slower process[10]. After 10 min $4 \cdot 10^{-3}$, $8 \cdot 10^{-3}$, $1 \cdot 10^{-2}$ and $1.6 \cdot 10^{-2}$ M bromo[^{14}C]acetate was added, and incubation further proceeded. At different times of incubation an aliquot was gel filtered to separate free bromoacetate. The amount of bound [^{14}C]carboxymethyl groups was determined.

The effect of ATP treatment on the velocity of carboxymethylation of the SH groups is illustrated in Fig. 4, after the addition of 0.01 M bromo[^{14}C]acetate to the sample. At different reagent concentrations no difference was found in the rate constant of carboxymethylation, which indicates that the process is limited by a first-order reaction. It has been shown that in the native enzyme only Cys-149 can be rapidly carboxymethylated at pH 8.5 and 0 °C. Since we found that 1 SH group per subunit is alkylated within a few minutes both in the control and in the ATP-treated enzyme, we assume that this SH group belongs to Cys-149.

In the control experiment the rapid reaction of Cys-149 is followed by a slow first-order reaction with rate constant of $1.8 \cdot 10^{-2} \cdot h^{-1}$. If alkylation is carried out during ATP treatment a biphasic reaction occurs, the first order rate constants of the

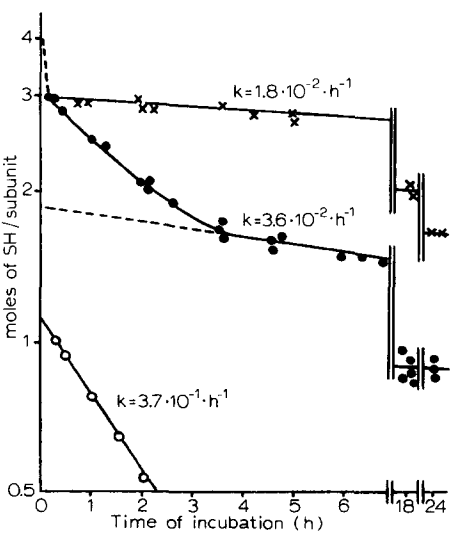

Fig. 4. Effect of ATP on the accessibility of SH groups of glyceraldehyde-3-phosphate dehydrogenase to bromoacetate. Experimental conditions as in legends to Fig. 1. After 10 min of incubation with and without ATP 0.01 M bromo[^{14}C]acetate was added to the samples, and incubation continued. For the determination of bound [^{14}C]carboxymethyl groups (see Methods). ×, control; ●, ATP-treated enzyme; ○, the fast part of the biphasic kinetics of alkylation of the ATP-treated enzyme.

2 processes being $3.6 \cdot 10^{-2} \cdot h^{-1}$ and $3.7 \cdot 10^{-1} \cdot h^{-1}$, respectively. Since in the native enzyme the second most reactive group towards DTNB and PCMB is SH-II it is to be expected that the slow process of alkylation of the native enzyme reflects the reaction of SH-II with the reagent. Furthermore, we assume that the slow part of the biphasic reaction which takes place if alkylation is carried out during ATP treatment also reflects the reaction of SH-II, because the rate constants are rather similar. In fact, as it will be shown analytically below, if samples were incubated for 5 h with bromo[^{14}C]acetate with and without ATP, a distinct label was found in the same CM-Cys peptide of both enzyme samples. This suggests that the same cysteinyl residues are alkylated during the slow processes.

In addition to the alkylation of Cys-149 and SH-II, during ATP treatment one further cysteinyl residue per subunit is alkylated but much more rapidly, $k = 3.7 \cdot 10^{-1} \cdot h^{-1}$. The rate constant of alkylation agrees well with that of the oxidative loss of SH groups (*cf.* inset of Fig. 1). Therefore we assume that this 1 mole of alkylated cysteine represents Cys-III and/or Cys-IV, *i.e.* the cysteinyl residues which in the native tetramer are buried and become accessible to the reagent during the ATP-induced dissociation.

As also seen in Fig. 4 there is 1 SH per subunit which is not alkylated even after prolonged incubation with bromoacetate in the presence of ATP. In order to test whether the SH groups which are not alkylated become oxidized during incubation in spite of the great excess of alkylating agent, or that they are intact but buried in the protein molecule, we titrated the number of residual SH groups in the presence of 6 M urea. We found that at any time of incubation with ATP and bromoacetate the non-reacted cysteinyl residues represent intact but conceivably buried SH groups.

The above result suggests that under the conditions used the rate-limiting step of alkylation is the exposure of the buried SH groups, which renders the process of alkylation independent of bromoacetate concentration.

Identification of the less-reactive SH groups of glyceraldehyde-3-phosphate dehydrogenase

Some of the further problems to be solved were as follows: (1) Whether these SH groups which are masked in the native tetramer and become exposed during ATP-induced dissociation are identical with SH-III or SH-IV or both, and what their position is in the amino acid sequence. (2) Location of SH-II in the sequence.

