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PHOTOINACTIVATION OF MICROSOMAL GLUCOSE-6-PHOSPHATASE

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SUMMARY

1. Rat liver microsomal glucose-6-phosphatase (D-glucose 6-phosphate phosphohydrolase, EC 3.1.3.9) is relatively insensitive to protein modifying reagents but is rapidly and completely inactivated by photooxidation in the presence of appropriate concentrations of methylene blue or rose bengal.

2. Inactivation is strictly dependent upon both light and the presence of dye.

3. A competitive inhibitor, P_i partially protects against the photoinactivation; although the binding is weak the apparent K_D for protection agrees reasonably well with the K_i for inhibition.

4. The pH dependence of the photoinactivation corresponds to a titration curve of an ionic group with a pK value between 6.5 and 6.9.

INTRODUCTION

The occurrence of glucose-6-phosphatase (D-glucose 6-phosphate phosphohydrolase, EC 3.1.3.9) in the microsomal fraction of liver and kidney has long been recognized, and this enzyme has proven useful as a "marker" identifying microsomal fractions. This close association of the enzyme with a membrane fraction, although advantageous for purposes of identification, has nevertheless somewhat hampered the chemical characterization of the enzyme protein. The catalytic properties of the enzyme have extensively investigated[1], but because of its particulate nature little is known about the chemistry of the protein or its active site.

In conjunction with our studies on the mechanism of action of microsomal glucose-6-phosphatase we have made several attempts to convert the enzyme to a soluble form which could be purified and characterized. The most successful of these, which involved treatment of the microsomal membranes with 1% digitonin, resulted in an enzyme preparation which would not sediment at 200 000 × g and which could be partially fractionated on Agarose gel columns. By other criteria, however, the enzyme remained particulate and resistant to purification. For example, it was observed that the enzyme in 1% digitonin did not penetrate the polyacrylamide matrix

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utilized for gel electrophoresis. Moreover, even in the presence of phospholipids, which have been shown to restore activity to inactivated preparations[2,3], the enzyme rapidly and irreversibly lost activity when subjected to other mild purification techniques such as isoelectric focussing.

The enzyme is not only resistant to solubilization and purification, but the catalytic activity is insensitive or sluggish to several types of protein-modifying reagents[4,5]. We have observed, for example, that incubation for several hours with sulfhydryl group reagents or "active serine" reagents under conditions where these reagents severely inhibit other enzymes has no perceptible effect on microsomal glucose-6-phosphatase. Possibly the membraneous environment protects the enzyme from most externally applied reagents, especially those which are ionically charged[6]. This insensitivity to protein modifying reagents makes it difficult to obtain meaningful information about the nature of chemical groups on the enzyme which are important for catalysis. We have found that the enzyme is relatively sensitive to photooxidation, being rapidly inactivated by light in the presence of methylene blue or rose bengal. Some of the characteristics of this photoinactivation are described in this paper. The accompanying paper correlates these observations with other mechanistic studies on this enzyme[7].

METHODS

Preparation and assay of microsomal glucose-6-phosphatase

Microsomes were prepared from the liver of male white rats, 200–300 g, by modifications of the technique of Schneider[8]. The microsomal fraction which remained after removal of mitochondria by centrifugation at 6000 × *g* was obtained as a crude pellet by centrifugation for 1 h at 45 000 × *g*. After washing by resuspension in 0.25 M sucrose, 0.1 mM EDTA and recentrifugation, the microsomes, approx. 25 mg protein/ml, were stored in small portions at −20 °C.

Immediately before being used in experiments, microsomes were thawed and activated[9] by addition of 0.1 ml 1 M NH_4OH to 0.9 ml undiluted microsomes. After 10 min at 2–3 °C the pH was adjusted by addition of appropriate buffer. The usual degree of activation observed was about 2-fold; longer exposures or higher temperatures for the alkali treatment gave no further activation.

Glucose 6-phosphatase activity was routinely assayed using a modification of the Fiske and SubbaRow technique[10] to measure P_i production from glucose 6-phosphate. The assay contained in a final volume of 1.0 ml: 0.1 M cacodylate (Na^+), pH 6.0; 2.5 mM glucose 6-phosphate (Sigma Chemical Company); and 0.4–2.0 mg of activated microsomal protein, which was added last to initiate the reaction after prior temperature equilibration. After 21 min incubation at 30 °C, the reaction was terminated by addition of 2.5 ml of a solution which was prepared fresh daily by mixing 1 vol. of 2.5 M H_2SO_4, 1 vol. of 3% (w/v) $NaHSO_3$–1% (w/v) *p*-methylaminophenol sulfate, 1 vol. of 2.5% (w/v) $(NH_4)_6Mo_7O_{24} \cdot 4\ H_2O$, and 4 vol. of water. After color development for 15 min at 30 °C the precipitated protein was removed by centrifugation in a desk top centrifuge, and the absorption of the supernatant fraction was determined at 660 nm in a spectrophotometer. Adequate zero time, zero enzyme and standard curve samples were included to ensure linearity of product formation with time and enzyme concentration under the conditions employed. In

some cases enzymatic activity was assayed by measurement of the hydrolysis of ^{32}P-labeled inorganic pyrophosphate, an alternative substrate[1], as described in the accompanying publication[7].

