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REGULATION OF THE ACTIVITY OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE BY $NADP^+$ AND NADPH

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SUMMARY

1. The saturation function of $NADP^+$ for glucose-6-phosphate dehydrogenase (D-glucose 6-phosphate:NADP oxidoreductase, EC 1.1.1.49) from human erythrocytes (electrophoretic type A) is sigmoid-shaped under certain experimental conditions. If the data are plotted in terms of Hill's equation (J. MONOD, J.-P. CHANGEUX AND F. JACOB, *J. Mol. Biol.*, 6 (1963) 306), the value of the interaction coefficient is $n = 1.69$ at 27° and pH 8.0.

2. These kinetic data can be interpreted as indicative of the existence of at least two $NADP^+$-binding sites on the enzyme, with a transition from low to high affinity for $NADP^+$ when the concentration of $NADP^+$ is increased. A simple method for calculating the two corresponding dissociation constants is presented, and the approximate values obtained are $K_{s1} = 45\ \mu M$, $K_{s2} = 13\ \mu M$ respectively.

3. NADPH inhibits erythrocyte glucose-6-phosphate dehydrogenase ($K_i = 16\ \mu M$). The kinetics of inhibition can be interpreted in terms of the superimposition of two effects of NADPH: (a) competition with $NADP^+$ for (possibly identical) binding site(s), and (b) enhancement of the affinity for $NADP^+$ of the remaining binding site(s). As a result, the cooperativity of $NADP^+$ molecules is decreased in the presence of NADPH, and NADPH has a paradoxical activating effect when the concentration of $NADP^+$ is very low.

4. Since the concentrations of $NADP^+$ and NADPH, in erythrocytes, are of the same order of magnitude, as the dissociation constants derived here, it appears that the changes in affinity induced by $NADP^+$ and the product inhibition by NADPH may represent a physiological mechanism for the regulation of glucose-6-phosphate dehydrogenase activity in the red cell.

INTRODUCTION

As part of a comparative investigation of some genetic variants of human erythrocyte glucose-6-phosphate dehydrogenase[1,2] (D-glucose 6-phosphate:NADP oxidoreductase, EC 1.1.1.49) it became important to determine a number of their kinetic properties. In the course of the study it became apparent that, under certain

experimental conditions, the dependence of the reaction rate on $NADP^+$ concentration does not conform to the Michaelis–Menten equation. This communication provides quantitative data on the deviation from standard kinetics for one of the common enzyme variants. The type of inhibition exerted by one of the products of the reaction, NADPH, has also been characterized.

EXPERIMENTAL

Materials

Glucose 6-phosphate, $NADP^+$ (Boehringer and Soehne) and NADPH (Sigma Chem. Co.) were all standardized spectrophotometrically at 340 mμ. Glucose-6-phosphate dehydrogenase of electrophoretic type A (refs. 3, 4) was partially purified from human red cells as described by CHUNG AND LANGDON[5], up to a specific activity of 5 units per mg of protein. Preparations were stored as precipitates under 70% $(NH_4)_2SO_4$. Before use the precipitate was dissolved in 0.05 M Tris–borate buffer (pH 8.0 at 25°), containing 0.1 mM EDTA and 2 μM $NADP^+$. The solution was dialyzed at 4° against 3 × 300 vol. of the same buffer, and finally clarified by centrifugation for 20 min at 15 000 × g.

Reaction kinetics

The reaction mixture used for enzyme velocity measurements contained 0.05 M Tris–borate buffer (pH 8.0), 4 mM glucose 6-phosphate, and variable amounts of $NADP^+$ (as shown in the figures) in a final vol. of 0.30 ml. The reaction mixture was prepared in a micro cuvette and was then equilibrated for 3 min at 27 ± 1° in a Gilford multiple absorbance recorder (model 2000), equipped with thermospacers. The reaction was initiated by inserting into the cuvette a small piece of plastic sheet (cut to fit loosely in the cuvette) to which 10 μl of enzyme solution had been applied. The sheet was gently moved up and down, twice, in order to achieve thorough mixing, and was then rapidly removed. The reaction was followed by recording the absorbance at 340 mμ with automatic blank compensation, at full scale settings ranging from 0.25 to 1.00 absorbance units. In the absence of either of the substrates the slope of the recording was 0, with random fluctuations of less than ±0.002 absorbance units over several minutes. The time interval between the addition of the enzyme and the beginning of the recording was less than 10 sec. When the $NADP^+$ concentration was less than 20 μM only one cuvette at a time was used, with the instrument programmed to record the absorbance of the reaction mixture every 15 sec. At higher $NADP^+$ concentrations 2 cuvettes at a time were used.

