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STUDIES ON THE SH GROUPS OF PHOSPHORYLASE *b*

REACTION WITH 5,5'-DITHIOBIS-(2-NITROBENZOIC ACID)

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SUMMARY

The reaction between 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) and the SH groups of phosphorylase *b* (α-1,4-glucan:orthophosphate glucosyltransferase, EC 2.4.1.1) has been investigated. At pH 6.8 and 25° approx. 2 SH groups, SH_I, reacted very rapidly, 4 SH groups, SH_{II}, reacted more slowly, and the remaining 10 SH groups, SH_{III}, reacted extremely slowly with DTNB. The second order rate constants for the SH_{II} and SH_{III} groups were found to be $3.1 \cdot 10^{-1}$ and $8.5 \cdot 10^{-2}$ $M^{-1} \cdot min^{-1}$, respectively, while the rate constant for the SH_I groups was estimated to be larger than $1 \cdot 10^5$ $M^{-1} \cdot min^{-1}$. The loss of enzymatic activity paralleled the decrease in the number of free SH_{II} groups. Reaction with SH_{II} also resulted in dissociation of the enzyme into subunits with $s_{20,w} = 5.6$ S. In the presence of sodium dodecyl sulfate approx. 16 SH groups reacted; the rate of reaction showed a maximum with respect to the concentration of sodium dodecyl sulfate, the molal ratio between sodium dodecyl sulfate and protein being approx. $3.5 \cdot 10^2$ at the maximum. AMP protected the SH_{II} groups but not SH_I or SH_{III} groups. Glycogen and glucose 1-phosphate, on the other hand, had little effect. The reactivity of the SH_{II} groups increased in the presence of salt, whereas the number of rapidly reacting SH groups was found to depend on the pH of the reaction mixture and reached a minimum at approximately pH 7.

The reactivity of the SH groups on apophosphorylase *b* was found to be quite different from that of the holoenzyme. 6 SH_I type groups were detected on the apoenzyme, and in the presence of AMP this number increased to approx. 8.

The enzymatic activity of the DTNB-treated enzyme could be fully restored by addition of reducing agents such as 2-mercaptoethanol. The reconstituted enzyme possessed the same sedimentation properties as the native holoenzyme.

Abbreviation: DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid).

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INTRODUCTION

The muscle phosphorylases (α-1,4-glucan:orthophosphate glucosyltransferase, EC 2.4.1.1) have for a long time been recognized as key enzymes in the glycogen metabolism. Despite the fact that these enzymes seem to be well characterized in terms of physical properties and kinetics, relatively little information is available as to the groups involved in the enzymatic reaction. It has been known for some time that the muscle phosphorylases are SH enzymes, *i.e.* enzymes whose activity are fully dependent on certain intact SH groups. Studies on the SH groups, particularly of phosphorylase *b*, have been carried out in a number of laboratories, but still it appears that the function of these groups remains somewhat uncertain.

The early work of CORI *et al.*[1] and of GREEN AND CORI[2] showed the importance of the SH groups of the phosphorylases with respect to activity and crystallization. The SH reagent *p*-mercuribenzoate has proved to be a useful tool in investigating the nature of the SH groups of these enzymes. In the absence of unfolding agents *p*-mercuribenzoate reacts with approx. 7 SH groups* in phosphorylase *b* and 14 in phosphorylase *a* (ref. 3). This reaction is accompanied by complete loss of activity, and furthermore the enzymes dissociate into subunits. The effect of *p*-mercuribenzoate could be completely reversed by the addition of excess cysteine. In the case of both phosphorylase *a* (ref. 4) and phosphorylase *b* (refs. 5, 6) some SH groups react rapidly with *p*-mercuribenzoate with no apparent loss of activity, while the remaining SH groups react more slowly producing complete inactivation of the enzymes. JOKAY *et al.*[7] observed that AMP protected against *p*-mercuribenzoate and *N*-ethylmaleimide inactivation of phosphorylase *b*, whereas Glc-1-*P* increased the rate of inactivation slightly. The most recent SH reagents to be employed are fluorodinitrobenzene and chlorodinitrobenzene[8,9]. In the case of phosphorylase *b* the results of GOLD[9] suggest that 4 SH groups react rapidly with these reagents without any apparent change in v_{max}, while the K_m for AMP and Glc-1-*P* decreased. Reaction with the remaining SH groups resulted in extensive inactivation.

In the present work DTNB has been used to study the reactivity of the SH groups of phosphorylase *b* and the mechanism of reaction with this reagent is described in detail. DTNB offers the advantage that it reacts rather specifically with SH groups, and the reaction can be easily measured. Previously we have used this reagent to determine the total number of SH groups in phosphorylase *b* (ref. 10). In the presence of denaturating agents a total of 16 SH groups were detected using both DTNB and *p*-mercuribenzoate. This value has recently been confirmed[9,11,12].

Some preliminary data from the present work have appeared[13,14].

MATERIALS

Chemicals

5,5′-Dithiobis-(2-nitrobenzoic acid) (DTNB), AMP, ATP, 2-mercaptoethanol, DL-β-glycerophosphate and sodium dodecyl sulfate were all products of the Sigma

* All numbers of SH groups have been calculated on the basis of the new molecular weight recently obtained by SEERY *et al.*[40] of 185 000. The values given may therefore differ from those originally published.

Chemical Comp. The purity of DTNB was checked spectrophotometrically[15]. Sodium dodecyl sulfate was recrystallized from 70% ethanol.