Photooxidation

The photooxidation[11] was conducted in a Standard Bath Assembly (Yellow Springs Instrument Company) equipped with four sample chambers which were stirred magnetically and temperature-controlled at 10 °C by a heating-cooling circulating unit (Precision Scientific Co. Lo-Temptrol 154). The light source was a 300-W Champion reflector floodlamp positioned 30 cm above the surface of the enzyme solution. A water barrier 1 inch thick protected the reaction vessel from the lamp's heat; a fan cooled the entire system. Four samples were photooxidized simultaneously, minimizing possibility for errors arising in different runs.

Microsomes were diluted and adjusted to the proper pH for the photooxidation experiments by adding 3.0 ml buffer to 1.0 ml activated microsomes (14.2 mg/ml). To minimize buffer effects over the pH range studied, buffer of 0.05 M cacodylic acid+ 0.05 M Tris free base was adjusted with NaOH or HCl to provide the correct pH and with NaCl to provide a final ionic strength of 0.20.

Photooxidation was initiated by the addition of 40 μl of dye to 4.0 ml of temperature-equilibrated enzyme at the appropriate pH. The light was then switched on and aliquots of 25 μl were removed from the vessels at times indicated and assayed immediately. There was no effect of methylene blue or rose bengal in the assay due to dilution of dye, protection by substrate, and relatively low light level. Because the rate of photooxidation varies with the volume of the reaction solution[11], a total of less than 5% of the initial reaction volume was removed for activity in order to minimize errors due to volume changes.

RESULTS

Comparison of photooxidation with rose bengal and methylene blue

Although rapid photoinactivation could be observed with either rose bengal or methylene blue, it was found that the concentration of rose bengal which resulted in a particular rate of inactivation was approximately two orders of magnitude lower on a molar basis than the concentration of methylene blue which gave a comparable rate of inactivation. This effect is noted in Fig. 1, where the apparent first order rate constant, k_{app} = inactivation rate/[dye], for the photoinactivation has a value of $3.1 \cdot 10^2\ M^{-1} \cdot min^{-1}$ for methylene blue and $2.2 \cdot 10^4\ M^{-1} \cdot min^{-1}$ for rose bengal under similar conditions. Fig. 1 also illustrates another difference between the two dyes; first order loss of activity was usually observed down to 95% or greater inactivation with methylene blue, but under conditions where a similar inactivition rate was observed with rose bengal, first order kinetics were obtained only to about 60% inactivation. At higher concentrations of rose bengal the rate of inactivation was apparently first order to at least 95%, but was too rapid to measure accurately. Because of the much greater extent of first order reaction at conveniently measurable rates, detailed analyses were made using methylene blue rather than rose bengal as the photooxidant.

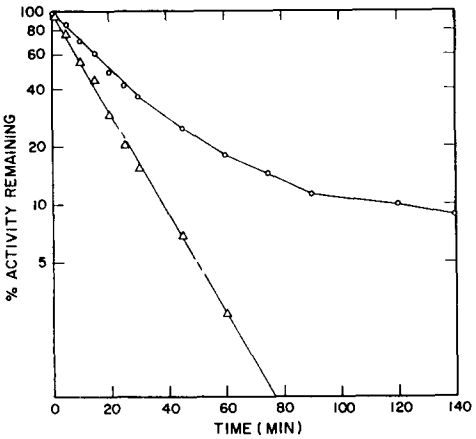


Fig. 1. Comparison of methylene blue and rose bengal as photooxidants. Methylene blue (△) was used at a concentration of $2 \cdot 10^{-4}$ M at pH 8.24 and rose bengal (○) at a concentration of $1.6 \cdot 10^{-6}$ M at pH 8.20 under identical conditions of ionic strength, temperature, and light intensity.

Effect of pH on the rate of inactivation

The observed rate of photoinactivation is dependent upon the pH of the solution, as seen from the representative data shown in Fig. 2, which compares the rapid rates observed at high pH with the somewhat slower rate at acid pH. In several replicate experiments the inactivation rates always closely approximated first order kinetics at all pH values. At acid pH (4.0–6.4), a slow first order loss of activity was observed in the control samples (absence of methylene blue or absence of light), in accord with previous reports of instability of the enzyme at acid pH[9]. All observed photoinactivation rates were routinely corrected for this relatively slow spontaneous

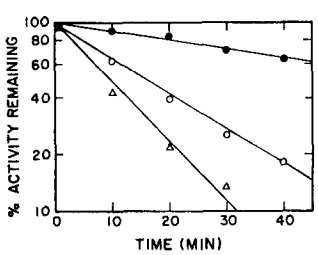


Fig. 2. Comparison of photoinactivation at acid and alkaline pH. Photoinactivation by methylene blue, $4 \cdot 10^{-4}$ M, at pH 9.16 (△) and pH 6.07 (○). A control sample at pH 6.10 in the absence of methylene blue (●) is also shown to illustrate the lability of the enzyme at acid pH. No loss of activity was observed in similar controls at pH >6.5.

