GLYCEROL 3-PHOSPHATE DEHYDROGENASE: LOSS OF DPNH BINDING FOLLOWING PHOTOOXIDATION

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1. Introduction

Recently Rose Bengal has been shown to mediate the photoinactivation of glycerol 3-phosphate dehydrogenase [1] and 6-phosphogluconate dehydrogenase [2]. In the case of 6-phosphogluconate dehydrogenase, inactivation occurred only when Rose Bengal was bound to the TPN binding site of the enzyme. The present communication demonstrates that photoinactivation of glycerol 3-phosphate dehydrogenase occurs when the dye is bound to the DPHN binding site and that photoinactivation is accompanied by the loss of the capacity of the enzyme to bind DPNH.

2. Methods

Glycerol 3-phosphate dehydrogenase was prepared as described previously [3] and was recrystallized three times. To remove DPNH, the enzyme was treated with dihydroxyacetone-P, to oxidize the DPNH to DPN, and then was passed through Sephadex G-25.

Photooxidation was carried out in 50 mM tris-HCl buffer (pH 7.5) in the presence of 1 mM EDTA, 0.1 mM dithiothreitol and Rose Bengal at the desired concentration. The reaction was performed at 20° with shaking in a Warburg apparatus (Gilson Medical Electronics) with a clear Lucite bottom. The enzyme solution was placed in a Warburg flask at a distance of 10 cm from the bottom of the bath and a total distance of 13 cm from a 150 W GE reflector spot.

Enzyme activity determinations were performed at 26° and pH 7.5 in the presence of 0.2 mM dihydroxyacetone-P and 0.2 mM DPNH except where indicated.

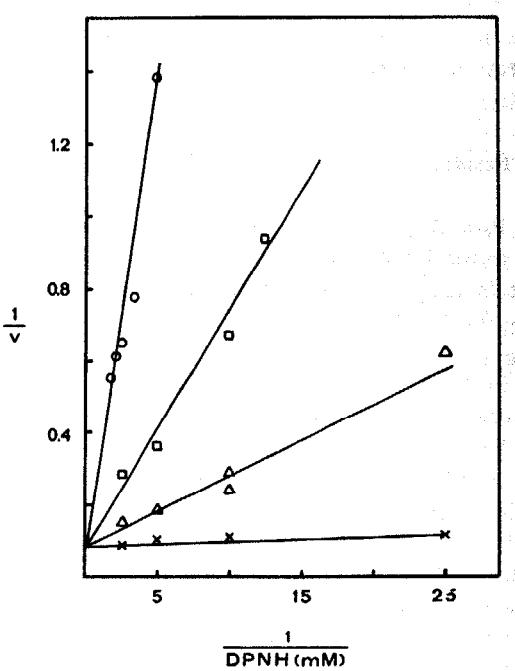

Fig. 1. Inhibition of glycerol 3-phosphate dehydrogenase by Rose Bengal. Assays performed as described in Methods with Rose Bengal at the following concentrations: ×, no addition; △, 5 μM; □, 10 μM; ○, 25 μM.

* This work was done during the tenure of an Established Investigatorship of The American Heart Association.

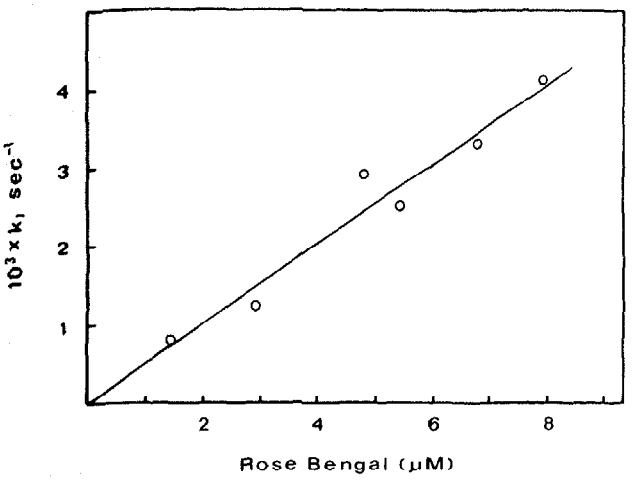

Fig. 2. Dependence of rate of photoinactivation on Rose Bengal concentration. Oxidations carried out as described in Methods with 1.0 mg/ml glycerol 3-P dehydrogenase. Rate constants determined from plots of ln (initial activity/activity at time) vs. time.

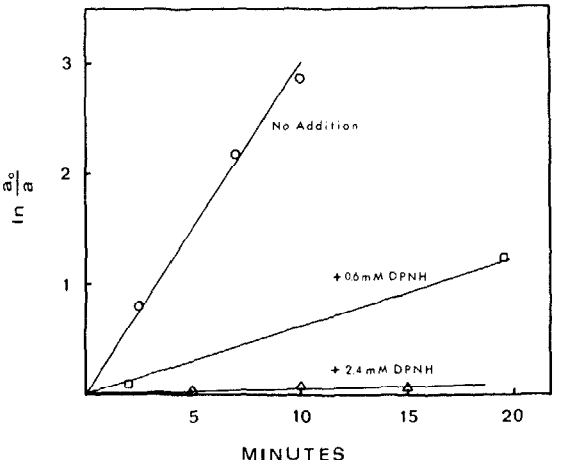

Fig. 3. Protection against inactivation by DPNH. Photoinactivation carried out as described in Methods with 1.0 mg/ml enzyme. Initial activity is designated by (a_0) and activity at any given time is designated (a).