Enzyme

Phosphorylase *b* was prepared from rabbit muscle according to FISCHER AND KREBS[16]. It was recrystallized 4 or 5 times. Cysteine and AMP were removed from the enzyme preparation immediately before use by passing it through a column of Sephadex G-100 (2.5 cm × 40 cm) equilibrated with 33 mM glycerophosphate, 1 mM EDTA (pH 6.8). The homogeneity of the enzyme sample was examined in the analytical ultracentrifuge. A single sedimenting peak was obtained. The specific activity of the enzyme was approx. 1600 units per mg protein and the ratio of absorbance at 260 and 280 mμ was 0.53.

The apoenzyme of phosphorylase *b* was prepared according to the procedure of SHALTIEL *et al.*[17]. Cysteine was removed from the enzyme in the same manner as for the holoenzyme.

METHODS

Reaction with DTNB

The reaction of DTNB with SH groups was carried out according to ELLMAN[18] by measuring the increase in absorbance at 412 mμ. In most experiments a Unicam SP 800 recording spectrophotometer equipped with constant temperature cell and automatic cell changer was employed. In a few experiments a Zeiss PMQ II spectrophotometer with constant temperature cuvette was used. All reactions were carried out in air-saturated buffers consisting of 33 mM glycerophosphate and 1 mM EDTA. The pH was 6.8, if not otherwise stated, and the temperature was 25°.

The molar extinction coefficient employed for the 2-nitrobenzoic acid thiol was that obtained by ELLMAN[18]: 13 600 $l \cdot mole^{-1} \cdot cm^{-1}$.

Enzyme assay

The activity of phosphorylase *b* was measured by determining the amount of P_i liberated in the reaction. The assay mixture contained 16 mM Glc-1-*P*, 1 mM AMP, 1% glycogen and approx. 0.2 μM phosphorylase *b* in a total volume of 0.8 ml. The buffer was 33 mM glycerophosphate, 1 mM EDTA (pH 6.8) and the temperature during the incubation was 30°. After incubating the mixture for 5 to 10 min, the reaction was stopped by adding 3.2 ml 10% trichloroacetic acid. The amount of P_i liberated was then determined according to TAUSSKY AND SHORR[19].

Determination of protein

Protein was determined according to the spectrophotometric method of APPLEMAN *et al.*[20] and by the method of LOWRY *et al.*[21].

Determination of sedimentation coefficient

The sedimentation studies were carried out in a Spinco analytical ultracentrifuge. All sedimentation studies were performed at 20°. The sedimentation

coefficients were calculated according to SCHACHMAN[22]. Corrections were made for the viscosity and density of the buffers employed.

Determination of pyridoxal 5'-phosphate

Pyridoxal 5'-phosphate was determined according to KENT *et al.*[23].

RESULTS

Time course of the reaction

The time course for the reaction of three different concentrations of phosphorylase *b* with DTNB is shown in Fig. 1. In the first few sec there is a very rapid increase in absorbance. Then follows a period where the absorbance rises more slowly, and after approx. 1–2 h it has leveled off, and the rate of increase has become constant, remaining the same for up to approx. 12 h. At this point the protein starts to precipitate out of solution. On the basis of reactivity it is thus possible to divide the SH groups of phosphorylase *b* into 3 different groups. These have been designated SH_I, SH_{II} and SH_{III}, and the apparent order of reactivity of these groups is as follows: $SH_I \gg SH_{II} > SH_{III}$. There are approx. 2 SH_I groups and 4 SH_{II} groups per molecule. Approx. 2 SH_{III} groups had reacted before the protein started to precipitate out of the solution. For the sake of simplicity, however, the remaining 8 SH groups have also been designated as SH_{III} groups. It seems quite likely that these would have reacted at the same rate as the first ones if the protein had stayed in solution. A total of 10 SH_{III} groups are, therefore, present in each phosphorylase *b* molecule, but these are all probably masked when the enzyme is in its native state.

The number of SH_I groups was found to vary slightly from one preparation to another. In general there were fewer SH_I groups in aged preparations of phosphorylase *b* than in the freshly prepared enzyme samples. It might be argued that SH_I groups are impurities of cysteine or cysteamine which could be present in small amounts in the preparations. This possibility was investigated by passing an enzyme sample through a column of Sephadex G-25 equilibrated with 33 mM glycerophosphate buffer (pH 6.8) containing 0.3 M KCl. The same sample had previously been put through a column of Sephadex G-100 in order to remove cysteine and AMP (see MATERIALS). Immediately after elution from the Sephadex G-25 column the reaction with DTNB was measured. No significant decrease in reactivity and number of SH_I groups was found compared with the sample which had not been passed through the column of Sephadex G-25. Thus it would appear that the SH_I groups are true SH groups on the enzyme and not artifacts.

Effect of DTNB concentration

The effect of DTNB concentration was investigated in detail, and the results are presented in Figs. 2 and 3. In the presence of small concentrations of DTNB only SH_I groups reacted. As the concentration of DTNB is further increased SH_{II} groups also started to react and at high concentrations of DTNB, SH_{II} groups reacted quite rapidly. SH_{III} groups reacted very slowly, but the rate of reaction with SH_{III} also increased with increasing concentrations of DTNB.

