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# THE ROLE OF CYSTEINE RESIDUES IN THE CATALYTIC ACTIVITY OF GLYCEROL-3-PHOSPHATE DEHYDROGENASE

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## Summary

Glycerol-3-phosphate dehydrogenase (*sn*-glycerol-3-phosphate:$NAD^+$ 2-oxidoreductase, EC 1.1.1.8) has been shown to be sensitive to inhibition by iodoacetate. The reaction of the enzyme with iodoacetate, which appears to be a simple bimolecular process, is accompanied by a corresponding loss of enzyme activity. In addition to changes in activity, the alkylation reaction was monitored by the incorporation of radioactivity from iodo[2-$^{14}$C]acetate, by changes in amino acid composition, and by changes in the content of free sulfhydryl groups. It is concluded that there are two cysteine residues in the native dimeric enzyme which are essential for enzymic activity. The rate of inactivation was relatively insensitive to the presence of various compounds with the exception of NADH which markedly inhibited the reaction. Kinetic and binding studies showed that the binding of NADH prevents alkylation and, conversely, alkylation prevents NADH binding. From the pH dependence of the alkylation reaction, the $pK_a$ of the essential sulfhydryl groups was calculated to be 8.5 and it is suggested that the binding of coenzyme is independent of the state of ionization of these groups.

## Introduction

Glycerol-3-phosphate dehydrogenase (*sn*-glycerol-3-phosphate:$NAD^+$ 2-oxidoreductase, EC 1.1.1.8) is a member of a class of several pyridine nucleotide-linked dehydrogenases which share common structural and functional charac-

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teristics [1—4]. Like many other dehydrogenases, glycerol-3-*P* dehydrogenase is sensitive to inhibition by compounds known to modify cysteine residues. The enzyme activity is abolished by reagents such as *p*-chloromercuribenzoate [5,6], alkyl maleimides [7], and haloacetates [8].

The stoichiometry of inactivation has been variable and dependent upon the modifying reagent. Anderson and coworkers [7] demonstrated that *N*-ethyl maleimide modifies all sulfhydryl groups of the enzyme. Likewise, *p*-chloromercuribenzoate appears to react with several sulfhydryl groups [6,9], although an early report by van Eys et al. [5] states that only one sulfhydryl group must be blocked to achieve complete inhibition. Bromoacetate was found by Sajgó and Telegdi [8] to be a more selective reagent. They concluded from radiolabeling experiments that two or more thiol groups are involved in the catalytic activity and they have determined the amino acid sequence around these sulfhydryl groups.

In this communication we report on some of the properties of the essential sulfhydryl groups of glycerol-3-*P* dehydrogenase and the role that these groups play in the binding of coenzyme.

## Experimental

Crystalline rabbit muscle glycerol-3-*P* dehydrogenase was obtained from PL Biochemical Co. (Milwaukee, WI, U.S.A.) or isolated from rabbit skeletal muscle as described below. Stock solutions were prepared fresh daily by dissolving the ammonium sulfate suspension in buffer followed by gel filtration through a Bio-Gel P30 column (Bio Rad Corp., Richmond, CA, U.S.A.) to remove small molecules. Iodo[2-$^{14}$C]acetic acid, 48 Ci/mol (lot 47), was from Amersham (Arlington Heights, IL, U.S.A.). All other biochemicals were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All inorganic chemicals were reagent grade. Iodoacetate was recrystallized from $CCl_4$ and dried before use.

Optical measurements were recorded using a Beckman model 25 ultraviolet-visible spectrophotometer. Enzyme activity was determined by following the oxidation of 0.1 mM NADH in the presence of 0.3 mM dihydroxyacetone phosphate at 30° C in a temperature-controlled cell compartment using a 1 ml solution containing 50 mM triethanolamine, 1 mM EDTA, 1 mM mercaptoethanol. Enzyme concentration was calculated on the basis of an extinction coefficient of 48 $mM^{-1} \cdot cm^{-1}$ [10]. Fluorescence measurements were recorded using a Farrand Model Mkl fluorescence spectrophotometer equipped with a temperature-controlled turret cell holder. All slit widths were set at 5 nm.

