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Affinity Labelling of the $NADP^+$-Binding Site of Glucose 6-Phosphate Dehydrogenase from *Candida utilis*

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1. Periodate-oxidized $NADP^+$ inhibits the catalytic activity of glucose 6-phosphate dehydrogenase from *Candida utilis*, competing with $NADP^+$. 2. Incubation of the enzyme with the coenzyme analogue causes partial reversible inactivation of the enzyme as a result of affinity labelling of the coenzyme-binding site. 3. Some kinetic values of the reaction were calculated. 4. The inactivation can be made irreversible by treatment with $NaBH_4$, which reduces a Schiff base formed between an aldehyde group on the coenzyme analogue and a lysine residue on the enzyme. 5. Complete inactivation can be correlated with the binding of only one molecule of inhibitor to each enzyme subunit. 6. The lysine residue involved in the binding of the inhibitor is present at the coenzyme-binding site.

The treatment of $NADP^+$ with periodate causes the cleavage of the bond between carbons 2′ and 3′ of the ribose ring bound to nicotinamide and the formation of two aldehyde groups at these carbons. We have reported that the reaction of 6-phosphogluconate dehydrogenase from *Candida utilis* with periodate-oxidized $NADP^+$ results in the reversible inactivation of the enzyme, as a result of formation of a Schiff base between an aldehyde group of the inhibitor and a lysine residue of the $NADP^+$-binding site of the enzyme (Rippa *et al.*, 1975; Dallocchio *et al.*, 1976). It has now been reported that periodate-oxidized $NADP^+$ also inactivates pigeon liver 'malic' enzyme (Chang & Huang, 1979).

We have now found that periodate-oxidized $NADP^+$ inactivates, by affinity labelling, glucose 6-phosphate dehydrogenase from *C. utilis*.

Materials and Methods

Materials

Glucose 6-phosphate, 6-phosphogluconate and $NADP^+$ were purchased from Boehringer. NaB^3H_4 was purchased from The Radiochemical Centre. Glucose 6-phosphate dehydrogenase (D-glucose 6-phosphate–$NADP^+$ oxidoreductase, EC1.1.1.49) from *C. utilis* was prepared and assayed as previously reported (Chilla *et al.*, 1973). Periodate-oxidized $NADP^+$ was prepared and purified as previously reported (Rippa *et al.*, 1975; Dallocchio *et al.*, 1976). Radioactive periodate-oxidized $NADP^+$ (1600c.p.m./nmol) was prepared by treating $NADP^+$ first with NaB^3H_4, which reduces and labels the nicotinamide ring, and then with periodate, which oxidizes that ring (which remains radioactive) and opens the ribose ring bound to nicotinamide.

The crystals of enzyme were dissolved in distilled water and the protein solution was subjected to gel filtration on Sephadex G-25 (column dimensions 0.8cm × 15cm) equilibrated in 50mM-sodium phosphate buffer, pH7.5, containing 1mM-EDTA. All experiments, unless otherwise stated, were carried out in this buffer and at room temperature.

Inactivation experiments

The enzyme was incubated with the coenzyme analogue. At timed intervals samples were withdrawn and immediately tested for residual catalytic activity; re-activation on dilution was negligible in the time (1min) required for the analysis. In order to make the inactivation irreversible, a few crystals of $NaBH_4$ were added to the solution containing the enzyme–inhibitor complex, which was then freed from excess of reagents by gel filtration in phosphate buffer, as described above, and assayed for residual enzyme activity.

Stoicheiometry of the inactivation

A sample of enzyme (3mg of protein/ml; 60nmol of subunit/ml) was treated with radioactive periodate-oxidized $NADP^+$ (final concentration 200nmol/ml). When the inactivation process had reached equilibrium, a few crystals of $NaBH_4$ were added and the enzyme, freed from excess of reagents by gel filtration in phosphate buffer, was assayed for protein concentration, residual activity and ra io-activity incorporated.

