

## Evidence for the Essential Arginine and Histidine Residues in Catalytic Activity of Glucose 6-Phosphate Dehydrogenase from *Streptomyces aureofaciens*

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### Abstract

Glucose 6-phosphate dehydrogenase (G6PD) was purified from *Streptomyces aureofaciens* and inactivated with butanedione and diethylpyrocarbonate. Incubation of the enzyme with butanedione resulted in a rapid activity loss (80%) within 5 min, followed by a slow phase using a molar ratio to enzyme concentration of 100. Fluorescence studies showed a conformational change in the butanedione-modified enzyme. NAD<sup>+</sup>, NADP<sup>+</sup> and glucose 6-phosphate protected the enzyme against inactivation. Diethylpyrocarbonate (2 mM) completely inactivated the enzyme after 2 min. Stoichiometry of the inactivation showed 2 moles of histidine residues per mole of enzyme with complete activity loss. Maximum emission spectrum of the enzyme decreased (23%) upon modification and the presence of NAD<sup>+</sup> or NADP<sup>+</sup> further decreased the fluorescence by 27% and 10.5%, respectively. The data suggest that essential arginine and histidine residues may be involved in the catalytic activity of *Streptomyces aureofaciens* G6PD.

**Keywords:** Glucose 6-phosphate dehydrogenase; Essential amino acids

### Introduction

Glucose 6-phosphate dehydrogenase (EC. 1.1.1.49, G6PD) catalyzes the first reaction in the hexose monophosphate pathway providing a major source for NADPH necessary for biosynthetic reactions [1]. In eukaryotes and many prokaryotes, G6PD consumes NADP<sup>+</sup> as coenzymes. In some prokaryotes, G6PD participates in two or more metabolic pathways. Such organisms consist either two distinct G6PD's, NAD<sup>+</sup> or NADP<sup>+</sup> preferring, or a single enzyme that is dual

coenzyme specific [2]. Among the later G6PD form *loconostoc mesenteroides* has been investigated extensively [1-3]. It has been clearly shown by kinetic [4], fluorescence [5], denaturation and renaturation [6], x-ray crystallography [7,8] and site directed mutagenesis [9,10] studies that in *L. mesenteroides* different conformational isomers of G6PD bind NAD<sup>+</sup> or NADP<sup>+</sup>.

Another G6PD that catalyzes both NAD<sup>+</sup> and NADP<sup>+</sup>-linked reaction is from *Streptomyces aureofaciens* [11]. For this enzyme two different forms

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have been proposed for  $\text{NAD}^+$  and  $\text{NADP}^+$  activities using fluorescence and kinetic studies [12,13]. Reassociation and reactivation of this enzyme after denaturation by urea [14] revealed that  $\text{NAD}^+$  and  $\text{NADP}^+$  binding pull the enzyme towards different conformational isomers. However, the mechanism by which *Streptomyces aureofaciens* G6PD selects coenzyme, is not well understood yet. Identification of amino acids involved in either substrate binding or catalytic activity of the enzymes may lead to the better understanding of this mechanism. Essential amino acids such as arginine residues in mandelate dehydrogenase [15], 17- $\beta$ -hydroxysteroid dehydrogenase [16] and human erythrocyte G6PD [17]; histidine in alkaline phosphatase [18] and sucrose phosphate synthase [19] and other amino acids in phenylalanine lyase [20] have been reported.

We have recently shown that two lysine residues are essential for substrate binding to *Streptomyces aureofaciens* G6PD [21]. In the present study the roles of arginine and histidine residues in the catalytic activity of this enzyme are reported.

## Materials and Methods

### Materials

Glucose 6-phosphate,  $\text{NAD}^+$ ,  $\text{NADP}^+$ , butanedione, diethylpyrocarbonate, DEAE-Cellulose and Sephadex G-100 were purchased from Sigma Co. (U.S.A.). All other chemicals were reagent grade. *Streptomyces aureofaciens* (#1119) was obtained from Iranian Scientific Research Organization (Tehran).

### Enzyme Assay

G6PD activity was measured in 40 mM Tris-HCl buffer pH 7.4 containing 1 mM  $\text{NADP}^+$ , 5 mM glucose 6-phosphate, 40 mM  $\text{MgCl}_2$  and appropriate amount of the enzyme, as described before [11], using a Perkin-Elmer spectrophotometer model 55 IS at 25°C.