The measurement of sulfhydryl groups in the enzyme was carried out by using the optical method based on the reaction with 5,5′-dithiobis-(2-nitrobenzoic acid) as described by Ellman [11].

The rates of reaction of glycerol-3-*P* dehydrogenase with iodoacetate were measured by two procedures: (a) the inactivation was followed by withdrawing aliquots of the reaction solution, diluting the mixture in 20 vol. cold 50 mM Tris buffer, 1 mM mercaptoethanol, 1 mM EDTA (pH 7.5) and immediately assaying the diluted enzyme and (b) the incorporation of [$^{14}$C]alkyl groups was followed as described above except 100-μl aliquots were removed and quenched in 3 ml cold 5% trichloroacetic acid and, after a 30 min incubation

in the cold, collecting the $^{14}C$-labeled enzyme on 0.45 μm Millipore filters (HAWPO24). The filters were washed twice with 3 ml 5% trichloroacetic acid, once with an equal volume of water and then ethanol, dried, and counted in a Packard Tri-Carb scintillation spectrometer using 10 ml scintillator/filter. The scintillation solution consisted of 3 g PPO, 72 mg POPOP, 600 ml toluene and 300 ml Triton X-100.

*Enzyme purification.* Glycerol-3-*P* dehydrogenase was purified from rabbit muscle by a combination of affinity and ion-exchange chromatography. Muscle tissue was homogenized in a Waring blender in 0.1 M phosphate buffer (pH 7.8), 5 mM 2-mercaptoethanol, 2 mM EDTA. The homogenate was centrifuged for 15 min at 10 000 rev./min to remove fat. The homogenate was then brought to 80% saturation with $(NH_4)_2SO_4$ and the mixture centrifuged. The resulting precipitate was dissolved in 50 mM triethanolamine buffer (pH 7.5), 5 mM 2-mercaptoethanol, 1 mM EDTA. This was then put on a trinitrobenzene affinity column prepared and operated according to the method of Kornbluth et al. [12]. The protein eluted from the column (with 0.2 mM NADH) was equilibrated with 5 mM phosphate buffer (pH 8.2), 5 mM 2-mercaptoethanol. The enzyme was applied to a DEAE-cellulose column and eluted with a NaCl gradient. The enzyme was then put on a 6-phosphogluconic acid affinity column, prepared and operated as described by McGinnis and deVellis [13].

*Gel electrophoresis.* Polyacrylamide gel electrophoresis was performed as described by Davis [14]. All gels were stained for protein by using a 0.1% Coomassie blue solution in 7% acetic acid and destained in 10% acetic acid. Gels were stained for enzyme activity by the method of Fondy et al. [15].

*Amino acid analysis.* Automated amino acid analysis was performed as described by Spies [16] on a Jeol model JLC-6AH amino acid analyzer. The hydrolysis of the enzyme was conducted using 3 M methanesulfonic acid according to the procedure of Liu [17].

## Results

Glycerol-3-*P* dehydrogenase was isolated using a combination of ammonium sulfate fractionation, ion-exchange, and affinity chromatography. This simple purification scheme, which takes advantage of the specificity of the enzyme for NADH and glycerol-3-*P*, was found to produce the enzyme in relatively high yields. A summary of the results are shown in Table I. The purified enzyme was

TABLE I

PURIFICATION OF GLYCEROL-3-*P* DEHYDROGENASE

TNB refers to elution from a trinitrobenzene-bound agarose column [10]. 6-Phosphogluconate refers to elution from a 6-phosphogluconate-bound agarose column [13].

| Purification step | Protein (mg) | Activity (units) | Specific activity (units/mg) | Percent yield | Purification (-fold) |
|---|---|---|---|---|---|
| 1. Homogenate | 28 400 | 20 000 | 0.70 | 100 | 1.0 |
| 2. $(NH_4)_2SO_4$ | 26 600 | 20 000 | 0.75 | 100 | 1.1 |
| 3. TNB | 350 | 8 700 | 25 | 44 | 36 |
| 4. DEAE-cellulose | 116 | 7 800 | 67 | 39 | 96 |
| 5. 6-Phosphogluconate | 48 | 7 100 | 148 | 36 | 211 |

