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Rose Bengal as a Specific Photosensitizer for a Histidine Residue at the Triphosphopyridine Nucleotide Binding Site of 6-Phosphogluconate Dehydrogenase*

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SUMMARY

Rose Bengal is a potent inhibitor of 6-phosphogluconate dehydrogenase from *Candida utilis* and mediates the photoinactivation of the enzyme. The experiments reported in this paper indicate that photoinactivation occurs only when the dye is bound to the TPN binding site of the enzyme.

Since the photoinactivation is correlated to the specific oxidation of only 2 residues of histidine, it can be assumed that this amino acid is located at the TPN binding site of 6-phosphogluconate dehydrogenase.

These results are a new application of the technique of active site-specific photooxidation.

It has been known for many years that certain amino acids are susceptible to photochemical oxidation catalyzed by dyes (1–3). The elegant procedure of photochemical oxidation mediated by methylene blue, introduced by Weil, Gordon, and Buchert (4), has provided information on the presence of photooxidizable amino acids in the active center of some enzymes (5–7). Ray (8) has reviewed the advantages and the limitations of this method.

Since the light-excited dye is able to react with any photooxidizable amino acid residue with which it comes in contact during its short lifetime, the photochemical oxidation of amino acids is specific neither for the nature nor for the number of the amino acid residues involved (8, 9). In many cases, indeed, various types of amino acids are destroyed and the use of the "all or none" assay was suggested (7) to identify the nature of the amino acid involved in the loss of the catalytic activity of enzymes.

In some special cases (10), the loss of enzymatic activity could be correlated with the specific oxidation of a particular type of amino acid; this could be a fortuitous combination, in which this particular amino acid residue, because of its environment, has a particularly high reactivity.

Westhead, in 1965, introduced a new photosensitizer, Rose Bengal, suggesting that this dye could be more specific than methylene blue (11). However, this dye is not specific (12) and in some cases the inactivation of enzymes was correlated with the destruction of many amino acids (13).

A solution for the problem of making the photochemical oxidation a highly specific method for the detection of amino acid residues in the active center of enzymes could be a suitable choice of sensitizers specific for a particular type of amino acid (14–18).

An alternate and more promising solution is to bind a dye selectively to the active center of the enzyme. In this case only those photooxidizable amino acid residues that are close to the photosensitizer, and hence at, or near, the active center of the enzyme, are destroyed. This technique of active center labeling and subsequent photooxidation not only indicates the presence at the active center of a photooxidizable amino acid residue, but also indicates that this residue and that one involved in the binding of the dye are at a measurable distance in the quaternary structure of the protein molecule in solution.

In a previous paper we have reported the first application of this new strategy. We have indeed shown (19) that pyridoxal 5′-phosphate mediates the selective photooxidation of a histidine residue only when bound to a lysine residue at the 6-phosphogluconate binding site of 6-phosphogluconate dehydrogenase. The oxidation of only 1 histidine residue for enzyme subunits caused the complete inactivation of the enzyme. Since then other applications of this method have been published (20–22).

In the present paper we report another application of the active center specific photooxidation, with Rose Bengal, and show that this dye mediates the oxidation of a histidine residue of 6-phosphogluconate dehydrogenase only when bound to the TPN binding site of the enzyme.

MATERIALS AND METHODS

Enzyme—6-Phosphogluconate dehydrogenase (6-phospho-D-gluconate:NADP oxidoreductase (decarboxylating), EC 1.1.1.44), type I, crystalline, was isolated from *Candida utilis* as previously described (10, 23). Prior to the experiments the enzyme was freed from ammonium sulfate and other salts by dissolving the crystals in a minimum amount of 10 mM Tris-HCl buffer, pH 7.5, containing 0.1 mM EDTA, and passing the enzyme solution through a Sephadex G-25 (Pharmacia, Uppsala) column equilibrated with the same buffer. The enzymatically active fractions were pooled and diluted to an enzyme concentration of 1 mg per ml, unless otherwise specified.