When the percent remaining SH_{II} groups were plotted against time in a semilogaritmic manner, shown in Fig. 3A, straight lines were obtained suggesting that

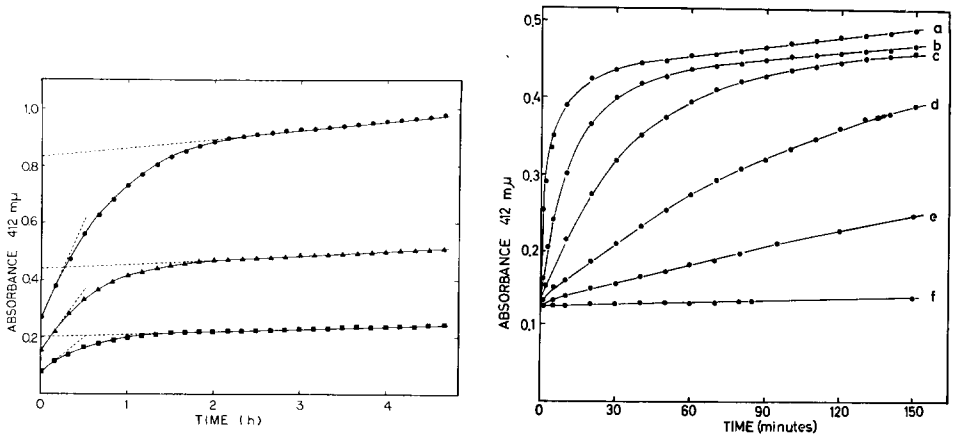


Fig. 1. Time course of reaction of phosphorylase *b* with DTNB. The reaction mixture contained: 1.33 mM DTNB, various amounts of phosphorylase *b*, 33 mM glycerophosphate, 1 mM EDTA in a total vol. of 3 ml at pH 6.8 and 25°. The concentration of phosphorylase *b* (mg/ml) was: ●—●, 2.2; ▲—▲, 1.1; ■—■, 0.55. For each experiment the intersections between the two dotted lines and the ordinate axis represent absorbance due to SH_I and $SH_I + SH_{II}$, respectively.

Fig. 2. Effect of DTNB concentration. The reaction mixture contained: 1.1 mg phosphorylase *b* per ml, 33 mM glycerophosphate, 1 mM EDTA, different concentrations of DTNB. The final vol. was 3 ml, and the reaction took place at pH 6.8 and 25°. The concentration of DTNB (mM) was: a, 6.6; b, 2.66; c, 1.33; d, 0.66; e, 0.33; f, 0.06.

the reaction with DTNB is pseudo first order with respect to SH_{II}. The apparent rate of reaction with SH_{II} increased linearly with DTNB concentration as shown in Fig. 3B, except for a very short lag period initially. This indicates that the reaction is also pseudo first order with respect to concentration of DTNB. The overall reaction is therefore a second order reaction. Similar experiments suggested that reactions

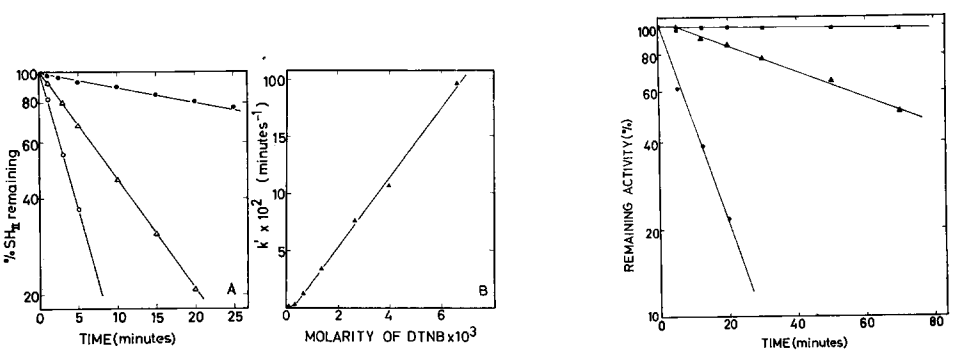


Fig. 3. Semilogaritmic plot of reaction with SH_{II} at different concentrations of DTNB (A) and effect of DTNB concentration on the pseudo first order rate constant for SH_{II} (B). The experimental conditions were as in Fig. 2. The concentration of DTNB (mM) was: ○—○, 6.6; △—△, 2.66; ●—●, 0.66.

Fig. 4. Effect of DTNB on the enzymatic activity of phosphorylase *b*. The reaction mixtures contained: phosphorylase *b*, 1.1 mg/ml; 33 mM glycerophosphate, 1 mM EDTA. Total vol., 3 ml; temp., 25°; pH 6.8. The concentration of DTNB was: ■—■, control (no DTNB); ▲—▲, 0.66 mM; ●—●, 2.66 mM. Aliquots of 50 μl were withdrawn at various times and diluted 100-fold with 33 mM glycerophosphate buffer. The activity of this solution was then immediately determined as described in METHODS.

with SH_I and SH_{III} groups also were second order reactions. The apparent second order rate constants for the reaction with SH_{II} and SH_{III} were found to be $3.1 \cdot 10$ $M^{-1} \cdot min^{-1}$ and $8.5 \cdot 10^{-2}$ $M^{-1} \cdot min^{-1}$. Due to the extremely rapid reaction between DTNB and SH_I groups the rate constant for this reaction could not be determined accurately using the present spectrophotometric technique. This rate constant was, however, estimated to be larger than $1 \cdot 10^5$ $M^{-1} \cdot min^{-1}$. All rate constants are given at 25° and pH 6.8.

Effect of DTNB on enzymatic activity

The effect of DTNB on the enzymatic activity is shown in Fig. 4. The enzymatic activity decreased linearly with time when plotted in a semilogaritmic manner. The two different concentrations of DTNB employed in this experiment, were the same as two of those used in Fig. 3A. By comparing the two figures it is evident that the rate of decrease of free SH_{II} groups is approximately the same as the rate of decrease in activity. When DTNB was allowed to react with SH_I only, using small concentrations of DTNB, there was no decrease in activity. One must therefore conclude that the catalytic property of phosphorylase *b* is fully dependent on the integrity of SH_{II} groups.