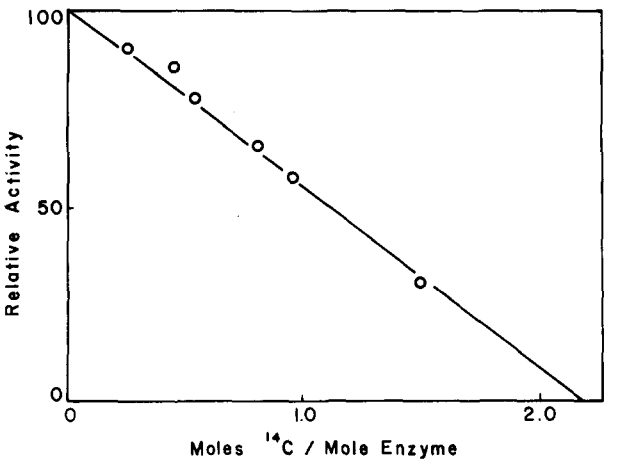


Fig. 1. The activity of glycerol-3-*P* dehydrogenase as a function of the mol $^{14}C$ incorporated/mol enzyme. 2 $\mu$M enzyme was incubated with 11 mM iodo[2-$^{14}C$]acetic acid and at various times aliquots were removed and assayed for enzyme activity or radioactivity.

found to be homogenous as judged by polyacrylamide gel electrophoresis and had a specific activity characteristic of the homogenous enzyme [10,18].

The time-dependent alkylation of glycerol-3-*P* dehydrogenase by iodoacetate was followed by measuring the loss of catalytic activity. Incubation of the enzyme with a large excess of iodoacetate resulted in a complete loss of catalytic activity. The time course for this inactivation was found to be kinetically (pseudo) first order as expected for a simple bimolecular reaction carried out in the presence of excess alkylating reagent. Moreover, the rate of inactivation was a linear function of the concentration of iodoacetate (up to 6 mM). The inactivation rate was found to be independent of enzyme concentration in the range 0.06—0.6 $\mu$M when iodoacetate was maintained at 100—1000-fold excess.

To determine if the rate of inactivation was identical to the rate of alkylation and to determine the stoichiometry of the reaction, iodo[2-$^{14}C$]acetic acid was used to measure the rate of incorporation of carboxymethyl groups into the enzyme. The enzyme was incubated with iodo[2-$^{14}C$]acetic acid and aliquots were removed at various times for measurement of enzyme activity and bound radioactivity. It was found that the loss of enzyme activity was parallel to the incorporation of radioactivity as shown in Fig. 1. There is a near-linear dependence of activity on the mol ratio of [$^{14}C$]carboxymethyl groups bound to the enzyme. Complete loss of enzyme activity occurs when approx. 2 mol [$^{14}C$]carboxymethyl groups are incorporated/mol of enzyme.

Automated amino acid analysis and titration with dithionitrobenzoate revealed that the incorporation of carboxymethyl groups was accompanied by the loss of cysteine residues. The amino acid composition of the native and carboxymethyl-enzyme, presented in Table II, shows that the reaction with iodoacetate results in the loss of 0.9 cysteine residue/enzyme subunit. These results were supported by titrations with dithionitrobenzoate which revealed the presence of eight sulfhydryl groups/subunit in the native enzyme and seven sulfhydryl groups/subunit in the carboxymethyl-enzyme.

TABLE II

AMINO ACID COMPOSITION OF NATIVE AND CARBOXYMETHYLATED GLYCEROL-3-*P* DEHYDROGENASE

Enzyme (48 $\mu$M) was reacted with 10 mM iodoacetic acid in 50 mM Tris, pH 7.5, for 8 h at room temperature. The reaction was terminated by the addition of trichloroacetic acid to a final concentration of 10% and the precipitated enzyme was washed and subjected to hydrolysis. The unmodified enzyme was treated similarly without the addition of iodoacetic acid.