Reagents—6-Phosphogluconate, TPN, TPNH, DPN, 2′-

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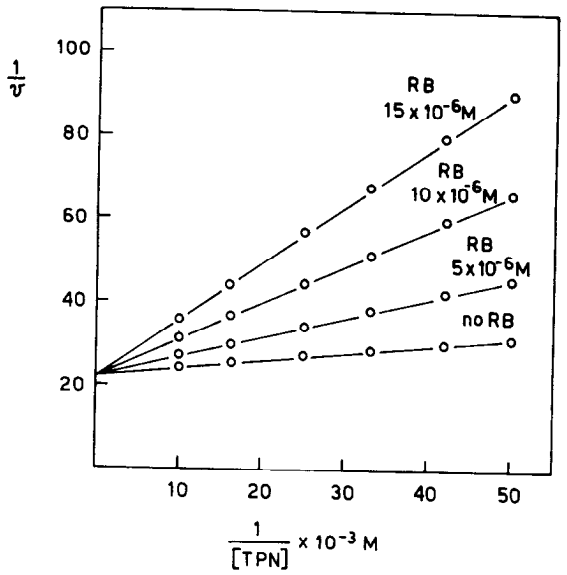

FIG. 1. Inhibition by Rose Bengal as a function of TPN concentration. The reaction mixture contained 20 mM triethanolamine buffer (pH 7.5), 0.3 mM 6-phosphogluconate, and TPN and Rose Bengal at the concentrations indicated in the figure.

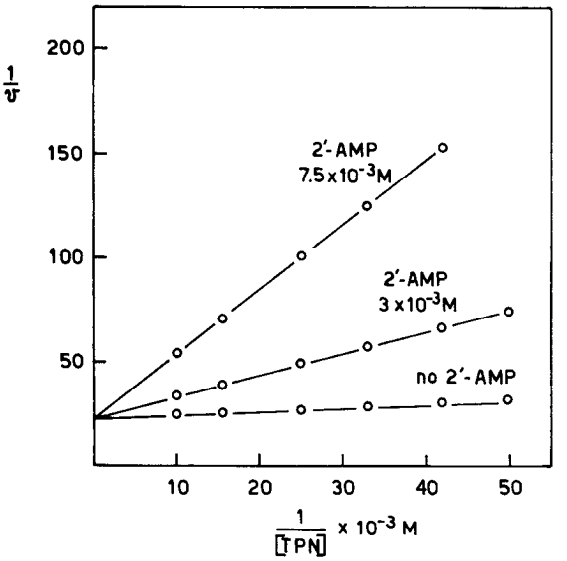

FIG. 2. Inhibition by 2'-AMP as a function of TPN concentration. Reaction conditions as described in the legend of Fig. 1.

AMP, 3'-AMP, 5'-AMP, EDTA, and pyridoxal 5'-phosphate were purchased from Sigma. Rose Bengal (tetraiodotetrachlorofluorescein) was obtained from British Drug Houses, Poole, England. This dye was purified as described by Brand, Gohlke, and Rao (24). An extinction coefficient of 9.5×10^4 liters per mole cm at 545 mμ was used (24).

Procedure for Photooxidation—Photooxidation was carried out in the apparatus previously described (10). Rose Bengal was added to the enzyme solution in the dark; the sample was then divided into two equal parts, one was kept in the dark as a control, and the other exposed to the light. Aliquots were removed from the control and from the illuminated solution at the desired time intervals, diluted 50-fold in 10 mM Tris-HCl buffer, pH 7.5, and tested for enzymatic activity.

TABLE I

Affinity and inhibition constants for substrates and TPN competitive inhibitors

Substrates	K_m	Reference	Inhibitors	K_i
	$\times 10^{-6}$ M			$\times 10^{-6}$ M
6-Phosphogluconate	52	23	Rose Bengal	0.2
TPN	20	23	2'-AMP	440
TPNH	0.47	25		