Fluorescence measurements were carried out with an Aminco-Keirs spectrophosphorimeter employing an excitation wavelength of 340 nm and measuring emission at 455 nm.

3. Results

Rose Bengal was found to be a potent inhibitor of glycerol 3-phosphate dehydrogenase, the inhibition being competitive with respect to DPNH (fig. 1). The K_m for DPNH has been reported to be less than 10 μM [4]. From the results in fig. 1 the K_i for Rose Bengal appears to be less than 1 μM, indicating a very tight binding to the DPNH site.

When the dehydrogenase and low concentrations of Rose Bengal were irradiated with white light, an irreversible loss of enzyme activity occurred. There was no loss of activity when the enzyme was incubated with Rose Bengal in the dark or when irradiated without the dye. The loss of activity roughly followed first order kinetics although in some instances the rate of activity loss appeared to increase slightly with time. Fig. 2 describes the apparent first order rate constant obtained from inactivation data at several concentrations of Rose Bengal. A reasonably linear relationship is indicated. It should be noted that the maximum Rose Bengal concentration employed (8 μM) was about one-fourth the concentration of the enzyme monomer present in the photooxidation mixture.

DPNH was a very effective inhibitor of the photoinactivation of glycerol 3-phosphate dehydrogenase (fig. 3), indicating that the binding of the dye to the DPNH site was required for photoinactivation. This observation suggests that the inactivation may be the result of the destruction of the DPNH site. Ankel [5] originally demonstrated the binding of DPNH to glycerol 3-phosphate dehydrogenase by the enhancement of DPNH fluorescence. Employing his technique, we have compared the DPNH binding ability of photooxidized enzyme (free of Rose Bengal) with that of the native enzyme. These results are shown in fig. 4. In this particular experiment, the maximum fluorescence enhancement in the presence of enzyme that had 20% of its original activity was about 25% of that observed with native enzyme. In a similar experiment, enzyme that had lost 50% of its initial activity retained about one-half of the original binding capability as measured by fluorescence enhancement. This indicates that the activity loss was due to the loss of the capacity of the enzyme to bind DPNH.

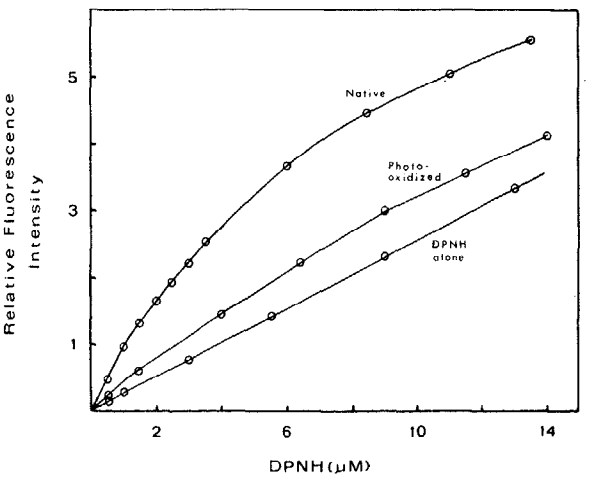

Fig. 4. Enhancement of DPNH fluorescence by glycerol 3-phosphate dehydrogenase. Photooxidation performed as described in Methods with 2.2 mg/ml enzyme (36.5 μM) and 2 μM Rose Bengal. After 50 min photooxidation, the enzyme had lost 80% of its initial activity. The Rose Bengal was removed by gel filtration on Sephadex G-25 in the presence of a buffer consisting of 50 mM TES, 1 mM EDTA, 150 mM KCl (pH 7.5). Fluorescence intensity was measured by adding small increments of DPNH to buffer alone and to both native and photooxidized enzyme. For the fluorescence measurements, both enzyme preparations were present at concentrations of 0.17 mg/ml (2.8 μM) in the buffer described above.

4. Discussion

The identity of the amino acid or amino acids oxidized during the inactivation of glycerol 3-phosphate dehydrogenase remains to be determined. The absorbance of the enzyme at 280 nm remains unchanged by photooxidation suggesting that tryptophan is unaffected. Apitz-Castro and Suarez [1] suggested that histidine is the site of photooxidation based upon the pH dependence of the rate of inactivation. Histidine was also identified as the site of oxidation in the inactivation of two other dehydrogenases, 6-P-gluconate dehydrogenase [2] and glyceraldehyde 3-P dehydrogenase [6]. If indeed histidine is the sole site of photooxidation, it is surprising that such a modification would so dramatically effect DPNH binding, since the binding involves at least three different interacting areas on the enzyme surface [7].

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