| Enzyme | Amino acid | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Trp | Lys | His | Arg | Asp | Thr | Ser | Glu | Pro |
| Unmodified enzyme | 2.1 | 21.1 | 7.1 | 7.0 | 22.8 | 11.8 | 11.6 | 36.5 | 13.5 |
| Carboxymethyl-enzyme | 1.7 | 20.8 | 6.7 | 6.8 | 23.1 | 10.4 | 10.9 | 35.8 | 14.0 |
| | Gly | Ala | Val | Met | Ile | Leu | Tyr | Phe | Cys |
| Unmodified enzyme | 29 | 22.4 | 25.3 | 5.7 | 20.3 | 25.1 | 5.2 | 11.9 | 7.8 |
| Carboxymethyl-enzyme | 29 | 24.4 | 26.3 | 6.8 | 21.1 | 24.2 | 4.8 | 12.2 | 6.9 |

In order to probe the role of the essential thiol groups in the catalytic mechanism, the alkylation reaction was carried out in the presence of substrates and other ligands. Table III shows the effect of various compounds on the rate of inactivation of the enzyme by iodoacetate. A slight but significant increase of the rate of inactivation was found in the presence of dihydroxyacetone phosphate whereas glycerol-3-*P*, $NAD^+$ and adenosine diphosphoribose showed smaller effects. By far the most striking result was the strong protective effect provided by low concentrations of NADH. The combination of NADH and glycerol-3-*P* together had essentially the same effect as that of NADH alone.

The simplest explanation for the protective effect of NADH is that NADH and iodoacetate compete for interaction with the essential thiols. That is, the

TABLE III

EFFECT OF VARIOUS COMPOUNDS ON THE RATE OF ALKYLATION OF GLYCEROL-3-*P* DEHYDROGENASE

The inactivation reactions were conducted at 30°C in the presence of 0.37 mM iodoacetate and 0.34 $\mu$M enzyme. All other conditions are as described in the legend to Fig 1. For the relative inactivation rate the rate in the absence of added ligands was set equal to 100. ADPR, adenosine diphosphoribose; DHAP, dihydroxyacetone phosphate.

| Compound | Concn. (mM) | Relative inactivation rate |
|---|---|---|
| 1. None | — | 100 |
| 2. NAD | 0.8 | 130 |
| 3. Glycerol-3-*P* | 15.3 | 123 |
| 4. NADH | 0.003 | 8 |
| 5. NADH + glycerol-3-*P* | 0.003 + 15.3 | 8 |
| 6. ADPR | 0.6 | 120 |
| 7. ADPR | 8.6 | 120 |
| 8. DHAP | 0.6 | 225 |

binding of NADH to the enzyme active site prevents reaction with iodoacetate and only unbound enzyme sites react with the reagent. Such a mechanism can be depicted as:

$$\begin{array}{l} \text{ESH + iodoacetate} \xrightarrow{k_0} \text{ES-carboxymethyl} \\ \text{NADH} \uparrow\downarrow \\ \text{ESH} \cdot \text{NADH} \end{array}$$

According to this mechanism, the rate of reaction in the presence of NADH is related to the rate of reaction in the absence of NADH by the factor $(1 + [\text{NADH}]/K_D)$ were $K_D$ is the dissociation constant and [NADH] is the concentration of unbound NADH. Thus

$$k_{app} = k_0 / \; 1 + \frac{[\text{NADH}]}{K_D}$$

where $k_{app}$ is the rate constant in the presence of NADH and $k_0$ is the rate constant in the absence of NADH. If such a mechanism applies to the alkylation reaction, then a plot of $(k_0/k_{app} - 1)$ versus NADH should be linear with a slope of $1/K_D$. Such plot is shown in Fig. 2. The calculated dissociation constant $K_D$ was found to be 0.3 $\mu$M. The linear plot indicates that it is unnecessary to assume the existence of more than one dissociation constant for the interaction of NADH with the possible active enzyme forms.