Velocity measurements at low $NADP^+$ concentrations

It will be shown that under the experimental conditions employed the velocity of the reaction at low $NADP^+$ concentrations was less than expected on the basis of Michaelis–Menten kinetics. Two possible artefactual causes for this behaviour have been considered and ruled out. (1) The enzyme velocity at low $NADP^+$ concentrations might have been underestimated if the initial slope of the absorbance curve had been missed due to substantial substrate depletion prior to commencement of recording. That this was not the case can be definitely proven by referring, for instance, to the lowest point of Curve A of Fig. 1a. Here the $NADP^+$ concentration

was 4 μM and the velocity observed was 0.0012 ΔA_{340}/min. Even if the 'initial' velocity had been 4 times greater (*i.e.*, that observed at 8 μM $NADP^+$), only 15% of the $NADP^+$ would have been consumed during the first minute. Since the delay in recording was only 10 sec, and 3 points were recorded within the first minute, it is not possible that an initial significantly higher slope of the reaction curve has been systematically missed. (2) The enzyme may have been inhibited by NADPH (produced in the course of the reaction) to a greater extent at low than at high $NADP^+$ concentrations. If we again refer to the same experimental point (lowest

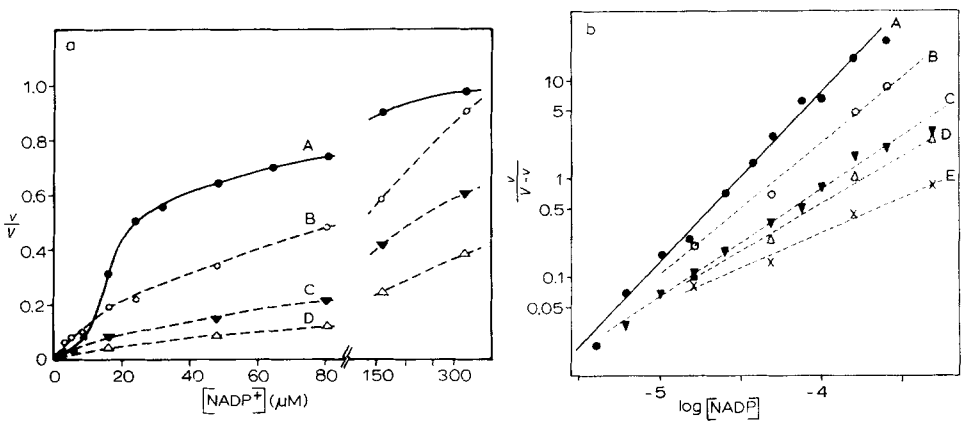

Fig. 1. Saturation function of red cell glucose-6-phosphate dehydrogenase with respect to $NADP^+$. The reaction mixture contained 0.05 M (final concn.) Tris–boric acid buffer (pH 8.0), 4 mM (final concn.) glucose 6-phosphate, $NADP^+$ as shown on abscissa and NADPH as indicated below. The maximal velocity, V, was 0.08 ΔA_{340}/min. (a) Plot of the reaction velocity (v) normalized to V. Curve A, in the absence of NADPH. Curve B, with 80 μM NADPH. Curve C, with 160 μM NADPH. Curve D, with 320 μM NADPH. (b) Hill plot (see ref. 6) of same data. Curve A, in the absence of NADPH. Curve B, with 40 μM NADPH. Curve C, with 80 μM NADPH. Curve D, with 160 μM NADPH. Curve E, with 320 μM NADPH. The values of the interaction coefficient n for the five curves are as follows: 1.69, 1.33, 1.09, 0.98, 0.75.

point on Curve A, Fig. 1a) by using the values of K_s and K_i derived below, we calculate that the inhibition caused by NADPH would be 17% after 4 min, assuming fully competitive behaviour. The error is therefore negligible before 4 min, when the slope is actually measured.