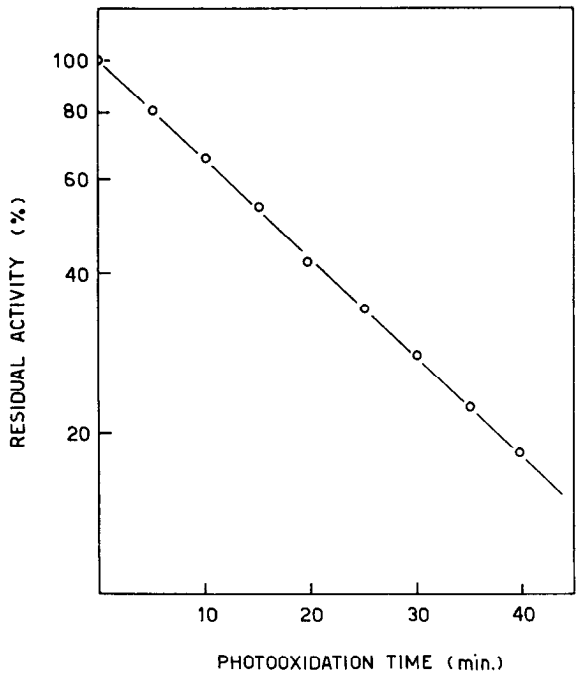

FIG. 3. Kinetics of the inactivation of the enzyme by photooxidation. Reaction conditions for the photooxidation: 0.5 ml of the solution exposed to the light contained 0.005 mM (0.5 mg) 6-phosphogluconate dehydrogenase, 10 mM Tris-HCl buffer, 0.1 mM EDTA, and 0.0015 mM Rose Bengal. The final pH was 7.5 and the temperature was kept at 30°. Aliquots (10 μl) were removed, at the indicated time intervals, and diluted 50-fold in Tris buffer. The diluted enzyme was immediately tested for enzymatic activity.

The amino acid analysis of the control and of the photooxidized enzyme was carried out as previously described (10).

RESULTS

Inhibition of Enzymatic Activity by Rose Bengal—The anionic dye Rose Bengal is a potent inhibitor of the 6-phosphogluconate dehydrogenase, the inhibition being competitive with respect to TPN (Fig. 1). A similar type of inhibition has been observed with 2'-AMP (Fig. 2). The data obtained from these kinetic studies are summarized in Table I, for a comparison with the known values of the affinity constants for the substrates.

The observed inhibition of the enzymatic activity by 2'-AMP seems to be highly specific, since, other compounds, such as 3'-AMP (10 mM), 5'-AMP (10 mM), and DPN (10 mM), do not affect the catalytic activity of the enzyme.

Rose Bengal-mediated Photoinactivation—When a solution containing 6-phosphogluconate dehydrogenase and low concentrations of Rose Bengal is exposed to light, an irreversible loss

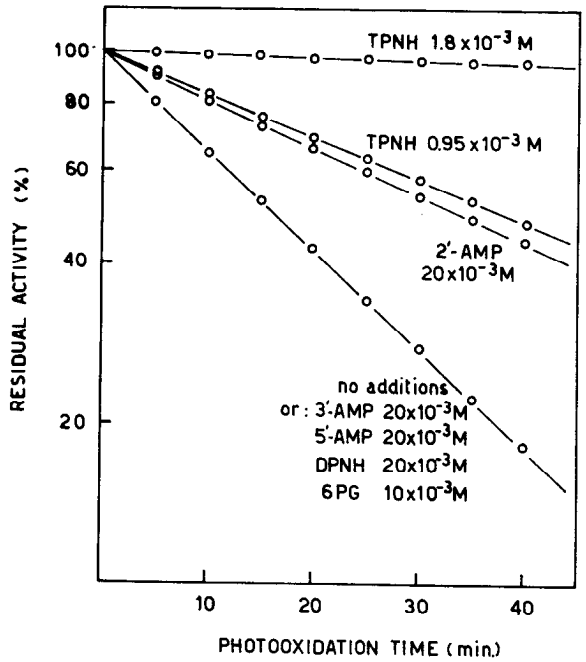

FIG. 4. Effect of TPNH and 2'-AMP on the rate of photoinactivation. Reaction conditions were as described in the legend of Fig. 3 except for the presence, in the photooxidation mixture, of the compounds indicated in the figure.