Identification of the amino acid residue involved in the binding of the inhibitor to the enzyme

The enzyme (60nmol of subunit/ml) was treated with periodate-oxidized NADP$^+$ (final concentration 200nmol/ml, non-radioactive). When the inactivation process had reached equilibrium, a few crystals of NaB^3H_4 were added and the radioactive protein, freed from excess of reagents by gel filtration, was treated as described by Dallocchio *et al.* (1976) for the identification of the amino acid residue involved in the binding of periodate-oxidized NADP$^+$ to the 6-phosphogluconate dehydrogenase. T.l.c. was carried out on glass plates covered with a layer (250μm thick) of silica gel (type 60, Merck) in a solvent of propan-1-ol/aq. 25% (w/w) NH_3 (67:33, v/v). After chromatography, the plates were dried and sprayed with ninhydrin to detect the positions of the markers. Marks were made every 0.5cm, and the gel in these sections was removed and counted for radioactivity in a Packard liquid-scintillation counter.

Results

Inhibition by periodate-oxidized NADP$^+$

Periodate-oxidized NADP$^+$ is an analogue of the coenzyme of glucose 6-phosphate dehydrogenase and thus could be an inhibitor of the enzyme. Fig. 1 shows that it does inhibit the catalytic activity of the enzyme, competing with the NADP$^+$. In our experimental conditions the K_m for NADP$^+$ and the K_i for the periodate-oxidized analogue are respectively 40 and 145μM. These values are approximate, since the experiments were done at only one concentration of the inhibitor.

The coenzyme analogue is not reduced by glucose 6-phosphate in the presence of glucose 6-phosphate dehydrogenase and is therefore not active as a coenzyme for this enzyme.

Inactivation by periodate-oxidized NADP$^+$

Treatment of glucose 6-phosphate dehydrogenase with periodate-oxidized NADP$^+$ results in inactivation of the enzyme (Fig. 2). Although the presence, in the inactivation mixture, of glucose 6-phosphate (0.7mM) or 6-phosphogluconate (0.7mM) does not change the kinetics of inactivation, the NADP$^+$ (0.2mM) fully protects the enzyme against the inactivation.

The rate and extent of inactivation are a function of the concentration of the inhibitor (Fig. 3). The catalytic activity decreases to an equilibrium value within 20–40min, the value of the residual activity being a function of the concentration of the inhibitor. In the first 8min the inactivation follows pseudo-first-order kinetics, but then deviates from linearity, as expected, since the inactivation is analysed in terms of approach to 100% reaction rather than of approach to equilibrium (Chen & Engel, 1975). A plot of the inactivation half-time ($t_{0.5}$) against the reciprocal of

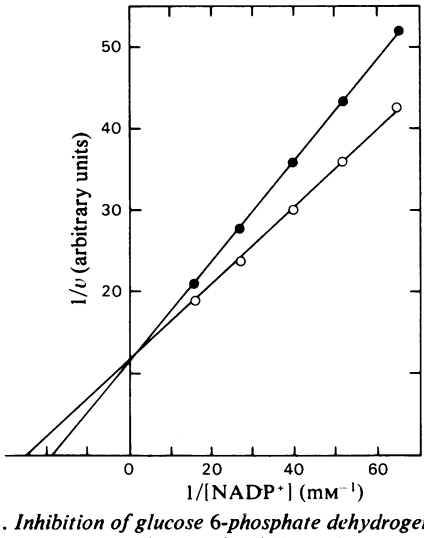

Fig. 1. *Inhibition of glucose 6-phosphate dehydrogenase by periodate-oxidized NADP$^+$*

The assay mixture contained 50mM-triethanolamine hydrochloride buffer, 0.1mM-EDTA, 1mM-glucose 6-phosphate, the enzyme and NADP$^+$ at the concentrations indicated in the abscissa; the final pH was 7.5. ○, No further addition; ●, in the presence of 58μM-periodate-oxidized NADP$^+$.