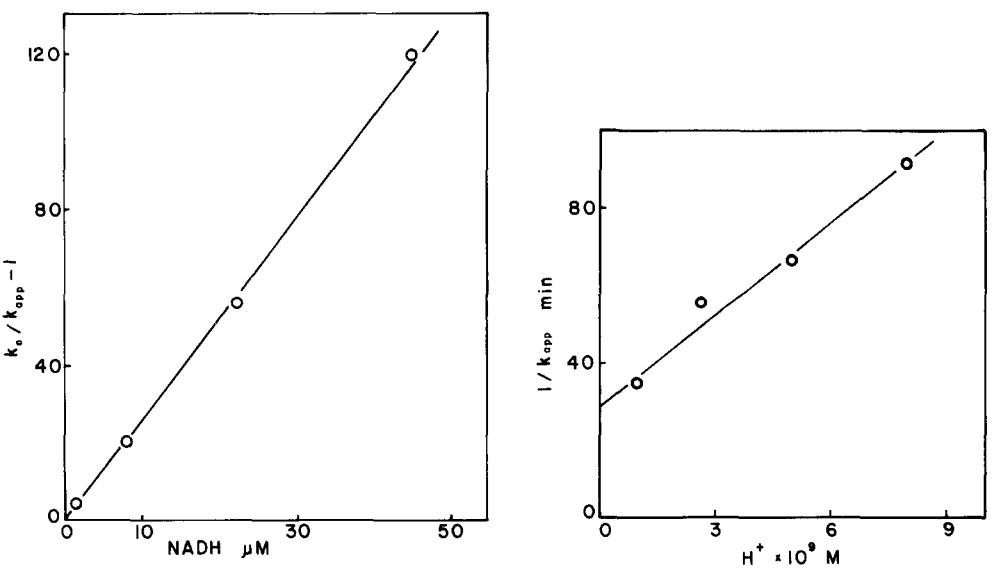


Fig. 2. The effect of NADH on the rate of inactivation of glycerol-3-*P* dehydrogenase by iodoacetate. 0.2 $\mu$M enzyme was incubated with 6 mM iodoacetate in the presence of various concentrations of NADH and the rate of inactivation was measured. The rate in the absence of NADH is indicated by $k_0$ and the rate in the presence of NADH is indicated by $k_{ap}$. All other conditions are indicated in the legend to Fig. 1.

Fig. 3. Plot of $1/k_{app}$ versus the $H^+$ concentration. 0.85 $\mu$M enzyme was incubated with 16.3 mM iodoacetate and the apparent rate constant for inactivation ($k_{app}$) was measured at various pH values. The buffer used was 50 mM triethanolamine. All other conditions are as indicated in the legend to Fig. 1.

To determine if the alkylated enzyme has lost the ability to bind NADH, fluorescence and equilibrium bindings studies were carried out. It is well established [19] that binding of NADH to glycerol-3-*P* dehydrogenase results in the enhancement of the NADH fluorescence. Using fluorescence as a measure of NADH binding, the carboxymethyl-enzyme was titrated with NADH. It was found that the fully carboxymethylated enzyme was incapable of enhancing the fluorescence of NADH. From these findings it was concluded that NADH does not bind to dicarboxymethylated enzyme. These results were supported by equilibrium dialysis experiments which failed to show binding to the modified enzyme. A minimum dissociation constant of 60 $\mu$M was estimated from the binding studies.

The ionization properties of the essential sulfhydryl groups were examined by measuring the rate of alkylation as a function of pH. Fig. 3 shows a plot of the inverse of the apparent rate constant as a function of the $H^+$ concentration in triethanolamine buffer. From this plot a $pK_a$ of 8.5 was calculated. This value presumably applies to the ionization of the essential thiol groups. The same value of the $pK_a$ was found when Tris was used as the buffer.

## Discussion

The sulfhydryl groups of glycerol-3-*P* dehydrogenase, like those in many other dehydrogenases, appear to play an essential role in the catalytic process. Anderson et al. [7] have shown that a series of *N*-alkyl maleimides readily inactivate the enzyme by reacting with sulfhydryl groups. They found that *N*-ethyl maleimide reacts with all cysteine residues. Chloromercuribenzoate is another sulfhydryl reagent which as been shown to inhibit the enzyme [6,9]. In this case a total of 6—7 mol of inhibitor are required for complete inactivation. Sajgo and Telegdi [8] found that bromoacetate was more selective than the above reagents in that the carboxymethylation of 1.4—2.0 sulfhydryl groups resulted in a 70—75% loss of enzyme activity. We have now found, by utilizing radiolabeling methods, amino acid analysis and titration with dithionitrobenzoate, that iodoacetate specifically alkylates two cysteine residues/(dimeric) enzyme. Moreover, these cysteine residues appear to be essential for enzyme activity.