Sedimentation and gel-filtration studies

The mechanism of reaction of DTNB with phosphorylase *b* was investigated further by sedimentation studies in the analytical ultracentrifuge. In these experiments DTNB was allowed to react with the enzyme for various lenıths of time, the mixture was then passed through a column of Sephadex G-25 in order to remove excess DTNB, and after elution from this column, it was immediately subjected to ultracentrifugation. The protein concentration in these sedimentation experiments was approx. 2 mg/ml, *i.e.* of the same order of magnitude as that employed in the

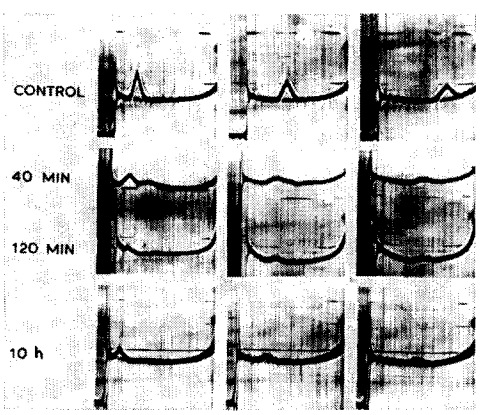


Fig. 5. Sedimentation of DTNB-treated phosphorylase *b* in the analytical ultracentrifuge. Three different samples of phosphorylase *b* (3.3 mg/ml) were allowed to react with DTNB (2 mM) for various lengths of time. The reaction was stopped by removing excess DTNB by gel filtration on a column of Sephadex G-25, and then immediately subjecting the sample to ultracentrifugation. The pictures were taken 16, 40 and 64 min after the centrifuge had reached the full speed of 59 780 rev./min. The temperature during the run was 20°. The protein was dissolved in 33 mM glycerophosphate buffer, 1 mM EDTA (pH 6.8). The concentration of protein in the control was approx. 3 mg/ml, in the other samples approx. 2 mg/ml.

previous experiments with DTNB. The results are shown in Fig. 5. After 40-min reaction time when 80% of SH_{II} groups had reacted with DTNB, the major component sedimented slower than phosphorylase *b*. The sedimentation coefficient for this component was estimated to be $s_{20,w} = 5.6$ S, suggesting that this component is a subunit of phosphorylase similar to that obtained when the enzyme is treated with *p*-mercuribenzoate[3]. A minor component sedimented faster than phosphorylase *b* and had a sedimentation coefficient of 11.6 S. The sedimentation coefficient for phosphorylase *b* was found to be 8.3 S, in good agreement with that reported earlier by KELLER AND CORI[24]. After 120-min reaction time all the SH_{II} groups had reacted and in this case only the subunits were observed with perhaps a slight trace of heavy sedimenting material. The same results were obtained after 10-h reaction time.

Molecular sieve chromatography on a column of Sephadex G-100 (2.5 cm × 40 cm) also indicated the same reaction mechanism. Using this technique it was possible to separate the subunits from the heavy molecular weight component. SH group analysis revealed that the heavy molecular weight component contained slightly fewer free SH groups than the subunits, suggesting that the heavy molecular weight component may contain inter- or intramolecular disulfide bonds formed during the reaction.

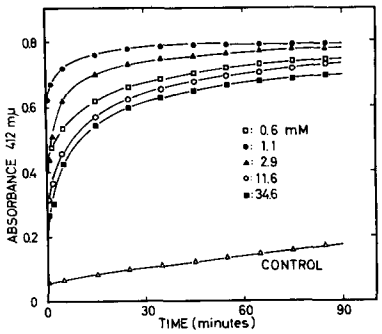
The pyridoxal 5'-phosphate content of DTNB-treated protein samples was also determined, and the results suggested that this group was not split off from the enzyme during the reaction with DTNB in the absence of denaturating agents.

Altogether the results of these experiments suggest that the reaction with DTNB is accompanied by dissociation of the enzyme. This may also account for the loss of the enzymatic activity. Similar results have recently been obtained by KASTENSMIDT *et al.*[25].

Effect of sodium dodecyl sulfate

Ionic detergents such as sodium dodecyl sulfate are known to be very efficient unfolding agents for proteins. During the unfolding process masked groups become exposed to the surrounding medium. Previously we have employed sodium dodecyl sulfate and urea to determine the total number of SH groups in phosphorylase *b* (ref. 10). In the present work the effect of sodium dodecyl sulfate has been examined in detail.

As shown in Fig. 6 the reaction between DTNB and phosphorylase *b* proceeded quite rapidly in the presence of sodium dodecyl sulfate and the reaction rate appeared to be maximal at approx. 1 mM sodium dodecyl sulfate, or when the molal ratio of sodium dodecyl sulfate to protein was about 350. After this maximum the rate decreased slightly with increasing concentrations of sodium dodecyl sulfate. The final plateau value reached after 2–3 h and corresponding to approx. 16 SH groups per molecule was, however, the same in all cases. The reason for the maximum observed with respect to sodium dodecyl sulfate is probably that sodium dodecyl sulfate is known to bind to proteins[26] and thus some of the SH groups may be sterically hindered from reacting with DTNB even though the molecule is in the unfolded state. The unfolding process was quite rapid and was complete in less than 30 sec at 1 mM sodium dodecyl sulfate. Preincubation of the protein with sodium dodecyl sulfate for longer periods before addition of DTNB did not result in any significant changes with respect to the reaction rate. Control experiments, performed with 2-mercapto-



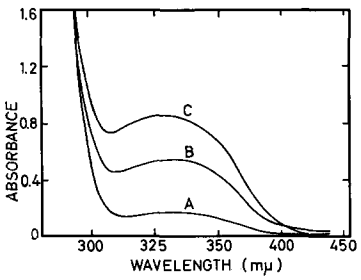


Fig. 6. Effect of sodium dodecyl sulfate. The reaction mixture contained: 0.7 mg/ml phosphorylase *b*, 0.66 mM DTNB, 33 mM glycerophosphate, 1 mM EDTA and sodium dodecyl sulfate at concentrations as indicated in the figure. Temperature, 25°; pH ,6.8. Sodium dodecyl sulfate was added 1 min prior to DTNB.