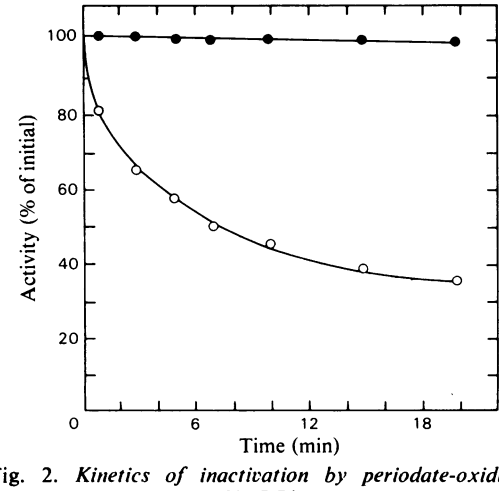

Fig. 2. *Kinetics of inactivation by periodate-oxidized NADP$^+$*

A solution of enzyme (0.50nmol of subunit/ml) in phosphate buffer was treated with periodate-oxidized NADP$^+$ (final concentration: 9.4nmol/ml). ○, No further addition; ●, in the presence of 0.2mM-NADP$^+$.

the concentration of the inhibitor (Fig. 4) shows that the inactivation obeys saturation kinetics (Laidler, 1958; Meloche, 1967; Trundle & Cunningham, 1969; Wold, 1977), providing evidence that the enzyme and the inhibitor form a dissociable complex before inactivation. Thus (Wold, 1977) the inactivation is due to affinity labelling. It can be calculated from Fig. 4 that the $K_{inact.}$ is 4.5 μM and that the maximum inactivation rate constant is 0.136 min^{-1}. The choices of concentration of the coenzyme analogue are not optimal for separation of kinetic constants, and more reliable estimates would have been obtained by using lower concentrations; but, in our case, in these conditions the determination of the inactivation half-time was not reliable. The inactivation is never complete, even at higher concentrations of inhibitor.

The enzyme partially inactivated by treatment with periodate-oxidized NADP+ has the same K_m for NADP+ (results not shown) as the native enzyme; this indicates that the inactivation is due to a decrease in the amount of the active enzyme present rather than being partial inactivation as a result of alteration of the properties of the native enzyme.

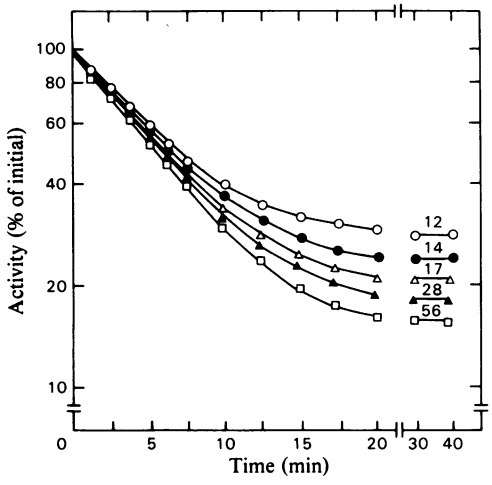

Fig. 3. *Effect of the concentration of periodate-oxidized NADP+ on the rate and extent of inactivation*
A solution of enzyme (0.5 nmol of subunit/ml) was treated with periodate-oxidized NADP+ at the final concentrations (μmol/l) indicated on the Figure.

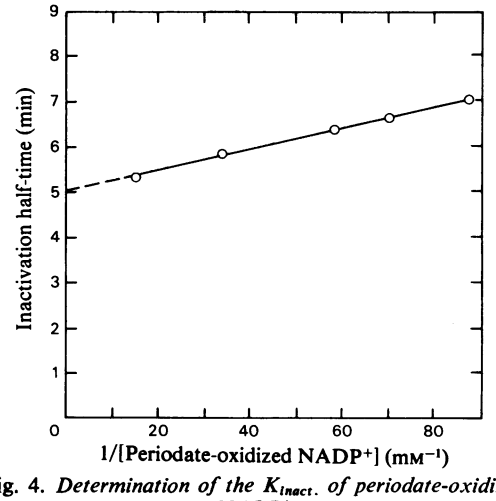

Fig. 4. *Determination of the $K_{inact.}$ of periodate-oxidized NADP+*
The inactivation half-times were calculated from Fig. 3.