### Enzyme Purification

*Streptomyces aureofaciens* cells were cultured according to Behal [22]. The cell were suspended in 0.2 M Tris-HCl buffer pH 7.4 containing 15% (v/v) glycerol, 1 mM  $\beta$ -mercaptoethanol, 2 mM EDTA and 0.05 mM phenylmethylsulfonyl fluoride. The suspension was sonicated at 22 kHz for 30 sec and repeated 13 cycles to break the cells. The disrupted cells were diluted in the above buffer (1:3), centrifuged at 20,000 g for 30 min. The supernatant was kept for the

enzyme purification. The enzyme was purified by ammonium sulfate precipitation and chromatography on DEAE-Cellulose and Sephadex G-100 columns as described before [14].

### Arginine Modification

G6PD (2  $\mu\text{M}$ ) was incubated with different concentrations of 2,3-butanedione in 50 mM borate buffer pH 7.5 in the dark at 25°C. Butanedione solutions were prepared immediately before use. The enzyme activity was measured at time intervals indicated in Figure 1.

### Histidine Modification

The enzyme (0.5 mg/ml) was incubated with different concentrations of diethylpyrocarbonate in 40 mM Tris-HCl buffer pH 7.4 at room temperature and at the indicated time intervals (see Fig. Legends) the enzyme activity was measured. Diethylpyrocarbonate solutions were freshly prepared just before use by diluting in absolute ethanol. The concentration of each solution was precisely determined by reacting with 10 mM imidazole solution (pH 7.5) using molar absorbance of  $3 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$  at 230 nm [19]. The number of histidine residues per mole of the enzyme was determined using molar absorptivity of  $3.2 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$  at 242 nm for N-carboxyhistidyl derivative formation [18]. Molecular weight of the enzyme dimer was 107,000 Da [14]. Control experiments were routinely carried out in which diethylpyrocarbonate was omitted.

### Other Methods

Fluorescence studies were done in 40 mM Tris-HCl buffer pH 7.4 at 25°C using a Perkin-Elmer model LS-3B spectrophotofluorimeter. Protein concentration was determined by the method of Lowry [23].

## Results

The purified enzyme showed a specific activity of 1-9 U/mg protein corresponding to our previous report [14].

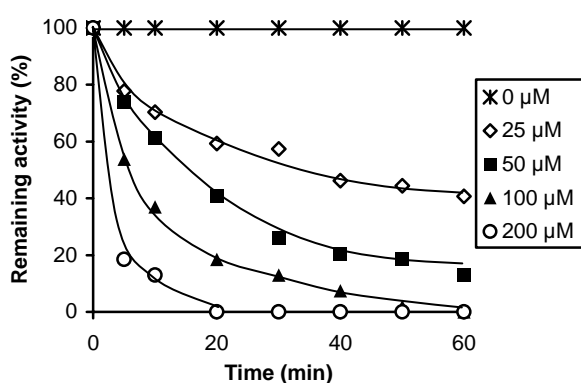
### Inactivation by Butanedione

Incubation of G6PD with butanedione rapidly inactivated the enzyme (Fig. 1). With a molar ratio to enzyme concentration of 100 complete inactivation was achieved in 20 min. The activity of the control sample

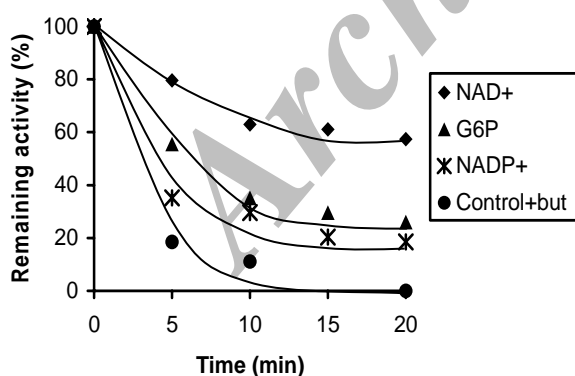
did not change during this period. Neither dilution nor dialysis of the modified enzyme caused enzyme reactivation. The maximum fluorescence of the intrinsic protein of the enzyme increased (44%) upon modification with butanedione and subsequent dialysis (Fig. 2).