RESULTS

The velocity of the enzyme reaction as a function of $NADP^+$ concentration does not show a simple hyperbolic dependence (Fig. 1a, Curve A). Rather, the sigmoid shape of the curve suggests that the affinity for $NADP^+$ is low at low concentrations, and increases sharply when the concentration of this substrate is increased. It should then be possible to determine 2 dissociation constants for the enzyme–substrate complex, at low and high $NADP^+$ concentrations respectively (see APPENDIX). In practice, a rough estimate of the latter can be obtained from a Lineweaver–Burk plot, ignoring the points at concentrations below 80 μM: this gives a value of K_s = 20 μM. A method for determining this constant more accurately, as well as for

estimating the low affinity constant is presented in the APPENDIX. The degree of cooperation among substrate molecules can be conveniently expressed by way of Hill's empirical equation (see ref. 6). Under the experimental conditions described, the value of the interaction coefficient n has been found to lie, in 5 experiments, between 1.6 and 1.8 (see Fig. 1b, Curve A). The value of n varies with the composition of the reaction medium. Thus, it was higher (1.85) in 0.05 M Tris–HCl and lower (1.21) in the presence of 20 mM $MgSO_4$. The coefficient of interaction also increases with increasing temperature. Thus, $n = 1.86$ at 37° and 2.02 at 46°.

It is known that NADPH is a competitive inhibitor of yeast glucose-6-phosphate dehydrogenase[7]. A similar behaviour is also observed in the case of the red cell enzyme type A. The inhibition constant calculated by the method of DIXON[8] is $K_i = 16\ \mu M$ (see Fig. 2b). Deviations from standard 'full competition' kinetics are

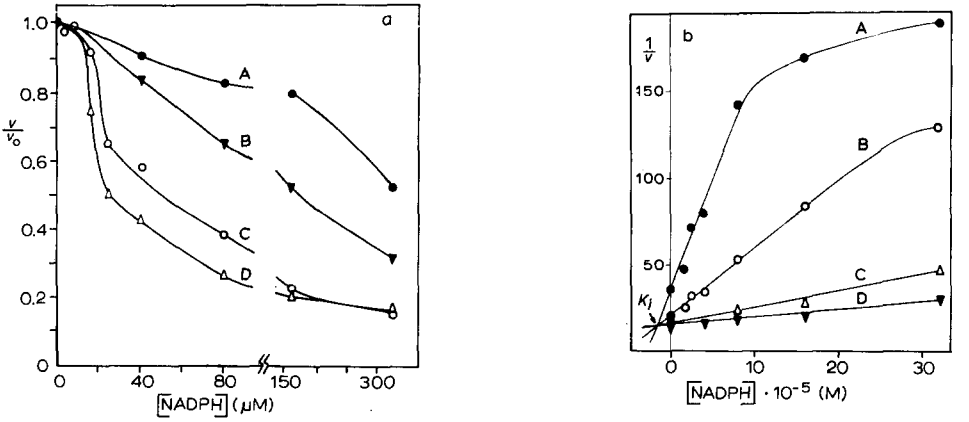

Fig. 2. Inhibition of red cell glucose-6-phosphate dehydrogenase by NADPH. Data as from Fig. 1. Concentration of NADPH as shown on abscissa and concentration of $NADP^+$ as indicated below. (a) Plot of the reaction velocity (v) normalized to the velocity of the uninhibited reaction (v_0). Curve A, with 480 μM $NADP^+$. Curve B, with 160 μM $NADP^+$. Curve C, with 48 μM $NADP^+$. Curve D, with 16 μM $NADP^+$. (b) Same data plotted according to DIXON[8]. Curves as in (a). The intersection of the four lines yields $K_i = 16\ \mu M$.

apparent. Thus, the inhibitor saturation curves are sigmoid in shape (Fig. 2a). By contrast, NADPH reduces and eventually abolishes the sigmoid shape of the substrate saturation function (Curves B–D, Fig. 1a). Further, at low $NADP^+$ concentrations and high NADPH concentrations the inhibition is less than expected, as shown by deviations from linearity in the reciprocal plots of Fig. 2b (Curves C and D). When the concentrations of both $NADP^+$ and NADPH are varied systematically, it is even possible to find conditions under which NADPH actually activates the reaction (*cf.* Curve B with Curve A in Fig. 1a). This is reminiscent of the observations made by GERHARDT AND PARDEE[9] on the effect of a substrate analogue on aspartate transcarbamylase.