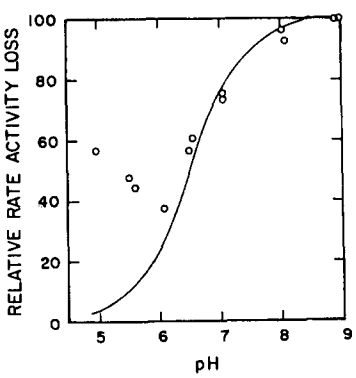


Fig. 3. Relative rate of photoinactivation as a function of pH. Photoinactivation by $4 \cdot 10^{-4}$ M methylene blue as described in Methods. Data (○) from two different experiments are included and are normalized to 100% at pH 9.0. The line is a theoretical curve for the ionization of a group with pK 6.5.

inactivation, illustrated in Fig. 2. At pH values greater than 6.5, the enzyme lost no activity during the time course of the reaction either in the absence of the dye in the light or in the presence of dye in the dark, indicating that both light and dye are necessary for inactivation.

The rate of photoinactivation as a function of pH is shown in Fig. 3; above pH 6 a theoretical titration curve with a pK of 6.5 provides a reasonable fit for the data. Because of the anomalously high rates of photoinactivation at pH values below 6, a somewhat better fit to a titration curve is obtained (Fig. 4) by assuming that the protonated form of the ionizing group retains 45% of its maximum sensitivity to

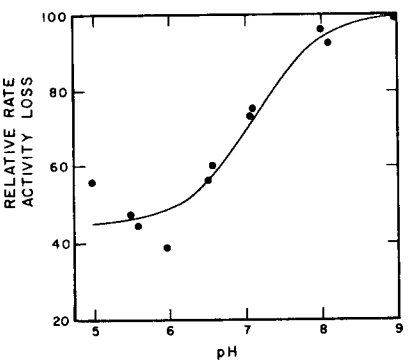


Fig. 4. Fit of experimental data (photoinactivation rate *vs* pH) to modified titration curve. Same data (○) as in Fig. 3. The line is a theoretical titration curve which is different from that in Fig. 3 in two ways: (a) it is assumed that the protonated form (pH 5) of the ionizable group involved is 45% (instead of 0%) as reactive toward photoinactivation as it is at pH 9, and (b) the pK is shifted to 6.9.

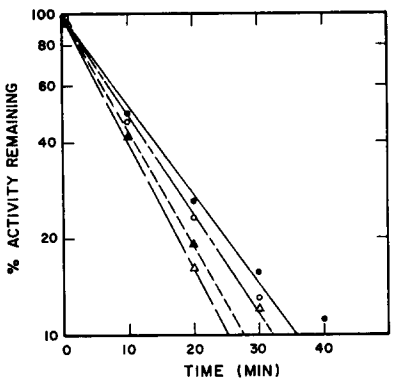
photoinactivation rather than being completely insensitive[12]. In this case a pK value of 6.9 provides the best fit to the data.

Photooxidation in the presence of a competitive inhibitor

If photoinactivation is the result of modification of a group at the active site of the enzyme, then the presence of bound substrate might afford at least partial protection against the inactivation process. Because of the hydrolytic nature of the reactions catalyzed by this enzyme it is difficult to maintain a substrate molecule at the active site. A competitive inhibitor such as P_i[13] might serve the same purpose, however, obviating the problem of enzyme turnover continually removing substrate from the site.

It was observed that P_i does protect the enzyme from photoinactivation. The protection at pH 7.0 by increasing concentrations of P_i is illustrated in Fig. 5. The data were obtained with a radioisotope assay[7] because the added P_i interferes with the conventional spectrophotometric assay. A dissociation constant for the orthophosphate–enzyme complex may be calculated[14] from the data of Fig. 5, as shown in Fig. 6. Under these conditions a dissociation constant of 0.081 M was observed.