### The Effects of Ligands on 2,3-Butanedione Inactivation

The presence of  $\text{NAD}^+$  ( $9.2 K_m$ ),  $\text{NADP}^+$  ( $13 K_m$ ) or glucose 6-phosphate ( $10 K_m$ ) in the incubation mixture protected the enzyme inactivation by butanedione by 65%, 26%, and 37%, respectively (Fig. 3). The  $K_m$  values were as reported before [11].



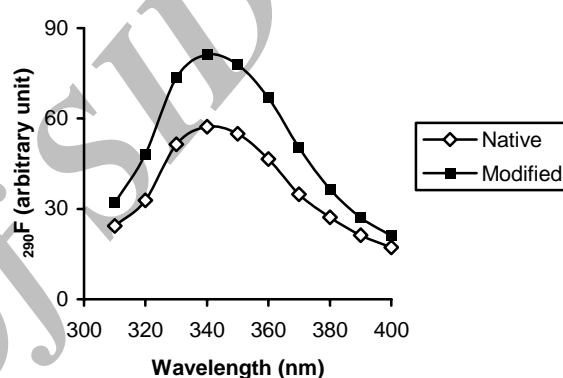
**Figure 1.** Inactivation of G6PD with butanedione. Enzyme ( $2 \mu\text{M}$ ) was incubated in 50 mM borate buffer pH 7.5 with butanedione at concentrations of 0, 25, 50, 100, and 200  $\mu\text{M}$ . The enzyme activity was measured at the indicated time intervals. Each point represents the mean of 2 independent experiments. For details see text.



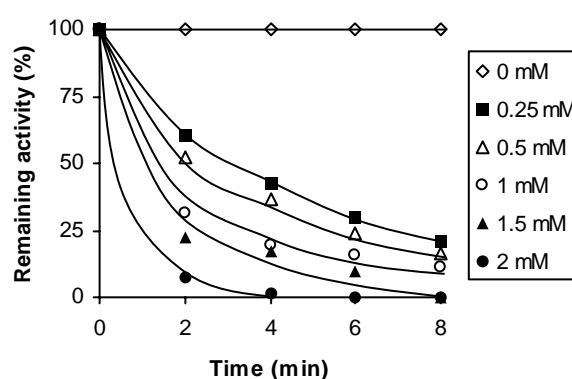
**Figure 3.** Protective effects of ligands on G6PD inactivation by butanedione. The enzyme ( $2 \mu\text{M}$ ) was incubated in 50 mM borate buffer pH 7.5 with butanedione (200  $\mu\text{M}$ ) (Control+but) and in the presence of 2.5 mM  $\text{NAD}^+$ , 8 mM glucose 6-phosphate (G6P) or 1 mM  $\text{NADP}^+$ . The enzyme activity was measured at the indicated time intervals. Each point represents the mean of 2 independent experiments.

### Inactivation by Diethylpyrocarbonate

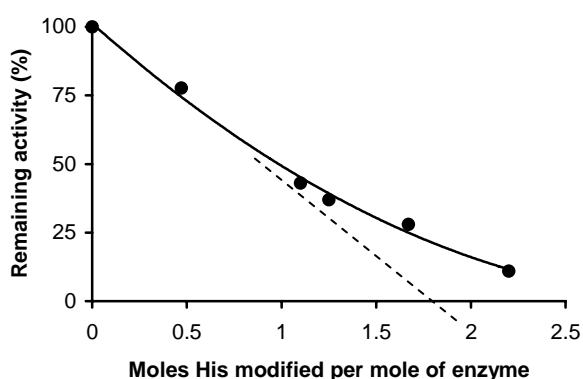
Modification of G6PD with diethylpyrocarbonate caused enzyme inactivation which was time and concentration dependent (Fig. 4). Complete inactivation was achieved within 6 min at 2 mM diethylpyrocarbonate. Stoichiometry of the reaction showed that 2 moles of histidine residues per mole of the enzyme were modified when 100% of the enzyme activity was lost (Fig. 5). The maximum fluorescence of the diethylpyrocarbonate-modified enzyme was decreased by about 20% and the presence of  $\text{NAD}^+$  and  $\text{NADP}^+$  further decreased the fluorescence by 40% and 37%, respectively (Fig. 6).



