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Half-site reactivity in 6-phosphogluconate dehydrogenase from human erythrocytes

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6-Phosphogluconate dehydrogenase from human erythrocytes was purified by an improved procedure. Binding studies showed that the dimeric enzyme binds 2 mol of $NADP^+$/mol but only 1 mol of NADPH/mol, and that the bindings of oxidized and reduced coenzyme are mutually exclusive. From initial-rate kinetics and inhibition studies, a sequential random-order mechanism is proposed. Double-reciprocal plots with $NADP^+$ as varied substrate show a downward curvature, indicating a negative co-operativity. We suggest that the negative co-operativity observed kinetically is a result of the half-site reactivity for the NADPH. The different binding stoichiometries for $NADP^+$ and NADPH generate a non-linear relationship between the apparent dissociation constant for the NADPH and the concentrations of the $NADP^+$, resulting in a regulatory mechanism highly sensitive to the changes in the $NADP^+$/NADPH ratio.

The pentose phosphate shunt is one of the most important metabolic pathways in the mature erythrocyte, providing the reducing power needed to keep the glutathione in the reduced state and to protect the cell from oxidizing agents. Since its discovery, interest has been focused on the first enzyme of the cycle, glucose-6-phosphate dehydrogenase, which is widely recognized as the key enzyme in the regulation of this pathway.

Only little information is available on 6-phosphogluconate dehydrogenase from erythrocytes, the second dehydrogenase of the pentose phosphate shunt. This enzyme catalyses the $NADP^+$-dependent oxidative decarboxylation of 6-phosphogluconate, and was purified from human erythrocytes by Pearse & Rosemeyer (1974*a*). The protein is composed of two apparently identical subunits, and has an M_r of 104000 (Pearse & Rosemeyer, 1974*b*). The enzyme, like glucose-6-phosphate dehydrogenase, is strongly inhibited by the reaction product NADPH, which is present in the erythrocytes at fairly high concentrations.

In the present paper we report kinetic and binding studies on 6-phosphogluconate dehydrogenase from human erythrocytes, and we suggest some interesting regulatory properties of this enzyme.

Materials and methods

Materials

6-Phosphogluconate, $NADP^+$ and NADPH were purchased from Boehringer Mannheim; gluconate 6-sulphate was prepared by Br_2 oxidation of glucose 6-sulphate (Horecker, 1955); periodate-oxidized $NADP^+$ and periodate-oxidized [^{3}H]$NADP^+$ were prepared as previously described (Dallocchio *et al.*, 1976; Bellini *et al.*, 1979). All experiments were carried out in 50mM-triethanolamine/HCl buffer, pH 7.4, containing 1mM-EDTA, at room temperature (22°C).

Assay of enzyme activity

Enzyme activity was assayed as described by Rippa & Signorini (1975). One unit of activity is the amount producing 1 μmol of NADPH/min.

Enzyme purification

6-Phosphogluconate dehydrogenase was purified from human erythrocytes by a modification of a previously described procedure (Morelli, 1979). Purification was carried out at 4°C, and in all steps 50mM-sodium phosphate buffer, pH 7.6, containing 25mM-NaCl, 1mM-EDTA and 0.1% 2-mercaptoethanol was used.

Washed erythrocytes (100ml) were haemolysed with 5vol. of distilled water, and the cellular fragments were removed by centrifugation. Adenosine 2′,5′-bisphosphate–Sepharose (2.5g; Pharmacia) was added to the haemolysate, which was then stirred for 20min. The suspension was poured into a Buchner funnel, and the resin was washed with buffer. The resin was then resuspended and poured into a column (1.1cm × 10cm), where the washing was continued until the A_{280} of the eluate was zero. The resin in the column was then washed with 10ml of buffer containing 5mM-NAD^+, to elute the lactate dehydrogenase bound to the resin. The elution was then started with 3ml of buffer containing 1mM-$NADP^+$, followed by 7mM-$NADP^+$. Glucose-6-phosphate dehydrogenase is eluted first, well separated from 6-phosphogluconate dehydrogenase and glutathione reductase, which are eluted unresolved. Pooled fractions containing 6-phosphogluconate dehydrogenase activity were concentrated with Centriflo CF-25 cones (Amicon), to a final concentration of about 2mg/ml, and the solution was made 55% saturated with $(NH_4)_2SO_4$. The precipitate, containing the glutathione reductase activity, was discarded, and in the supernatant remained pure 6-phosphogluconate dehydrogenase with a specific activity ranging from 26 to 28 units/mg, and an overall yield of 36–38%. The enzyme appears to be homogeneous on the basis of sodium dodecyl sulphate/polyacrylamide-gel electrophoresis.