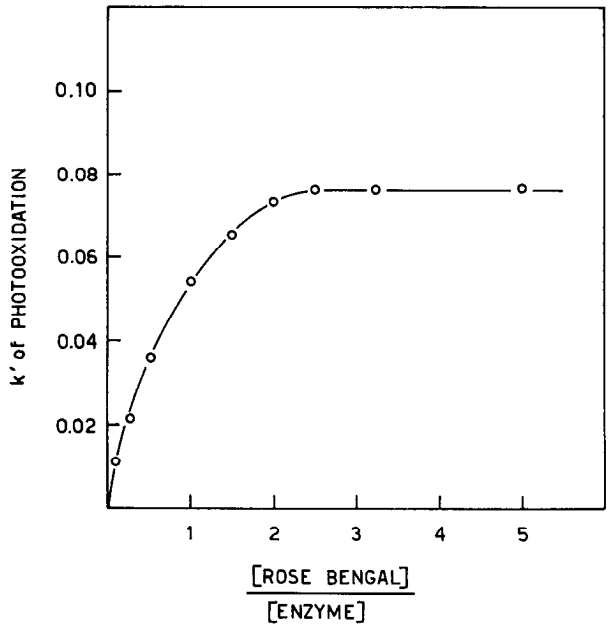

FIG. 5. Effect of the enzyme concentration on the rate of photoinactivation. The apparent first order velocity constants, k', of the photoinactivation process, were obtained graphically from semilogarithmic plots similar to that reported in Fig. 3. Reaction conditions: 0.5 ml of the reaction solution contained 0.58 μM Rose Bengal and the enzyme in decreasing concentrations. The rates of photoinactivation were followed as described in the legend of Fig. 3. When the enzyme concentration was lower than 0.1 mg per ml, the enzyme activity was tested without prior dilution. *Abscissa*, molar ratio of Rose Bengal to enzyme; *ordinate*, first order velocity constant of the photoinactivation process.

of the catalytic activity of the enzyme occurs. The process of photoinactivation follows first order kinetics until the enzyme is completely inactivated (Fig. 3). The enzyme undergoes no irreversible loss of catalytic activity when incubated with Rose Bengal in the dark, or when exposed to the same illumination in the absence of the dye. In order to determine whether the presence of substrates or of compounds structurally related to TPN altered the rate of the photoinactivation process, TPNH, 6-phosphogluconate, 2'-AMP, 3'-AMP, 5'-AMP, and DPNH were added to the enzyme prior to the exposure to the Rose Bengal mediated photooxidation. The results, reported in Fig. 4, indicate that only TPNH and 2'-AMP protect, to different degrees, against the loss of enzymatic activity.

Effect of Concentrations of Rose Bengal and of Enzyme on Rate of Photoinactivation—At constant enzyme concentration, there is a direct proportionality between the concentration of Rose Bengal present in solution and the rate of photoinactivation of the enzyme. This proportionality was expected, being similar to the one observed in photoinactivation experiments with methylene blue or with pyridoxal 5'-phosphate (10, 19).

The rate of photoinactivation, at constant Rose Bengal concentration, depends on the concentration of the enzyme. The plot of the apparent first order velocity constant, k', of the photoinactivation process, against the ratio between the concentrations of Rose Bengal and of the enzyme indicates (Fig. 5) that a maximal velocity is obtained when this ratio reaches an approximate value of 2.

Assuming that a maximal rate of photoinactivation is attained only when all enzyme molecules are bound to Rose Bengal, from the dissociation constant reported in Table I, it appears that a complete saturation of the enzyme and hence a maximal rate of photoinactivation should be achieved only at a higher Rose Bengal-enzyme ratio. This apparent discrepancy between the theoretical and the experimental ratios is explained by the observation that the formation of the dye-enzyme complex is reversible, so that there is always dye free to bind other enzyme molecules. Such an explanation seems correct since, also when the enzyme is in excess with respect to the dye, a complete photoinactivation is achieved, although at a slower rate.

TABLE II

Recovery of amino acids after photoinactivation

The values reported are expressed as moles of amino acid per mole of enzyme.

Amino acid	Control enzyme	Photoinactivated enzyme	
		Untreated	*p*-Hydroxymercuribenzoate-treated
Histidine	12	10.1	10.2
Cysteine	8	5.7	8
Tyrosine	36	36	36
Tryptophan	12	12	12
Methionine	16	16	16

The relation existing between the rate of photoinactivation and the ratio of Rose Bengal to enzyme is another indication that the dye mediates the photoinactivation only when bound to the protein.