Fig. 7. Absorption spectra of DTNB-treated protein samples. A, phosphorylase *b* control; B, sample treated with 2 mM DTNB for 40 min and excess reagent removed by gel filtration; C, sample treated with 2 mM DTNB and 8 mM sodium dodecyl sulfate for 2 h and excess reagents then removed by gel filtration. The protein was dissolved in 33 mM glycerophosphate, 1 mM EDTA (pH 6.8) at a concentration of 2.1 mg/ml.

ethanol, indicated that sodium dodecyl sulfate did not affect the color development. The above findings are not in accord with those presented by BATELL *et al.*[27] who found only 11.2 SH groups in the presence of sodium dodecyl sulfate. The reason for this discrepancy is probably due to the different conditions employed.

Analysis for pyridoxal 5'-phosphate in the sodium dodecyl sulfate-treated protein samples showed that the prosthetic group was split off during the sodium dodecyl sulfate treatment.

Spectra of DTNB-treated enzyme and estimation of number of different disulfide bonds formed

Fig. 7 shows the spectra of DTNB-treated enzyme samples in the near ultraviolet region. The DTNB-treated protein samples possess a distinct maximum in the region 325–330 mμ, *i.e.* at approximately the same wavelength as DTNB at pH 6.8. The untreated phosphorylase *b* also has a maximum at the same wavelength region due to the pyridoxal 5'-phosphate group.

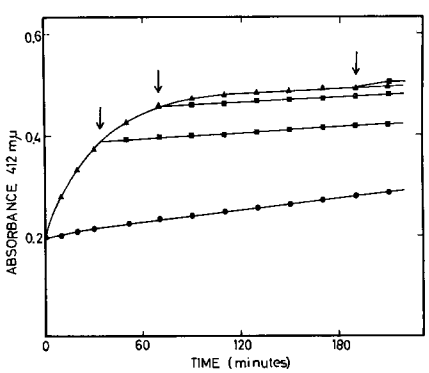
DTNB is a symmetrical molecule which contains 2 nitrobenzoate groups per molecule. If one assumes that the molar extinction coefficient for the protein bound nitrobenzoate thio group is half that of DTNB at the maximum, *i.e.* 10 300 $M^{-1} \cdot cm^{-1}$, then it is possible to calculate the number of mixed disulfide bonds which contain this group in the protein, and then correlate this with the experimental value obtained by titrations. Evidence for the validity of the above assumption is presented in the paragraph dealing with the effect of reducing agent on the DTNB-treated protein. In the case of the 40-min sample it was estimated that approx. 3.6 nitrobenzoate thio groups were present per molecule of enzyme. The titration data indicated that approx. 4 SH groups should have reacted with DTNB. Therefore approx. 0.2 inter- or intramolecular disulfide bonds must have been formed due to the exchange reaction protein–S–S–R + protein–SH⇌ protein–S–S-protein + RSH where R stands for the

nitrobenzoate group and "protein" for monomer, dimer or a polymeric species. It must be emphasized that the net result with respect to SH-group determination is the same whether or not this reaction takes place. In the case of D-glyceraldehyde-3-*P* dehydrogenase[28] it is well established that reactions of this type take place when the enzyme is treated with DTNB. The amount of protein–S–S–protein disulfide bonds did not increase significantly when phosphorylase *b*, in the absence of sodium dodecyl sulfate, was treated for longer times with DTNB. These results are thus in good agreement with the gel-filtration data. When the enzyme was allowed to react with DTNB in the presence of sodium dodecyl sulfate, however, approx. 4 protein–S–S–protein bonds were found per molecule of enzyme.

It therefore appears that very few protein–S–S–protein disulfide bonds are formed during the reaction with DTNB in the absence of sodium dodecyl sulfate, thus suggesting that the present conclusions with respect to reactivity of SH groups in this enzyme are valid. The relatively high number of protein–S–S–protein disulfide bonds found in the sodium dodecyl sulfate sample treated with sodium dodecyl sulfate is to be expected, since in this case the peptide chains are unfolded, and thus more SH groups are available for this type of exchange reaction.

Effect of AMP and substrates

AMP is an allosteric effector of phosphorylase *b*. 2 moles of AMP are bound per mole of enzyme[29]. Previously we have shown that AMP protected some SH groups[13]. In the present study we have characterized these further. The results are summarized in Fig. 8. AMP was found to protect SH_{II} groups but not SH_I or SH_{III} groups. When AMP was added at various times after addition of DTNB the rate of reaction with SH_{II} groups decreased to approximately the same level as when added at zero time. As expected AMP also protected the enzymatic activity. The data therefore suggest that SH_{II} groups may be involved in the binding of AMP; another possibility is that AMP protects the enzyme by preventing it from dissociating into subunits. AMP did



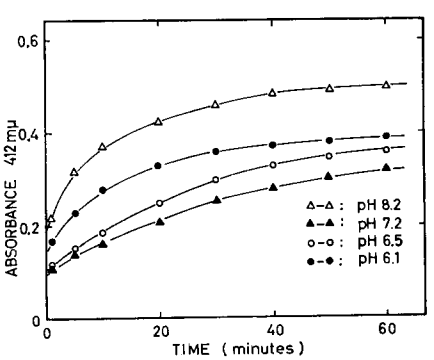


Fig. 8. Effect of AMP on the DTNB reaction. The reaction mixture contained: phosphorylase *b*, 1 mg/ml; 1.33 mM DTNB, 1 mM AMP when added, 33 mM glycerophosphate and 1 mM EDTA in a total vol. of 3 ml, at pH 6.8 and 25°. ▲—▲, control (no AMP present); ●—●, AMP added at zero time; ■---■, AMP added at arrow.