DISCUSSION

The data presented indicate that the affinity of red cell glucose-6-phosphate dehydrogenase for $NADP^+$ varies with the concentration of $NADP^+$. This is to be

expected if the enzyme molecule bears multiple binding sites for $NADP^+$, and if the binding of the first molecule of $NADP^+$ modifies the affinity of one or more other sites for this substrate. In the simplest case, where there are two sites (or any number of equivalent pairs*), the expression for the reaction velocity as a function of substrate concentration can be readily derived (see APPENDIX): the function is found to be sigmoid, its exact shape depending on the values of the two different enzyme–substrate dissociation constants involved. No additional assumption is necessary in order to account for the kinetic data reported. With appropriate values for these constants Curve A of Fig. 1a can be fitted precisely (see APPENDIX).

As to the mechanism by which substrate binding affects enzyme–substrate affinity, the possibility of a conformational change in the sense of MONOD, WYMAN AND CHANGEUX[12] presents itself forcefully as the most convincing model thus far developed. It is of course impossible, on the basis of kinetic data alone, to distinguish between such a model and one in wich 'cooperation' between two substrate molecules depends—for instance—on their interaction at overlapping binding sites, without a conformational change in the enzyme. However, it may be recalled that KIRKMAN AND HENDRICKSON[13] and TSUTSUI AND MARKS[14] have independently reported that the sedimentation of erythrocyte glucose-6-phosphate dehydrogenase in sucrose density gradients is affected by the $NADP^+$ concentration. The forms of the enzyme having lower and higher sedimentation rates may be involved in a monomer–dimer equilibrium or may represent interconvertible conformational forms without difference in their state of aggregation. In either case, it is conceivable that the change in sedimentation behaviour induced by $NADP^+$ also corresponds to the transition from low to high affinity for $NADP^+$, observed in our experiments.

The effect of NADPH can be interpreted as resulting from the combination of two effects: direct competition with $NADP^+$ for (possibly identical) binding sites, and enhancement of the affinity for $NADP^+$ of the remaining binding sites. Since competition is very strong, the second effect becomes apparent, by causing enzyme 'activation', only when the concentrations of $NADP^+$ and NADPH are both low (Fig. 1a, Curve B**). It is also revealed by the fact that the slope of the substrate saturation function (in the form of Hill's plot) decreases when the concentration of NADPH increases (Fig. 1b): the substrate analogue, by replacing substrate molecules, obscures the cooperative effect among them***. This is consistent with, and is a specific prediction of the model of MONOD, WYMAN AND CHANGEUX[12].

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* The highest value for the interaction coefficient n observed in this study was 2.02 at 46°. This indicates either that cooperation in binding is 'infinite' at this temperature, or that the binding sites are more than two in number. The number of molecules of bound $NADP^+$ per molecule of erythrocyte glucose-6-phosphate dehydrogenase has been estimated as being 2, or 6 (refs. 10, 11) but the relationship between 'bound' $NADP^+$ and 'substrate' $NADP^+$ is not yet known.

** In 1897 HALDANE AND SMITH[15] observed that a small percentage of CO in inhaled air helped animals to resist the effect of a very low O_2 pressure; and in 1935 HALDANE AND PRIESTLEY[16] interpreted this phenomenon in terms of the peculiar double-bended form of the dissociation curves of oxyhaemoglobin and CO-haemoglobin. It appears that the interaction between $NADP^+$ and NADPH in the binding to glucose-6-phosphate dehydrogenase can be qualitatively interpreted in the same way.

*** At 320 μM NADPH the value of the interaction coefficient n becomes actually less than 1 ($n = 0.75$, Curve E in Fig. 1b). This is difficult to explain, if the velocity of the reaction is always proportional to the concentration of the enzyme-substrate complex (or complexes), *i.e.* if K_{s_1}, K_{s_2} and K_i (see APPENDIX) have the meaning of true dissociation constants. The assumption that this is so, which has been made throughout the paper, may not be uniformly valid.

In conclusion, it has been found that the kinetics of erythrocyte glucose-6-phosphate dehydrogenase (electrophoretic type A) are not compatible with the Michaelis–Menten model but are compatible with a model involving two possible states of the enzyme, with low and high affinity for $NADP^+$ respectively. The transition from one state to the other can be brought about not only by NADP but also by NADPH, which, in addition, is a competitive inhibitor with respect to the former. The low dissociation constant for NADPH ($K_i = 16\ \mu M$) suggests that this reaction product may be an efficient feedback controller of the glucose-6-phosphate dehydrogenase activity. Moreover, it may be noted that the total concentration of NADP (oxidized + reduced) inside the erythrocyte is about 25 μM (from BISHOP, RANKINE AND TALBOTT[17]). If the ratio between $[NADP^+]$ and [NADPH] in the human red cell is similar to that found in other animals[18], *i.e.* about 2:1, then both lie in the range in which the transition of the enzyme from low to high substrate affinity takes place. Thus, the interplay between $NADP^+$ and NADPH, leading to enzyme activation or inhibition according to their absolute and relative concentrations, may constitute a refined mechanism for the regulation of the activity of glucose-6-phosphate dehydrogenase and therefore of the pentose-phosphate pathway in the red cell.