Correlation between Inactivation and Amino Acid Destruction—Comparison of the amino acid composition of the control and

of the photoinactivated enzyme revealed no differences in the content of any amino acid residue, other than cysteine and histidine. In Table II (Column 3) are reported the values of those amino acids known to be sensitive to photooxidation. It appears that a 90% loss of the enzyme activity is reached when 2.3 eq of cysteine and 1.9 eq of histidine are destroyed. If photooxidation is carried out following the protection of the —SH groups with *p*-hydroxymercuribenzoate (10, 19), a 90% loss of enzyme activity is obtained when only 1.8 residues of histidine are destroyed (Table II, Column 4).

DISCUSSION

The anionic dye Rose Bengal strongly inhibits, as a result of a competition with the TPN, the 6-phosphogluconate dehydrogenase from *C. utilis*. The dye apparently binds to the coenzyme binding site twice as tightly as does TPNH and approximately 100 times more strongly than TPN.

Kinetic studies revealed that 2′-AMP also inhibits the enzyme, while other compounds, structurally related to the TPN, such as DPN, 3′-AMP, 5′-AMP, are without effect. Such differences indicate the importance of the phosphoric group at position 2′ of the TPN and strongly suggest the presence at the TPN binding site of a positively charged group.

Rose Bengal mediates the photoinactivation of the enzyme. The amino acid analysis of the protein inactivated by photooxidation indicates that the loss of the catalytic activity is correlated with the destruction of only 2 residues of histidine per molecule of enzyme.

This result is of special significance when correlated with the existence in 6-phosphogluconate dehydrogenase of two subunits and two TPNH binding sites (25).

This apparent stoichiometry and the fact that TPNH and 2′-AMP protect against the photoinactivation suggest a high degree of specificity and indicates that, in our experimental conditions, Rose Bengal acts as a photoinactivating agent only if bound to the TPN binding site.

A further characterization of the Rose Bengal binding site is provided by the observation that 2′-AMP competes with TPN, protects against the Rose Bengal-mediated photoinactivation, and causes (as does TPN) changes in the absorption spectrum of the Rose Bengal-enzyme complex (26). These mixed competitions for the same site suggest that the minimal requirement of the TPN for the binding to the enzyme is the 2′-AMP moiety, and that Rose Bengal presumably binds to the enzyme region which is also responsible for the interaction with the 2′-AMP portion of the TPN. According to this hypothesis and to the topographical specificity of the photooxidation, we may postulate that the histidine residue involved in the enzyme inactivation is located in this region of the protein.

A conformational change induced by Rose Bengal and stabilized by the photooxidation could also explain the inactivation of the enzyme. This hypothesis, however, seems unlikely; prior to photoinactivation the enzyme is active and still able to bind TPNH, and so, if conformational changes occurred, upon interaction with the dye, they should be reversible and minimal. A remote possibility is that these hypothetical, reversible, slight conformational changes are enhanced and stabilized by the oxidation of only 1 residue of histidine per protein subunit.

In a previous report (19) we have shown that the photoinactivation of the 6-phosphogluconate dehydrogenase, mediated by pyridoxal 5′-phosphate, also resulted in the oxidation of 2 histidine residues presumably located at the 6-phosphogluconate binding site. Studies are in progress in this laboratory to determine whether the same or different histidine residues are oxidized by the pyridoxal phosphate- and by the Rose Bengal-mediated photoinactivations.

We have shown that pyridoxal 5′-phosphate is a powerful enzyme inhibitor specific for lysine residues (27–29). Since then more than 30 enzymes have been reported to be inhibited by this reagent (19, 30). Rose Bengal is an inhibitor of 6-phosphogluconate dehydrogenase and alcohol dehydrogenase (24).

Rose Bengal and the pyridoxal phosphate bind to a specific region of 6-phosphogluconate dehydrogenase (and of other enzymes) that can be localized from kinetic experiments and in the case of pyridoxal phosphate also directly from the identification of the amino acid residue. Since, at low concentration, they photosensitize only if bound to the protein, their action is restricted to a narrow, predictable enzyme region, and the amino acid residue involved in the photoinactivation must be located in the close proximity to the dye binding site.

Another advantage of the use of the active site-specific photosensitizers is the possibility of detecting, from kinetic experiments, in the case of an enzyme requiring a coenzyme, whether the amino acid destroyed is at the substrate or at the coenzyme binding site.

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