Reversibility and stabilization of the inactivation

The inactivation by periodate-oxidized NADP+ can be reversed by diluting the enzyme–inhibitor complex (Fig. 5); 2 h after the dilution more than the 85 % of the original activity is recovered. The re-activation reaction follows, in the first minutes, first-order kinetics, with a rate constant of 0.023 min^{-1}.

No recovery of activity is obtained if the enzyme–inhibitor complex is treated with $NaBH_4$ before

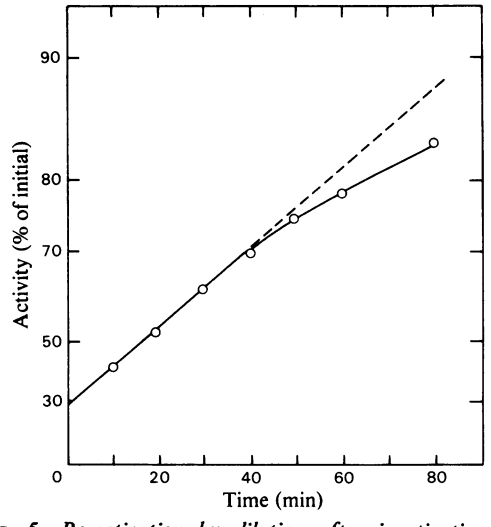

Fig. 5. *Re-activation by dilution after inactivation by periodate-oxidized NADP+*
A solution of enzyme (122 nmol of subunit/ml) in phosphate buffer was treated with periodate-oxidized NADP+ (final concentration 200 nmol/ml). When the activity was 28 % of the initial value, the protein was freed from excess of reagents by gel filtration in phosphate buffer and diluted 100-fold in the same buffer. At timed intervals the enzyme activity was tested. The graph is plotted on a semilogarithmic scale.

dilution; thus the reduction with $NaBH_4$ stabilizes the inactivation.

A second treatment of the enzyme, partially and irreversibly inactivated by treatment with periodate-oxidized $NADP^+$ and $NaBH_4$, with periodate-oxidized $NADP^+$ causes a further analogous incomplete inactivation (Fig. 6).

Stoicheiometry of the inactivation

A sample of enzyme was treated with radioactive periodate-oxidized $NADP^+$ and $NaBH_4$, as described in the Materials and Methods section. Radioactivity measurements indicate that the enzyme, when 74% irreversibly inactivated, contained 0.7 molecules of inhibitor bound to each subunit of enzyme. This stoicheiometry indicates that complete inactivation of the enzyme can be correlated with the irreversible binding of 1 molecule of inhibitor to each subunit of enzyme.

Identification of the amino acid residue involved in the binding of the inhibitor to the enzyme

A sample of enzyme was treated with non-radioactive $NADP^+$ and then with NaB^3H_4 to label the Schiff base. The reduced-enzyme–inhibitor complex was then subjected to the treatments required for the identification of the amino acid residue involved in the binding of the inhibitor to the enzyme (Dallocchio *et al.*, 1976). Chromatography of the protein hydrolysate (Fig. 7) shows only two radioactive spots, with R_F values equal to those of the compounds indicated in the Figure. This suggests that the two aldehyde groups of the inhibitor can form a reducible Schiff base with a lysine residue present at the $NADP^+$-binding site of the enzyme.