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APPENDIX

The approximations involved in the use of both the Michaelis–Menten and Hill's equations can be evaluated by considering a simple model for the binding of two molecules of substrate by the enzyme molecule, with different dissociation constants, thus:

$$E + S \rightleftharpoons ES \qquad K_{s_1} = [E]\,[S]/[ES] \tag{1}$$

$$ES + S \rightleftharpoons ES_2 \qquad K_{s_2} = [ES]\,[S]/[ES_2], \tag{2}$$

with $K_{s2} < K_{s1}$. Then the velocity v as a function of the substrate concentration $[S]$ would be*:

$$v = [S]^2 V/([S]^2 + [S]K_{s_2} + K_{s_1}K_{s_2}) \tag{3}$$

In reciprocal form:

$$V/v = 1 + K_{s_2}/[S] + K_{s_1}K_{s_2}/[S]^2 \tag{4}$$

* The following expression neglects the contribution that might be given to v by molecules of ES decomposing directly to $E + S$. Such contribution is expected to be small, considering that ES, compared to E, has greater affinity for S.

It is obvious that at high [S] the quadratic term becomes negligible and (4) reduces to the usual Michaelis–Menten form, with $K_m = K_{s_2}$. Therefore a reciprocal plot at high [S] will yield K_{s_2} (in our case 20 μM). Since most data on glucose-6-phosphate dehydrogenase have been obtained at relatively high concentrations of $NADP^+$, it is likely that the K_m values usually quoted really represent K_{s_2}. Theoretically one could measure $K_{s_1}K_{s_2}$ (and thus K_{s_1}) by plotting $1/[S]^2$ against $1/v$ at very low [S]. In practice on such a plot the error in the intercept is so great that only the order of magnitude of K_{s_1} can be obtained. In our case it can be deduced in this way that K_{s_1} lies between 5 and 25 μM. Alternatively, we may use known values of v and [S] and obtain K_{s_1} and K_{s_2} directly from equation (4). From Curve A (Fig. 1a) we find that, for instance, at $v/V = 1/10$, $[S]_{1/10} = 9\ \mu$M; at $v/V = \frac{2}{3}$, $[S]_{2/3} = 52\ \mu$M. If we now insert these two pairs of values in equation (4) rewritten twice, we have a set of two equations for the two unknowns K_{s_1} and K_{s_2}. The solution is:

$$K_{s_1} = 45\ \mu\text{M}; \qquad K_{s_2} = 13\ \mu\text{M}.$$

When these values of the dissociation constants are re-inserted in equation (4) and the function $v[S]$ is drawn by points, the sigmoid curve obtained is almost superimposable on the experimental Curve A, *i.e.* it never deviates from it by more than 10%.

In logarithmic form (4) becomes:

$$\log \frac{v}{V - v} = \log [S] - \log \left(1 + \frac{K_{s_1}}{[S]}\right) - \log K_{s_2} \tag{5}$$

There is no simple relationship between (5) and the usual form of Hill's equation (ref. 6),

$$\log \frac{v}{V - v} = n \log [S] - n \log K \tag{6}$$

Thus the constant K, which lies between K_{s_1} and K_{s_2}, cannot be used to calculate either. On the other hand, n clearly remains a very convenient way to express the existence, at one time, of multiple binding sites and of different dissociation constants. In our case $K = 27\ \mu$M and $n = 1.7$ in the absence of NADPH.

It has been shown above that even at low $NADP^+$ concentration the enzyme has a measurable affinity for this substrate. This corresponds to the model with 'non-exclusive ligand binding' discussed by RUBIN AND CHANGEUX[19], with a high ratio between the two dissociation constants, $c = K_{s_2}/K_{s_1} = 0.3$. It is noteworthy that under these conditions (see bottom curve of Fig. 2b of their paper) the value of the Hill interaction coefficient n is expected to decrease when the substrate concentration required for half-saturation increases under the influence of an effector. This is exactly verified in our case upon addition of increasing amounts of NADPH (see Fig. 1b).

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