This procedure allows a simultaneous purification of 6-phosphogluconate dehydrogenase, glucose-6-phosphate dehydrogenase, glutathione reductase and lactate dehydrogenase.

The excess of $NADP^+$ was removed by gel filtration on a Sephadex G-50 column (1cm × 24cm) equilibrated in 50mM-triethanolamine/HCl buffer, pH7.4. Before the experiments the enzyme was checked for $NADP^+$ content as described by De Flora *et al.* (1974*a*), to ensure that the coenzyme was fully removed.

Kinetic measurements

Initial-rate measurements were performed either spectrophotometrically, with a Zeiss PM 2K spectrophotometer, or fluorimetrically, with a Perkin–Elmer MPF-3L spectrofluorimeter. With the first method the increase of absorbance at 340nm was measured; with the second method the increase of fluorescence emission of the NADPH at 460nm, on excitation at 340nm, was measured. The arbitrary units of fluorescence intensity were converted into μmol of NADPH by using a calibration curve obtained with solutions of known concentrations of the coenzyme.

The concentrations of $NADP^+$ and 6-phosphogluconate were determined enzymically; the concentrations of NADPH were measured spectrophotometrically; the concentrations of gluconate 6-sulphate were determined by spectrophotometric measurement of the reduction of periodate in neutral solution, with 6-phosphogluconate as standard; linear plots were fitted by the least-squares method, and non-linear plots were fitted by the Gauss–Newton method.

Binding of coenzymes

The binding of $NADP^+$ and NADPH to 6-phosphogluconate dehydrogenase was measured fluorimetrically. For $NADP^+$, the quenching of protein fluorescence (excitation 280nm, emission 330nm) was measured. For NADPH, the enhancement of the coenzyme fluorescence (excitation 340nm, emission 460nm) was measured. When the binding of the NADPH was studied at low enzyme concentrations, the excitation was at 290nm (emission 460nm): the fluorescence due to energy transfer from protein to the bound coenzyme was measured, and the large blank due to the fluorescence of free NADPH was avoided.

The fluorescence values were corrected for the inner filter (Parker & Rees, 1960).

The number of binding sites was measured by fluorimetric titration (48μM enzyme with $NADP^+$, 14.3μM enzyme with NADPH).

The dissociation constants were estimated by using Scatchard plots in the form:

$$\frac{\Delta F}{[\text{Coenzyme}]} = \frac{1}{K_d}(\Delta F_{max.} - \Delta F)$$

where ΔF is the observed enhancement (or quenching) of fluorescence, and $\Delta F_{max.}$ the enhancement (or quenching) at infinite coenzyme concentration. Experimental points were fitted by the least-squares method.

The enzyme concentration was calculated from the enzymic activity, taking 28units/mg as the specific activity of the pure fully active enzyme.

An independent measurement of the concentration of the enzyme was obtained by using the method of Kurganov *et al.* (1972) as described by Silverberg & Dalziel (1975). This method indicates that only the active enzyme binds the coenzymes.

Inactivation by periodate-oxidized $NADP^+$

Kinetics and stoichiometry of inactivation of 6-phosphogluconate dehydrogenase by periodate-oxidized $NADP^+$ were performed by using procedures previously described (Bellini *et al.*, 1979). Pseudo-first-order rate constants were obtained from semi-logarithmic plots of residual activity versus time. Stoichiometry of inactivation was obtained by measuring the incorporation of periodate-oxidized [^{3}H]$NADP^+$ after reduction of the enzyme–inhibitor complex with $NaBH_4$.

Results

Binding of the oxidized coenzyme

The binding of $NADP^+$ to 6-phosphogluconate dehydrogenase from human erythrocytes does not show any remarkable feature. Both titration of the binding sites and measurement of the dissociation constant (Fig. 1) show the presence of two independent equivalent binding sites, with $K_d = 9.8\,\mu M$. These data agree with the dimeric structure of the enzyme (Pearse & Rosemeyer, 1974*b*).