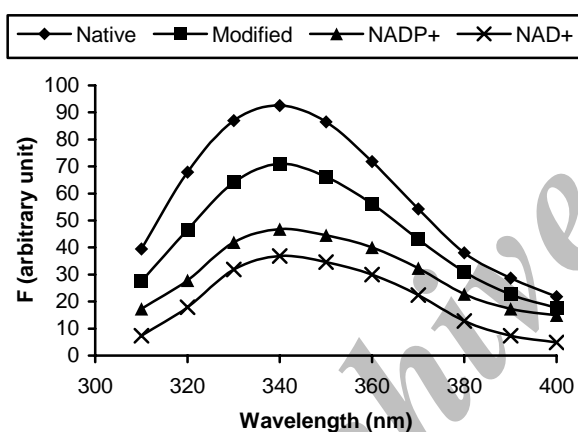
**Figure 2.** Emission spectra of the native and butanedione-inactivated enzyme. Both native and modified enzyme ( $2 \mu\text{M}$ ) samples were dialyzed vs. 0.4 mM Tris-HCl buffer pH 7.4 for 24 h at  $4^\circ\text{C}$  and the intrinsic protein fluorescence measured as described in text.



**Figure 4.** Inactivation of G6PD by diethylpyrocarbonate. The enzyme ( $2 \mu\text{M}$ ) was incubated in 40 mM Tris-HCl buffer pH 7.4 in the presence of 0, 0.25, 0.5, 1, 1.5, or 2 mM diethylpyrocarbonate at room temperature and the enzyme activity measured at the time intervals. Each point represents the average of 2 independent experiments.



**Figure 5.** Stoichiometry of G6PD inactivation by diethylpyrocarbonate. The enzyme (0.5 mg/ml) was incubated with different concentrations of diethylpyrocarbonate (0-1.5 mM) in 40 mM Tris-HCl buffer pH 7.5 for 8 min prior to the enzyme assay and then the number of histidine residues modified per mole of enzyme determined as described in Methods.



**Figure 6.** Emission spectra of the native and diethylpyrocarbonate-modified G6PD. The fluorescence of native and modified enzyme samples, each at 60  $\mu$ M concentration, was measured in 40 mM Tris-HCl buffer pH 7.5 by exciting at 290 nm. The fluorescence of the modified enzyme was also measured in the presence of NAD<sup>+</sup> or NADP<sup>+</sup>. Each point represents the average of 2 independent experiments.

### Discussion

Different amino acid side chains have been recognized to be essential for the activities of enzymes. The involvement of arginine residues in the catalytic activities of enzymes such as human erythrocyte G6PD [17], pyrophosphate fructose 6-phosphate 1-phosphotransferase [24] and ATPase [25] has been reported. In the present study butanedione, an arginine modifying

reagent, with a molar ratio to enzyme concentration of 100, about 80% of the enzyme activity was lost within 5 min, followed by a slow phase of inactivation. The slow phase could be due to a conformational change resulted from modification of essential arginines which affects the accessibility of the residues to the modification. This is also evident from the fluorescence change of the enzyme upon butanedione reaction. Several reports have suggested that essential arginine residues selective modification in various enzymes are presumed to be located in a special hydrophobic environment in the interior of protein structure [25,26].

Protection against butanedione inactivation by coenzymes and glucose 6-phosphate (Fig. 3) suggested that one or more arginine residues may be involved in the coenzyme or substrate binding sites. In human erythrocyte G6PD also arginine residue(s) play an important role in substrate binding [17]. The finding that NAD<sup>+</sup> exerts more protective effect than NADP<sup>+</sup>, is consistent with different conformational changes induced in the enzyme upon coenzyme binding [12,13].

Modification of *Streptomyces aureofaciens* G6PD with diethylpyrocarbonate, a known histidine modifying reagent, resulted in complete loss of the enzyme activity at concentrations higher than 1.5 mM. The activity loss was parallel with the modification of 2 histidine residues per mole of enzyme. Structural studies demonstrated that the active form of this enzyme is a dimer with two identical subunits [21]. Each subunit, therefore, may consist of one histidyl residue necessary for the enzyme activity. The imidazole ring of histidine was reported to be involved in the catalytic activity of rat liver phosphatidate phosphohydrolase [27].

The different effects of NAD<sup>+</sup> and NADP<sup>+</sup> on the intrinsic protein fluorescence of the diethylpyrocarbonate-modified enzyme revealed that each coenzyme binds to different conformational isomer which confirms the previous studies [12-14].

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