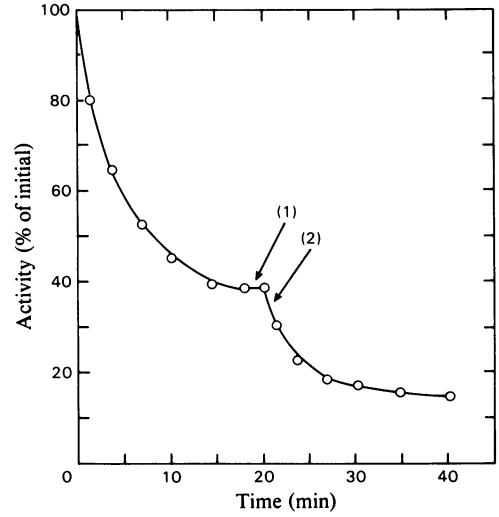

Fig. 6. *Repeated treatment of the enzyme with periodate-oxidized $NADP^+$*

A sample of enzyme was treated with periodate-oxidized $NADP^+$ as described in the legend of Fig. 5. When 38% of the original activity was reached, the sample was treated with $NaBH_4$, and the protein was freed from excess of reagents by gel filtration in phosphate buffer. The protein sample was treated again with periodate-oxidized $NADP^+$, at the same final concentration as in the first treatment. Arrow (1) $NaBH_4$ and gel filtration; arrow (2) re-inactivation.

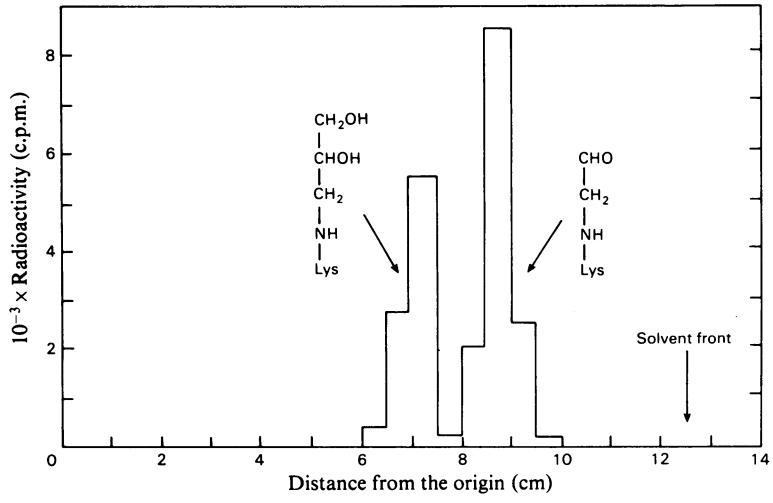

Fig. 7. *Chromatography of the acid hydrolysate of the reduced enzyme–inhibitor complex*

Details are given in the Materials and Methods section.

Discussion

The coenzyme analogue periodate-oxidized NADP+ inhibits the catalytic activity of glucose 6-phosphate dehydrogenase from *C. utilis*; it competes with NADP+ and reversibly inactivates the enzyme. These findings and the fact that the reaction follows saturation kinetics indicate that periodate-oxidized NADP+ can be used as an affinity label specific for the NADP+-binding site of the enzyme. The specificity of the inactivation is also supported by the stoicheiometry: the binding of only 1 molecule of inhibitor to each subunit of the enzyme causes complete inactivation. The inactivation is reversible, but can be stabilized by treatment with $NaBH_4$. Chemical analyses have shown that an aldehyde group on the inhibitor forms a reducible Schiff base with a lysine residue, which, for the topographical specificity of the binding of the inhibitor to the enzyme, should be present in the protein region that is in close contact with the ribose ring bound to the nicotinamide portion of the coenzyme.

The kinetic mechanism of the inactivation deserves a more detailed discussion, which can be based on the following points: (*a*) the enzyme is subjected to an instantaneous reversible competitive inhibition by periodate-oxidized NADP+ and thus there is the immediate formation of an enzyme–inhibitor complex; (*b*) inactivation follows saturation kinetics, indicating that the relatively slow inactivation follows faster formation of an enzyme–inhibitor complex and hence there are two different enzyme–inhibitor complexes; (*c*) the enzyme can be reactivated by dilution, thus there is chemical equilibrium between the active and the inactive enzymes; (*d*) the existence of this equilibrium is supported by the fact that a second treatment with periodate-oxidized NAD+ of the enzyme that had previously been partially and irreversibly inactivated by treatment with periodate-oxidized NADP+ and $NaBH_4$ results in further similar inactivation.