Similar results are obtained with the coenzyme analogue periodate-oxidized $NADP^+$. Either in the presence or in the absence of the substrate, 6-phosphogluconate, the stoichiometry of inactivation is 2 mol of inhibitor/mol of enzyme dimer (Fig. 2 inset). Again, the plot of the inactivation half-times against the reciprocal of the inhibitor concentrations is linear, showing that the two sites are fully independent (Fig. 2). The only effect of the substrate is a decrease of the K_i from 92 μM to 14.6 μM.

Binding of the reduced coenzyme

In contrast with the data obtained with $NADP^+$, the binding stoichiometry of NADPH is only 1 mol of coenzyme/mol of enzyme dimer (Fig. 3), with $K_d = 0.5\,\mu M$. A second binding site for NADPH

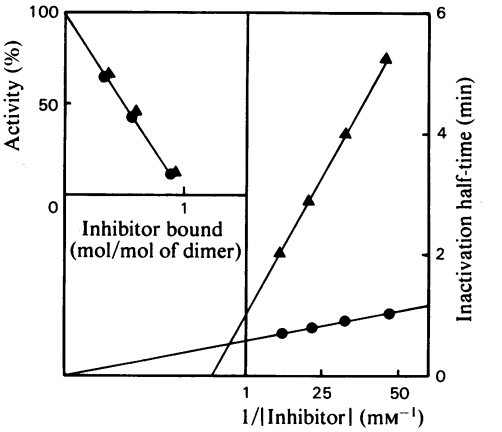

Fig. 2. *Inactivation of erythrocyte 6-phosphogluconate dehydrogenase by periodate-oxidized $NADP^+$*

The enzyme was inactivated at different concentrations of inhibitor, and the inactivation half-times were plotted against the reciprocal of the concentration of the inhibitor. Inset: stoichiometry of inactivation by periodate-oxidized [^{3}H]$NADP^+$. The enzyme was inactivated to different extents and the enzyme–inhibitor complex was stabilized by reduction with $NaBH_4$. After removal of the excess of reagents, the enzyme was checked for residual activity and for the bound radioactivity. ▲, In the absence of 6-phosphogluconate; ●, in the presence of 1 mM-6-phosphogluconate.

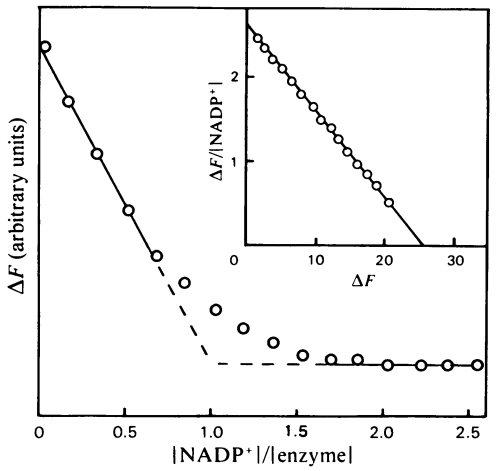

Fig. 1. *Binding of $NADP^+$ to erythrocyte 6-phosphogluconate dehydrogenase*

To a solution of enzyme (42 μM in subunits, 0.4 ml) in 50 mM-triethanolamine/HCl buffer, pH 7.5, were added portions of $NADP^+$ solution (2.85 mM, 1 μl each addition). The protein fluorescence (excitation at 290 nm, emission at 330 nm) was corrected for the dilution. Inset: Scatchard plot for the binding of the $NADP^+$ to the enzyme. The experimental conditions were the same as for the titration experiments, except for the enzyme concentration (3 μM in subunits).

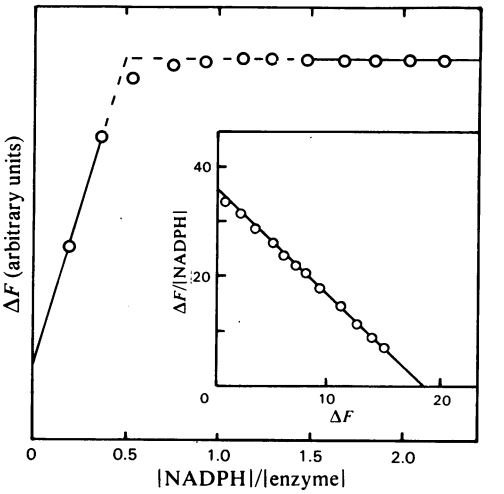

Fig. 3. *Binding of NADPH to erythrocyte 6-phosphogluconate dehydrogenase*

The experiments were performed as described in Fig. 1 legend, with an enzyme concentration of 14.3 μM and an NADPH concentration of 1.07 mM. The fluorescence of the NADPH was recorded (excitation at 340 nm, emission at 460 nm). Inset: Scatchard plot for the binding of NADPH to the enzyme. The enzyme concentration was 0.43 μM in subunits, the fluorescence was excited at 280 nm, and the emission was recorded at 460 nm.

could not be detected in the concentration range examined (up to 100 μM).