Fig. 9. Effect of pH on the reaction between phosphorylase *b* and DTNB. The reaction mixture contained: 0.8 mg protein/ml, 1.33 mM DTNB in 33 mM glycerophosphate and 1 mM EDTA. The pH varied as indicated. Temp., 25°.

not afford any protection of SH groups in the presence of sodium dodecyl sulfate.

ATP, which is a cofactor analog to AMP, is bound to the same site as AMP and inhibits the enzyme[30]. ATP was found to protect the SH_{II} in the same manner as AMP.

The effect of the substrates glycogen and Glc-1-*P* was also investigated. Employing the same concentrations as in the assay system, glycogen protected SH_{II} groups slightly, whereas Glc-1-*P*, on the other hand, caused a slight increase in the reaction rate of these SH groups. The latter effect may be due to the fact that Glc-1-*P* increases the ionic strength considerably (see paragraph on the effect of salts).

Effect of pH

The DTNB reagent can only be used in the pH region from pH 6 to approximately pH 8.5. Above pH 8.5 DTNB itself is slowly reduced, and below pH 6 the amount of nonionized nitrobenzoate thiol increases rapidly, thereby causing a change in the absorption spectrum[15]. Fig. 9 shows the effect of pH in this region on the reaction between phosphorylase *b* and DTNB. Control reactions were carried out employing 2-mercaptoethanol in the same pH region, but no significant effects of pH were observed. 2-Mercaptoethanol has a p*K* of 9.5 (ref. 31). The lowest reactivity both with respect to SH_I- and SH_{II}-type SH groups was found at about pH 7. On both sides of this pH value both the number of SH_I-type and reactivity of SH_{II}-type increased. At pH 8 the number of rapidly reacting SH groups is approx. 4 which is in good agreement with that obtained by GOLD[9] employing dinitrophenylating agents.

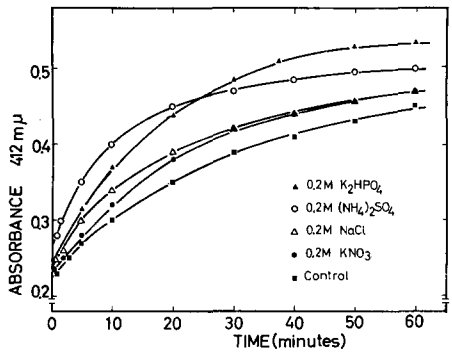
Phosphorylase *b* is known to exist exclusively as a dimer in this pH region[32], therefore dissociation into subunits cannot explain the above results. It is more likely that subtle changes in the conformation occurs as the pH is changed which could result in a change in reactivity of the SH groups.

Effect of salts

The reaction rate between SH groups and DTNB has been shown to increase in the presence of salts[33]. In the case of phosphorylase *b* the presence of salts also increased the reactivity of SH_{II} groups as shown in Fig. 10. Divalent anions such as P_i and SO_4^{2-} appeared to be more efficient in this respect than monovalent anions, suggesting that the reactivity of SH_{II} groups to some extent depends on the ionic strength of the reaction mixture.

Reaction of DTNB with apophosphorylase b

The apophosphorylase *b* has recently been crystallized by HEDRICK *et al.*[34]. At 25° and pH 7 the apoenzyme exist as a dimer with sedimentation coefficient of 7.1 S, whereas the holoenzyme has a sedimentation coefficient of 8.3 S. In addition also the stability of the tertiary and quarternary structure is considerably less than for the holoenzyme. This suggests that the apoenzyme possesses a different conformation from that of the holoenzyme. The possibility thus exists that that the reactivity of the SH groups of the two species differs. This was indeed found to be the case, as shown in Fig. 11. Approx. 6 SH groups reacted immediately with DTNB, whereas the reactivity of the remaining ones resembled that of SH_{II} and SH_{III}. AMP did not protect any SH groups as was the case for the holoenzyme. On the contrary an increase in the number of rapidly reacting SH groups was found when AMP was



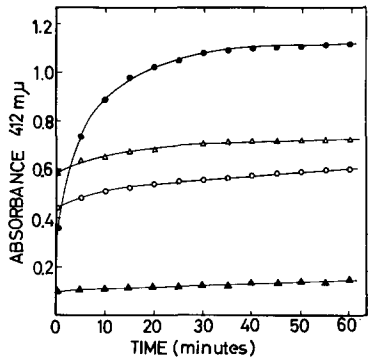


Fig. 10. Effect of salts on the reaction with DTNB. The reaction mixtures consisted of :phosphorylase *b*, 1.1 mg/ml; 0.66 mM DTNB, various amounts of salts as indicated, 33 mM glycerophosphate, 1 mM EDTA. The pH was 6.8, and the temp. 25°.