On these bases, it can be postulated that, when the periodate-oxidized NADP+ reacts with the enzyme, there is first rapid formation of a non-covalent enzyme–inhibitor complex, which then yields a covalent inactive enzyme–inhibitor complex in which the inhibitor is bound to the enzyme through a Schiff base (Scheme 1). This reaction is analogous with that between glutamate dehydrogenase and pyridoxal 5′-phosphate, for which Chen & Engel (1975) have done a detailed kinetic analysis. Applying that analysis to our data, we have calculated the values of some kinetic constants.

In Fig. 4 the intercept on the ordinate gives the value of $1/k_{+2}$ and the slope is $k_{-1}/k_{+1}k_{+2}$; it can be calculated that $k_{+2}=0.136\,min^{-1}$ and $k_{-1}/k_{+1}=4.5\,\mu M$. From Fig. 5 the value of k_{-2} is $0.023\,min^{-1}$. If it is assumed that the non-covalent complex dissociates immediately on dilution, and thus is measured as active enzyme, whereas the covalent complex dissociates slowly, it means that, even with very high concentrations of inhibitor, the assayable activity cannot decrease:

$$\frac{k_{-2}}{k_{+2}+k_{-2}} \times \text{initial activity}$$

in our case this is 14.4 % of the initial activity.

These values have been obtained from the rates of inactivation, but can also be obtained by using the values of residual activity under conditions of equilibrium and non-saturating concentrations of inhibitor. Indeed, if the reciprocal of the concentration of the inhibitor is plotted against the reciprocal of the percentage of inactivation (Fig. 8), the intercept on the ordinate gives the value:

$$\left(1+\frac{k_{+2}}{k_{-2}}\right)\frac{k_{+1}}{k_{-1}}$$

The intercept on the abscissa gives $1+(k_{-2}/k_{+2})$ and the slope is $k_{+1}k_{+2}/k_{-1}k_{-2}$. The values obtained are:

$$\text{Enzyme} + \text{inhibitor} \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} \text{enzyme–inhibitor} \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} \text{enzyme=inhibitor}$$

Non-covalent complex — Covalent complex

Scheme 1

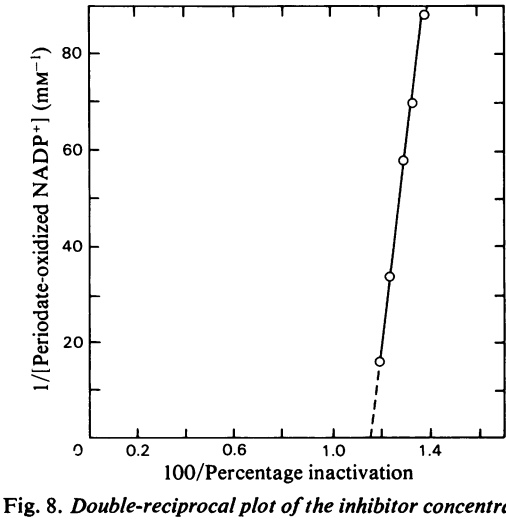

Fig. 8. *Double-reciprocal plot of the inhibitor concentration against percentage inactivation*
For the significance of this plot see the text.

$k_{-2}/k_{+2}=0.16\,\mu M$, $k_{-1}/k_{+1}=10.8\,\mu M$ and the minimum activity assayable is 13.7% of the initial. The first and the third values obtained from these equilibrium data are in good agreement with those obtained from the kinetic data; the second value is, however, higher. We have no explanation for this discrepancy, except that it may be inherent in the relative inaccuracy of the kinetic analyses as compared with the measurements of static equilibrium activity.

The value of k_{-1}/k_{+1} ($10.8\,\mu M$), which represents the dissociation constant of the non-covalent complex, is of the same order of magnitude as the K_m for $NADP^+$ ($40\,\mu M$).

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