The presence in the reaction mixture of the 6-phosphogluconate does not modify either the binding stoichiometry or the dissociation constant.

Since $NADP^+$ and NADPH show different stoichiometries, it was essential to establish whether the enzyme is able to bind, at the same time, both the oxidized and the reduced coenzyme.

We measured the apparent dissociation constant for NADPH ($K^{app.}_{NADPH}$) in the presence of different concentrations of $NADP^+$. If the binding of $NADP^+$ and NADPH is mutually exclusive, i.e. if the dimeric enzyme is able to bind only $NADP^+$ or NADPH, the $K^{app.}_{NADPH}$ should be given by:

$$K^{app.}_{NADPH} = K_{NADPH}\left(1+\frac{[NADP^+]}{K_{NADP^+}}\right)^2 \qquad (1)$$

If instead the binding of NADPH to one subunit does not impair the binding of the $NADP^+$ to the other subunit (and thus the two subunits are fully independent in this regard), the $K^{app.}_{NADPH}$ should be given by:

$$K^{app.}_{NADPH} = K_{NADPH}\left(1+\frac{[NADP^+]}{K_{NADP^+}}\right) \qquad (2)$$

Our data agree with eqn. (1) (Fig. 4), and therefore the enzyme is unable to bind, at the same time, both the oxidized and the reduced coenzyme.

Initial-rate kinetics

Initial-rate kinetics were studied with either 6-phosphogluconate or $NADP^+$ as varied substrate. The Lineweaver–Burk plot (Fig. 5) shows a set of intersecting straight lines with 6-phosphoglucon-

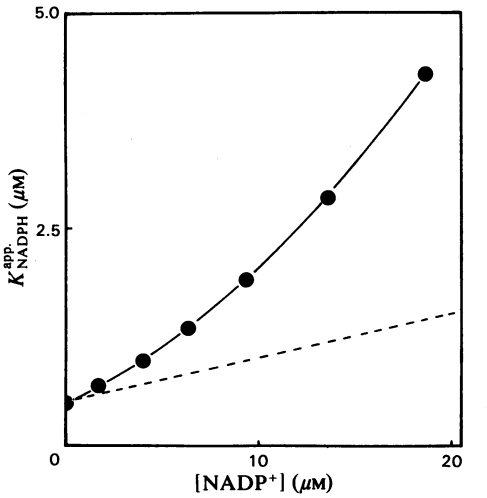

Fig. 4. *Effect of $NADP^+$ concentration on the $K^{app.}_{NADPH}$ of erythrocyte 6-phosphogluconate dehydrogenase*
The $K^{app.}_{NADPH}$ values were obtained from the Scatchard plot, as described in Fig. 3 legend, in the presence of the $NADP^+$ concentrations indicated on the abscissa. ●, Experimental values; ——, calculated from eqn. (1); – – – –, calculated from eqn. (2).