Fig. 11. Reaction of apophosphorylase *b* with DTNB. The reaction mixture contained: 0.9 mg protein/ml, 0.33 mM DTNB, 1 mM AMP and 20 mM sodium dodecyl sulfate when added. pH was 6.8; temp., 25°. ▲—▲, Control holoenzyme; ○—○, control apoenzyme; △—△, apoenzyme + AMP; ●—●, apoenzyme + sodium dodecyl sulfate.

added. Thus in the presence of 1 mM AMP approx. 8 SH groups reacted immediately. Addition of sodium dodecyl sulfate caused unfolding of the protein in a manner similar to that observed for the holoenzyme, and furthermore the total number of SH groups was also the same as for the holoenzyme.

AMP is known to cause both association and dissociation of the apophosphorylase *b* as shown by sedimentation experiments[34]. In the presence of 1 mM AMP 42% of the protein sedimented as subunits with $s_{20,w} = 5.8$ S, while the remainder were found to consist of higher molecular weight aggregates with $s_{20,w} = 14.1$ S. In view of these pronounced changes brought about in the quarternary structure of apophosphorylase *b* by AMP the present observations with respect to reactivity of SH groups are not surprising but would be expected as the subunits probably contain more SH_1-type SH groups than the polymer.

Effect of reducing agents on DTNB-treated phosphorylase b

When 2-mercaptoethanol or cysteine was added to a DTNB-treated enzyme sample an increase in absorbance at 412 mμ similar to that found when DTNB was added to SH compounds was observed. This is illustrated in Fig. 12A for a protein sample which had been treated with DTNB and sodium dodecyl sulfate and the excess of these reagents removed by gel filtration. In this particular sample approx. 8 SH groups existed as mixed disulfides with the nitrobenzoate thiol. With 10 mM 2-mercaptoethanol the reaction appears to be complete in approx. 30 min. The final amount of nitrobenzoate thiol liberated corresponded closely to that calculated from the absorbance at 330 mμ of the protein as discussed previously. When the reaction was plotted in a semilogaritmic manner shown in Fig. 12B, the curves for 10 and 1 mM 2-mercaptoethanol were linear two-step curves, suggesting that two types of disulfide groups exist which differ in reactivity towards 2-mercaptoethanol. In this particular experiment 3 disulfide groups reacted with a second order rate constant of $3.2 \cdot 10^2$ $M^{-1} \cdot min^{-1}$ and the remaining 5 with a rate constant of $0.65 \cdot 10^2$ $M^{-1} \cdot min^{-1}$.

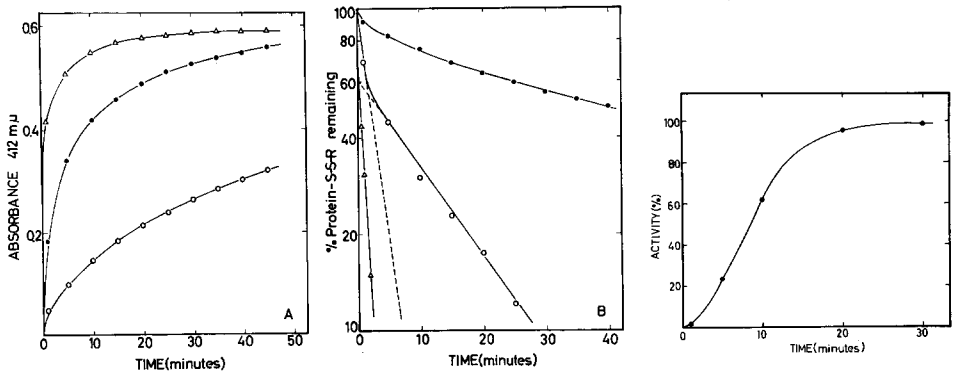


Fig. 12. Reaction of DTNB-treated phosphorylase *b* with 2-mercaptoethanol (A). Reaction plotted in a semilogaritmic manner (B). The reaction mixture contained: 0.8 mg DTNB-sodium dodecyl sulfate-treated enzyme/ml, various amounts of 2-mercaptoethanol, 33 mM glycerophosphate, 1 mM EDTA (pH 6.8). Temp., 25°. The concentration of 2-mercaptoethanol (mM) was: ○—○, 0.1; ●—●, 1; △—△, 10.

Fig. 13. Reactivation of DTNB-treated phosphorylase *b*. The reaction mixture contained: 0.8 mg DTNB-treated enzyme/ml in which 7 SH groups were blocked, 20 mM 2-mercaptoethanol, 33 mM glycerophosphate, 1 mM EDTA. Temp., 25°; pH, 6.8. The activity was determined as described in METHODS.

Similar results with respect to kinetics and rate constants were also obtained for protein samples not treated with sodium dodecyl sulfate.

The enzymatic activity of holoenzyme samples treated with DTNB in the absence of sodium dodecyl sulfate could be completely regained by addition of reducing agents as shown in Fig. 13. In the presence of 10 mM 2-mercaptoethanol the activity was fully restored after approx. 30 min for an enzyme sample which contained about 7 blocked SH groups per molecule. Thus there is good correspondance between rate of reduction of disulfide bonds and recovery of activity.

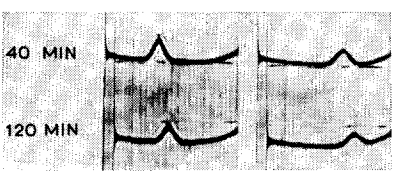


Fig. 14. Sedimentation properties of reconstituted phosphorylase *b*. The 40- and 120-min DTNB-treated samples from Fig. 5 were allowed to react with 20 mM 2-mercaptoethanol for 2 h, then subjected to sedimentation in an analytical ultracentrifuge. Photographs were taken 40 and 64 min after the full speed of 59 780 rev./min had been reached. The temperature during the run was 20°.