Carboxymethylation of one enzyme subunit evidently does not markedly influence the catalytic activity of the unmodified site. If one assumes that both of the NADH binding sites in the native enzyme are catalytically active, then a linear relationship between the enzyme activity and the mol ratio of carboxymethyl groups to enzyme would exist (cf. Fig. 1) only if the unmodified site had a specific catalytic activity insensitive to carboxymethylation of the adjacent site [20].

Further evidence that monoalkylation does not alter the unmodified site comes from studies of the effect of NADH concentration on the alkylation rate. The dependence of rate on NADH concentration is consistent with the idea that NADH acts as a simple competitor with iodoacetate for the thiol site, i.e NADH binding prevents alkylation. The dependence of alkylation rate on NADH concentration is predictable on the basis of an apparent NADH dissociation constant of 0.3 $\mu$M for interaction with essential thiols. This value is

similar to the NADH dissociation constant determined independently by fluorescence titration [19,21]. These results thus support the idea that there is only a single type of NADH binding site on the enzyme.

To see if a reciprocal relationship exists between NADH binding and carboxymethylation, the binding of NADH to dicarboxymethylated enzyme was measured. The results of the binding studies demonstrate that carboxymethylation prevents the binding of NADH, an effect which is sufficient to account for the loss of enzyme activity. The role of the sulfhydryl groups in NADH binding was further explored by examining the pH dependence of the alkylation reaction. This reaction showed a dependence on a group with a $pK_a$ of 8.5. Assuming a simple nucleophilic reaction between the cysteine thiolate anion and the iodoacetate, this $pK_a$ can be assigned to the ionization of the essential thiols. Since it has been shown [21] that the binding of NADH to the enzyme depends on a group (or groups) with $pK_a$ of approximately 7.2, it follows that the binding of NADH is independent of the ionization state of the essential thiols.

The rate of alkylation of glycerol-3-*P* dehydrogenase by iodoacetate was found to be relatively insensitive to the presence of various substrates and coenzymes with the striking exception of NADH. Even $NAD^+$ at high concentrations has little effect on the reaction rate. This phenomenon is not limited to the carboxymethylation reaction since Anderson et al. [7] found that NADH, and not $NAD^+$ or other nucleotides tested, protects the enzyme from inactivation by *N*-ethyl maleimide. These differences between $NAD^+$ and NADH may be accountable by the dissimilarity of the overall conformation of the oxidized and reduced coenzymes. Kaplan and Sarma [22] reported changes in the geometry of the pyridine ribose on reduction of the oxidized form as well as apparent alternation of the diphosphate backbone. The stereochemical differences in binding of the coenzymes may lead to direct steric hindrance of the thiol site in the case of NADH but not $NAD^+$. However, an induced conformational change accompanying binding cannot be ruled out as an explanation of the protection offered by NADH. Indeed, coenzyme binding is known to be accompanied by conformational alterations in some dehydrogenases. Especially well documented are the changes induced by coenzyme binding in lactate dehydrogenase [23,24] and glyceraldehyde-3-phosphate dehydrogenase [25].

Similarities between several pyridine nucleotide-linked dehydrogenases have been found in primary sequence [1], tertiary and quaternary structure and kinetic mechanism [4]. X-ray crystallographers have noted [2,3] strong similarities in the tertiary structure of the subunits of malate dehydrogenase and lactate dehydrogenase. In addition the coenzyme binding domains of these two enzymes as well as liver alcohol dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase have obvious similarities including the conformation and the position of the bound coenzyme with respect to the $\beta$-sheet structure of the protein subunit [26]. Another general feature of the pyridine nucleotide-linked dehydrogenases is their high reactivity toward sulfhydryl reagents [27—30]. In most cases, the stoichiometry of the inactivation reaction is one cysteine residue modified/enzyme subunit. Because of these similarities the essential cysteie residues have been used as a point of origin in searches for homology in the amino acid sequences of these enzymes. These results have been used to

suggest an evolutionary connection between these enzymes although Rossmann et al. [26] have pointed out that some of these comparisons are inappropriate since the essential sulfhydryl groups are not always located on the same structural domains and consequently are unlikely to have a common evolutionary origin.

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