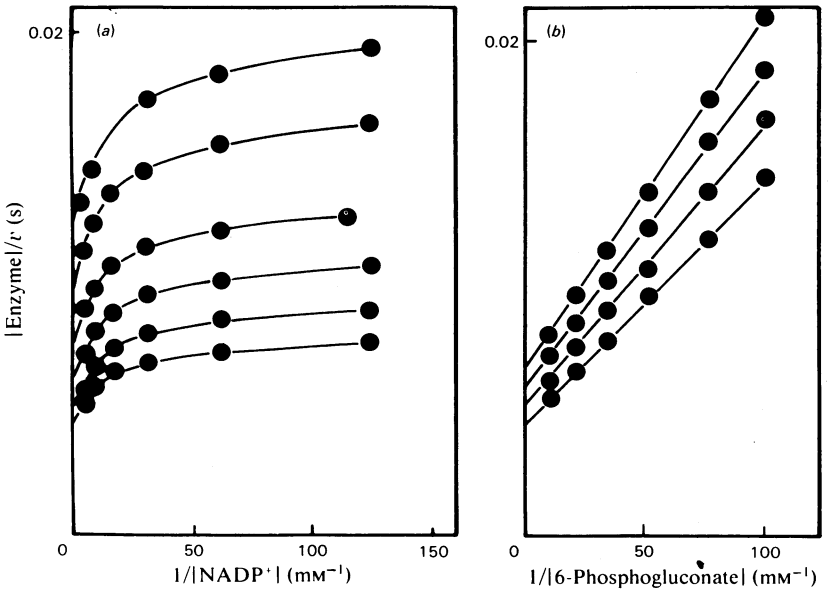

Fig. 5. *Lineweaver–Burk plots of initial-rate measurements for erythrocyte 6-phosphogluconate dehydrogenase*
(*a*) Plot of reciprocal of the reaction rates against the reciprocal of the concentration of $NADP^+$. The concentrations of 6-phosphogluconate are (from top to bottom) 10 μM, 13 μM, 20 μM, 30 μM, 50 μM and 100 μM. (*b*) Plot of reciprocal of reaction rates against the reciprocal of the concentrations of 6-phosphogluconate. The concentrations of $NADP^+$ are (from top to bottom) 4 μM, 16 μM, 60 μM and 170 μM.

ate as varied substrate, whereas the plot with $NADP^+$ as varied substrate shows a set of curves. Secondary plots as a function of the reciprocal of the $NADP^+$ concentrations are again curves (not shown).

These data suggest that 6-phosphogluconate dehydrogenase displays a Michaelian behaviour with 6-phosphogluconate (K_m 20 μM) and a negative co-operativity with $NADP^+$ (K_{m1} 0.4 μM, K_{m2} 172 μM).

Inhibition studies were performed to establish whether the binding of substrate and coenzyme is ordered or random. The curvature of double-reciprocal plots with $NADP^+$ as varied substrate does not allow a clear-cut definition of the inhibition pattern. The following statements are based on computer-fitted curves.

NADPH acts as a competitive inhibitor towards $NADP^+$ and as a mixed inhibitor towards 6-phosphogluconate (Fig. 6). Also, gluconate 6-sulphate acts as a competitive inhibitor towards 6-phosphogluconate and as a mixed inhibitor towards $NADP^+$ (Fig. 6). These results suggest that the enzyme follows a sequential random-order mechanism, in that a competitive inhibitor for each substrate acts as mixed inhibitor for the other substrate.

A similar random-order mechanism was suggested for the 6-phosphogluconate dehydrogenases from *Candida utilis* (Pontremoli *et al.*, 1962) and sheep liver (Dyson *et al.*, 1973), whereas an ordered mechanism was suggested for this latter enzyme (Silverberg & Dalziel, 1973) and for the enzyme from pig liver (Toews *et al.*, 1976).

Discussion

In the present paper we show that 6-phosphogluconate dehydrogenase from human erythrocytes displays a full-site reactivity with the oxidized