Apoenzyme of 10 μM concentration was treated with stoichiometric amount of ATP for 10 min and the control was incubated without ATP. After carboxymethylation of Cys-149 with non-radioactive bromoacetate the samples were further incubated in the presence of 0.01 M bromo[^{14}C]acetate for 5 h and dialyzed. The [^{14}C]CM-Cys enzyme derivatives were digested with trypsin and peptide maps were prepared by two-dimensional electrophoresis. The patterns of radioactive peptides were revealed by radioautography, and compared with that of glyceraldehyde-3-phosphate dehydrogenase fully alkylated in the presence of urea according to Harris and Perham[1].

Radioautographs of the peptide maps are presented in Fig. 5. In both cases radioactivity appeared in the peptide containing Cys-153 (*cf.* ref. 1) but the intensity of the spot was stronger with the ATP-treated enzyme. Furthermore, one additional intensive spot appeared on the radioautograph of the ATP-treated enzyme, which

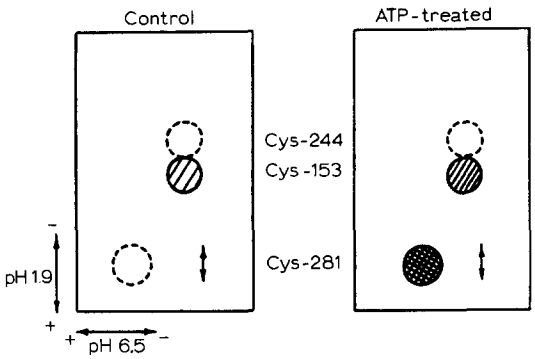

Fig. 5. Radioautograph of peptide map of [^{14}C]carboxymethylated glyceraldehyde-3-phosphate dehydrogenase. Experimental conditions as in legends to Fig. 1. To samples incubated for 10 min in the presence and absence of ATP 0.01 M unlabelled bromoacetate was added and incubation continued for further 10 min. Then 0.001 M bromo[^{14}C]acetate was added and incubation was continued for 5 h. Peptide maps were prepared by electrophoresis in pyridine–acetic acid–water (96:4:900, v/v/v) at pH 6.5, 40 V/cm and formic acid–acetic acid–water (20:80:900, v/v/v) at pH 1.9, 80 V/cm for 2 and 1.5 h, respectively. For the position of the peptides on the map *cf.* ref. 1.

corresponds to the carboxymethylation of residue Cys-281. With either ATP-treated or untreated enzyme the radioactive spot of the peptide containing Cys-244 occurred as a trace component. Comparison of these results with those presented in Fig. 4, shows that Cys-153 corresponds to SH-II and Cys-281 is the residue which becomes exposed on ATP treatment. We propose this latter residue to be named SH-III.

Molecular weight of the ATP-treated enzyme in urea

Since during the ATP-induced dissociation into dimers SH-I and SH-II are only slightly affected by spontaneous oxidation and only one of the two previously buried SH groups can be oxidized to disulfide, the S–S bridges must be formed between monomers within the dimers.

A direct evidence for this assumption could be obtained by the determination of molecular weight. After 5 h of incubation with and without ATP the samples were denatured with 6 M urea in the presence of a great excess of bromoacetate. The molecular weight of the untreated and ATP-treated enzyme were 33 000 $\pm$ 5000 and 60 000 $\pm$ 5000, respectively, as determined by equilibrium ultracentrifugation[19]. From this result it is obvious that the dimers formed by ATP treatment are stabilized by a covalent bond between the monomers.

It s known that on prolonged incubation with PCMB all the 4 SH groups of muscle glyceraldehyde-3-phosphate dehydrogenase can be blocked with the mercurial[9]. However, according to the data of Schachman's laboratory[8], blocking of all the SH groups requires dissociation into monomers. This dissociation is the result of a secondary effect of PCMB treatment[8]. The fact that during the dissociation into dimers caused by ATP one SH group per subunit remains in buried position allows us to assume that this SH group, namely Cys-244, is masked by monomer–monomer interaction in the dimer.

We have concluded that residue Cys-153, which is in the vicinity of the most reactive cysteinyl residue (Cys-149), corresponds to SH-II. We confirmed this result also by modification with spin-labeled SH reagents[20].

SH-I (Cys-149) and SH-II (Cys-153) are practically not affected during dissociation into dimers and inactivation caused by ATP-treatment but SH-III (Cys-281), which is masked in the tetramer, becomes unmasked and oxidized to form an intersubunit S–S bridge. We assume the dissociation into dimers to be the first and rate-limiting step which is simultaneous with or followed by the exposure and very rapid oxidation of Cys-281. The last step may be the formation of interchain S–S bridges, which should be connected with the inactivation of ATP-treated enzyme, since in the presence of 2-mercaptoethanol an active dimeric form of the enzyme can be detected[10]. It has not been clarified yet whether in the native tetrameric enzyme the Cys-281 residues are also sterically near each other. It may well be that the formation of interchain S–S bridges is prevented in native glyceraldehyde-3-phosphate dehydrogenase by the "quaternary constraint".

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