The reconstituted enzyme sedimented as a homogeneous protein (Fig. 14), and possessed the same sedimentation properties as the holoenzyme. The protein samples employed in this experiment were the same as the 40- and 120-min DTNB-treated samples shown in Fig. 5.

DISCUSSION

The results presented in this work indicate that phosphorylase *b* possesses three different types of SH groups differing in reactivity toward DTNB. At pH 6.8 and 25°, 2 SH groups (SH_I) reacted very rapidly with DTNB, 4 (SH_{II}) more slowly, and the remaining 10 SH groups (SH_{III}) reacted extremely slowly. Loss of enzymatic activity was found to parallel the decrease in free SH_{II} groups. The effect of DTNB resembles that observed for other SH-group reagents such as *p*-mercuribenzoate[5] and dinitrophenylating agents[9]. In analogy with *p*-mercuribenzoate, DTNB caused the enzyme to dissociate into subunits, and this reaction could be reversed by addition of reducing agents. The effect of DTNB on phosphorylase *a* was not investigated in the present work, but judging from the results obtained with *p*-mercuribenzoate[4], it is reasonable to assume that the mode of reaction with DTNB for this enzyme is similar to that observed for phosphorylase *b*.

The rate of reaction of DTNB with SH groups is thought to be influenced by the amount of the anionic form of the SH group present[15]. Different SH groups in the protein probably possess slightly different pK values, and thus the rate of reaction with DTNB should differ somewhat. It is doubtful, however, that this can be the full explanation for the marked difference in reactivity observed in the present case. It seems more likely that steric hindrance must play the most important role with respect to reactivity of the SH groups toward DTNB in proteins. The rather bulky DTNB molecule requires a certain amount of space in the proximity of the SH group in order to react. This would be possible if the SH group is located at or near the surface of the protein molecule. Based on this reasoning one can construct a model of phosphorylase *b* indicating the approximate position of the SH groups. It is assumed that phosphorylase *b* is made up of two subunits, each subunit having the same number of SH groups. Some evidence for this has recently been presented by ZARKADAS *et al.*[12]. Each subunit is therefore thought to contain 1 SH_I, 2 SH_{II} and 5 SH_{III} groups. It is further suggested that SH_I groups are located on the surface of the protein and are readily available for the reaction with DTNB, while the SH_{II} groups could be situated in the space between the two subunits. Most of the SH_{II} groups could only react with DTNB after dissociation into subunits or a change in conformation has taken place. The SH_{III} groups are so-called buried or masked SH groups which only will react with DTNB after unfolding of the molecule has occurred. It must be emphasized that the above model refers to the conformation of the holoenzyme at pH 6.8 only. The apoenzyme apparently possesses a different conformation from that of the holoenzyme[34], and therefore it is difficult to make any comparison between the two enzyme species with respect to location of SH groups. However, the finding that removal of the pyridoxal 5'-phosphate group caused a 3-fold increase in the number of rapidly reacting SH groups may suggest that SH groups are involved in the binding of the pyridoxal 5'-phosphate group as previously proposed by KREBS AND FISCHER[35].

The SH groups reacting with other SH reagents may also be predicted from this model. At pH 6.8 *p*-mercuribenzoate is known to react rapidly with 4 SH groups with no apparent loss of activity. 3 more SH groups react more slowly producing complete inactivation[5]. Furthermore, AMP protects the enzyme against inactivation[7]. It is suggested that *p*-mercuribenzoate reacts rapidly with the 2 SH_I groups and

2 SH_{II} groups. The groups reacting more slowly are the remaining SH_{II} groups and perhaps 1 or 2 SH_{III} groups. Steric considerations are less important for *p*-mercuribenzoate and dinitrophenylating reagents than for DTNB, therefore in the case of *p*-mercuribenzoate reactions with 2 SH_{II} groups could presumably take place without any concomitant dissociation of the enzyme with loss of activity. The results with the dinitrophenylating reagents at pH 8 (ref. 9) suggest that these compounds only react rapidly with the 4 SH_I-type groups which were observed at this pH value. The lack of effect of AMP also supports this conclusion.

The reaction between SH_{II} groups and DTNB may be an all-or-none reaction similar to that postulated for *p*-mercuribenzoate[36]. The mechanism of protection of AMP and the fact that the loss of activity parallels the decrease in number of free SH_{II} groups strongly support this view. Once one SH_{II} group has reacted with DTNB the molecule might dissociate into subunits thereby unmasking the remaining SH_{II} groups. The rate-limiting step in this process could be the reaction with the first SH_{II} group. The protective effect exerted by AMP could then be explained on the basis that AMP prevents the enzyme from dissociating into subunits, or alternatively one SH_{II} group could be located at or near the binding site for AMP and AMP would then protect merely by steric hindrance. In regard to the first possibility it should be mentioned that AMP causes a change in the optical rotatory dispersion spectra of phosphorylase *b* and furthermore it promotes association of the enzyme during crystallization[37,23]. According to MONOD *et al.*[38] and BUC[39] the enzyme exist in two different conformational states $R \rightleftharpoons T$. AMP might have a high affinity for the R state, whereas the other state, T, predominant in the absence of any ligands, has little affinity for AMP. The function of AMP would then be to freeze the enzyme in a certain conformational state where all SH_{II} groups are masked.

The DTNB reagent has proved to be very useful tool in studies on SH groups and conformational changes in proteins. The mechanism of reaction between DTNB and phosphorylase *b*, described in the present work, may apply to other disulfides as well. Experiments in this laboratory have shown that both cystamine and oxidized glutathione inactivate phosphorylase *b* (K. KLEPPE, unpublished observations, 1968). The rate of inactivation is, however, much slower than that observed for DTNB.

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