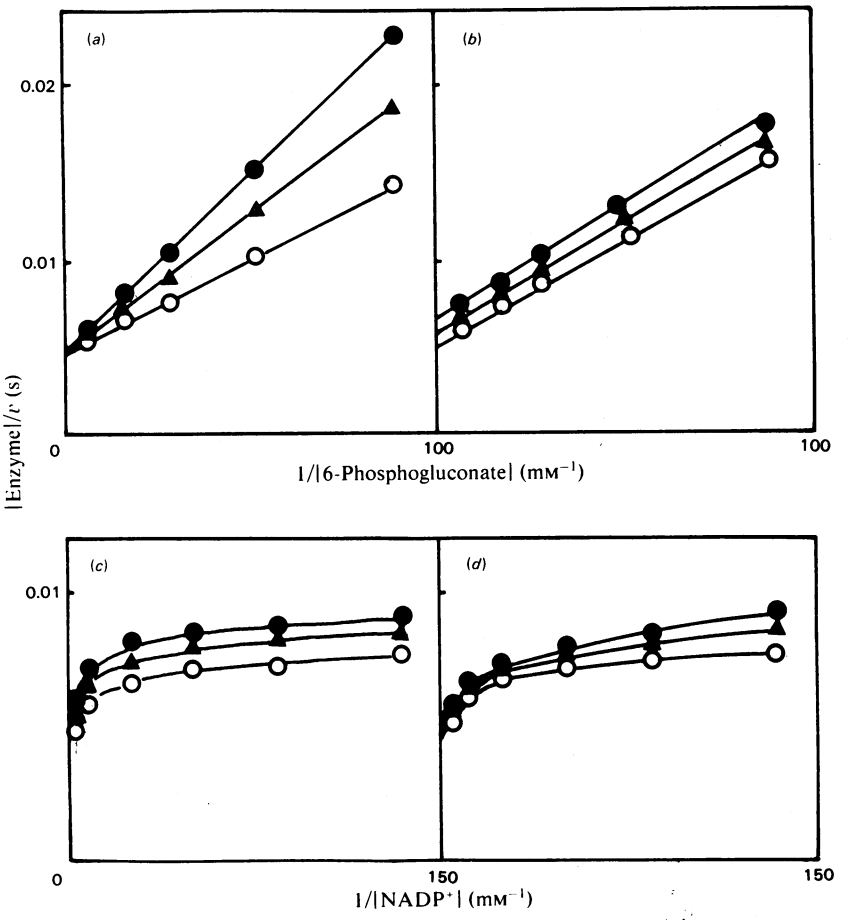

Fig. 6. *Inhibition of erythrocyte 6-phosphogluconate dehydrogenase by gluconate 6-sulphate and NADPH*
(*a*) and (*c*) Inhibitions by gluconate 6-sulphate: ○, none; ▲, 0.6 mM; ●, 1.5 mM. (*b*) and (*d*) Inhibitions by NADPH: ○, none; ▲, 42 μM; ●, 80 mM.

coenzyme and a half-site reactivity with the reduced coenzyme. We cannot exclude the binding of a second molecule of NADPH; however, this could only occur very far from physiological concentrations.

The most interesting kinetic feature is the negative co-operativity towards $NADP^+$. Furthermore we propose for this enzyme a sequential random mechanism, although further data are required for definitive conclusions. A steady-state treatment of a random Bi Ter mechanism with two non-independent sites is a difficult task, and it is outside of the aim of the present work. We report below only some simple considerations.

The negative co-operativity appears to be a result of the half-site reactivity for NADPH. Indeed, dehydrogenation of the substrate occurring on an enzyme fully saturated by $NADP^+$ will produce $NADP^+$ and NADPH bound on the two sites of the same molecule of dimeric enzyme. We have seen that the free enzyme is unable to bind $NADP^+$ and NADPH at the same time, and it is expected that the enzyme could show a non-Michaelian behaviour.

If $NADP^+$ and NADPH are never bound on the same enzyme molecule at the same time, then high $NADP^+$ concentrations would inhibit the reaction. In our case we observe a negative co-operativity, and this means that, although with some difficulty, the enzyme can simultaneously hold $NADP^+$ and NADPH during the catalytic cycle. The 'instability' of the enzyme with both $NADP^+$ and NADPH bound is reflected by the increased K_m for the $NADP^+$. This explanation is consistent with the model proposed by Dalziel & Engel (1968) for the enzyme glutamate dehydrogenase.

The different binding stoichiometries for the oxidized and the reduced coenzyme might have remarkable effects on the regulation of the enzyme activity, because the NADPH acts as a strong inhibitor for the 6-phosphogluconate dehydrogenase, and the enzyme activity is regulated by the $NADP^+$/NADPH ratio. As shown in the Results section (eqn. 1), the apparent dissociation constant for the NADPH depends on a second-degree term in $NADP^+$ concentration. This non-linear relationship can provide an amplification to the regulatory role of the $NADP^+$/NADPH ratio. Indeed, a small increase of the $NADP^+$ concentrations will be 'amplified' by the second-degree term, and the inhibition by NADPH will be decreased with a high efficiency.

It has been pointed out that, when the enzymic activity is regulated by an inhibitor competing with the substrate, a more sensitive regulation can be achieved by negative co-operativity (Lamb & Rubery, 1976). In the present case the enzyme shows an extreme negative co-operativity for the inhibitor, and this results in an increased sensitivity of the enzyme to the substrate/effector ratio.

The evidence here reported of a possible regulatory mechanism operating in 6-phosphogluconate dehydrogenase does not mean that this enzyme could have a physiological role in the regulation of the pentose phosphate pathway. However, it has been reported that glucose 6-phosphate dehydrogenase from erythrocytes shows a coenzyme-binding stoichiometry similar to that of 6-phosphogluconate dehydrogenase, i.e. full-site reactivity with $NADP^+$ and half-site reactivity with NADPH (De Flora *et al.*, 1974*b*). Thus the regulatory mechanism observed in 6-phosphogluconate dehydrogenase could be a general mechanism for the regulation of the pentose phosphate shunt in human erythrocytes.

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