For the purpose of comparison with K_D, we determined kinetically the K_i for P_i under similar conditions, again utilizing ^{32}P-labeled inorganic pyrophosphate as



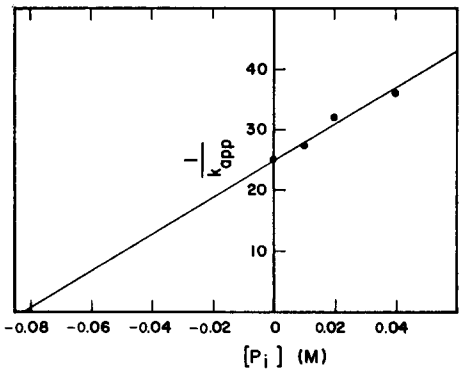


Fig. 5. Orthophosphate protection against photoinactivation. Photoinactivation by $4 \cdot 10^{-4}$ M methylene blue at pH 7.0. Enzymatic activity was measured with ^{32}P-labeled inorganic pyrophosphate as substrate[7]. Orthophosphate concentration: o (△); 0.01 M (▲); 0.02 M (○); and 0.04 M (●).

Fig. 6. Determination of K_D for P_i. Rate constants for photoinactivation were determined from the data of Fig. 5 and plotted *vs* total P_i concentration. The intercept on the abscissa is $-K_D$; the ordinal intercept is the reciprocal of the true rate constant for the photoinactivation reaction[14].

substrate. The value of K_i for P_i obtained by this method, 0.033 M, is within a factor of 2.5 of the above K_D for P_i observed by protection against photoinactivation. The agreement is considered satisfactory, considering the weakness of the binding and the errors in the determination of K_D caused by the high concentrations of P_i which diminish the accuracy of the assay.

DISCUSSION

Although dye-sensitized photooxidation of enzymes has been employed to determine the nature of amino acid residues at enzymic active sites, previous studies have utilized this technique exclusively with soluble, purified proteins[15–19]. In this study the membrane-bound microsomal glucose-6-phosphatase was shown to be comparable to soluble enzymes in its susceptibility to photoinactivation. Despite the opacity of the enzyme–dye reaction mixture and the multiplicity of proteins present in the preparation, reasonably linear first-order rates of inactivation were obtained using methylene blue as the photooxidant. Lack of linearity of inactivation rates in the presence of rose bengal might be due to depletion of the dye during the reaction, for the concentrations required for comparable rates of inactivation were relatively low so that reaction with extraneous proteins might consume a large portion of the dye.

The presence of other proteins in the membrane preparation also prevents correlation of activity loss with the destruction of various amino acids, for any critical amino acid lost is likely to be only a small fraction of the total destroyed. Therefore, the critical amino acid(s) cannot be positively identified.

The rate of photoinactivation as a function of pH resembles the titration curve of an ionizable group with a pK of 6.5–6.9. The precise value depends on whether and how the data is corrected for the anomalously rapid inactivation at low

pH. This aberrant sensitivity to photoinactivation at low pH has been observed with other enzymes[17,18]; it may be due to a pH-dependent conformation change which confers unusual reactivity on the critical residue, or to slower pH-independent destruction of some other critical amino acid. Weil[12] found for a methylene blue-dependent photooxidation of histidine, tryptophan, methionine, and their derivatives a pH profile similar to that observed here. Although these observations do not permit identification of the critical residue[2], they are consistent with earlier suggestions of a prominent role for the imidazole group of histidine in the catalytic mechanism of this microsomal enzyme[20,21]. The evidence presented here for the congruency of kinetic inhibition and protection against photoinactivation by orthophosphate is a further indication of, but not a proof for, the location of the critical residue at the active site of the enzyme.

We have previously presented in a preliminary report[22] independent evidence that a histidine residue on the enzyme is phosphorylated during an intermediate stage of the reaction. The accompanying paper provides extensive documentation of this finding and correlates evidence for the involvement of histidine in the catalytic mechanism of this enzyme[7]. Igarashi *et al.*[23] have presented similar types of evidence for the participation of phosphoryl histidine as an intermediate in a crystalline non-specific acid phosphatase from rat liver.

After this work was completed, Maddaiah *et al.*[24] reported that microsomal glucose 6-phosphate from rat and human liver could be rapidly inactivated by incubation with pyridoxal 5′-phosphate. Glucose 6-phosphate was effective in protecting against this inactivation. These authors suggested inactivation was the result of formation of a Schiff base between pyridoxal 5′-phosphate and an ε-amino group of lysine at the substrate binding site of the enzyme. However, such inactivation should be reversible upon dilution or removal of pyridoxal 5′-phosphate; the experimental technique employed by Maddaiah *et al.*[24] favored reversal of the inactivation but the results showed clearly that none occurred[24]. Recent reports that pyridoxal 5′-phosphate is a specific photosensitizing agent for oxidation of histidine residues in 6-phosphogluconate dehydrogenase[25] and muscle and spinach leaf aldolase[26] suggest that the irreversible inactivation observed by Maddaiah *et al.*[24] may have been due to photooxidation of a histidine residue. This interpretation, which appears not to be excluded by their report, would be fully consistent with the observations reported here.

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