Catalytic Properties of Activated and Nonactivated Phosphoglucomutase*

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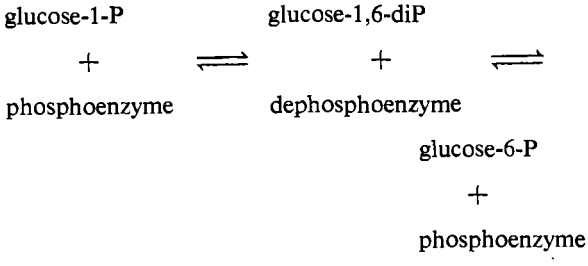
ABSTRACT: Phosphoglucomutase can be activated considerably by preincubation in Mg^{2+} and imidazole, both of which are also necessary for catalytic activity. The concentration of Mg^{2+} and imidazole used in the reaction mixture determines the *p*H optimum of the reaction.

The activated enzyme differs from the nonactivated form in several respects. The concentrations of Mg^{2+} and imidazole required for optimal activity are lower than those needed for the nonactivated form. The *p*H optimum is considerably lower and the resistance to thermal inactivation is greater than the nonactivated form. It is proposed that the activated enzyme is a Mg^{2+}–imidazole–protein complex and the activated substrate is a metallosubstrate complex composed of imidazole–Mg^{2+}–glucose 1-phosphate.

The enzyme phosphoglucomutase catalyzes the interconversion of glucose 1-phosphate (glucose-1-P) and glucose 6-phosphate (glucose-6-P) through a two-step mechanism which has been postulated to proceed as follows (Najjar and Pullman, 1954):



In earlier communications the time-dependent activation of phosphoglucomutase by preincubation with magnesium and imidazole was described (Robinson and Najjar, 1960, 1961). It was shown that the catalytic rate of the activated enzyme is increased 2- to 6-fold. The accompanying paper presents a detailed study of this phenomenon (Harshman *et al.*, 1965). The activation process appears to require both magnesium and imidazole. Neither alone is effective. The extent of activation varies directly with the products of their concentrations at any appropriate *p*H. This indicates that the activator is a complex composed of one Mg^{2+} and one molecule of imidazole^0 (unionized imidazole). In the light of these observations, a reevaluation of the Mg^{2+} and imidazole requirement for catalysis in the activated and nonactivated state was indicated. This paper reports such a study. The results confirm earlier indications that a Mg^{2+}-imidazole^0 complex is also an active component of the catalytic reaction (Robinson and Najjar, 1961). Enzyme activity was found to be influenced by the concentration of Mg^{2+}, imidazole, and hydrogen ions. The effect of hydrogen ions is exerted largely to the extent that it governs the concentration of imidazole^0. It is shown below that the requirement for Mg^{2+}-imidazole^0 complex during the catalytic reaction is considerably reduced in the activated state, i.e., when the enzyme has had prior exposure to the complex by preincubation in Mg^{2+} and imidazole. This is brought out by the lower *p*H and lower optimum concentrations of Mg^{2+} and imidazole required for optimal catalysis by the activated enzyme as compared to the nonactivated form.

Experimental Procedure

The methods and materials used for the preparation and assay of the enzyme have been described in the accompanying publication (Harshman *et al.*, 1965). In all studies involving the activated state, the enzyme was preincubated in $MgCl_2$ 1×10^{-3} M and imidazole 4×10^{-2} M at *p*H 7.5 and 0°. The final concentration of the enzyme varied between 10 and 20 μg/ml. Ten minutes of incubation sufficed to allow the maximum activation possible under these conditions (Robinson and Najjar, 1961). The level of activation is measured by transferring 0.1 ml of the enzyme to a standard reaction mixture having a final concentration of $MgCl_2$ 1×10^{-3} M, imadazole 4×10^{-2} M, and glucose-1-P 4×10^{-3} M at *p*H 7.5. The samples of substrate used contained sufficient amounts of glucose-1,6-diP to yield a final concentration in the reaction mixture of $5–10 \times 10^{-6}$ M. This is an appropriate range for the saturating concentration necessary for optimal catalysis. The reaction was run at 30°.

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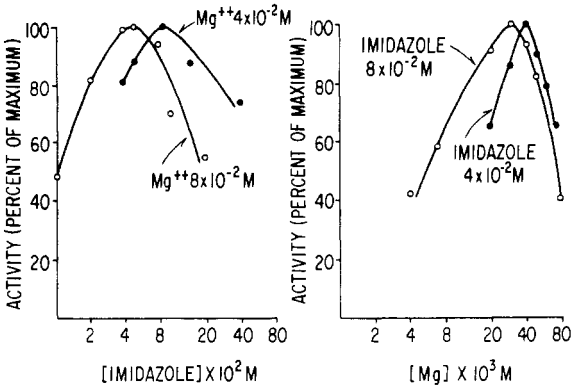

FIGURE 1: The effect of varying Mg^{2+} and imidazole concentrations on their requirements for optimal catalytic activity of the nonactivated enzyme. The log of the concentrations, ranging from 1×10^{-2} M, at the intersection of the ordinate, to 80×10^{-2} M for imidazole and 1×10^{-3} to 80×10^{-3} M for Mg^{2+}, are plotted against the per cent of maximal activity. The standard reaction mixture contained glucose-1-P 4×10^{-3} M. This contributed saturating quantities of glucose-1,6-diP 5–10 $\times 10^{-6}$ M, $MgCl_2$, and imidazole as indicated in the graph, *p*H 7.5, temperature 30°, final volume 0.5 ml. The reaction was started by the addition of about 1 μg of crystalline enzyme, appropriately diluted in water at 0° immediately prior to use. The reaction was stopped after 5 minutes by the addition of 1 ml of 5 N H_2SO_4, at which interval the rate of the reaction still maintains zero-order kinetics. The enzyme had a specific activity of 18 (Najjar, 1948) catalyzing the formation of 116 μmoles glucose-6-P/mg/min.

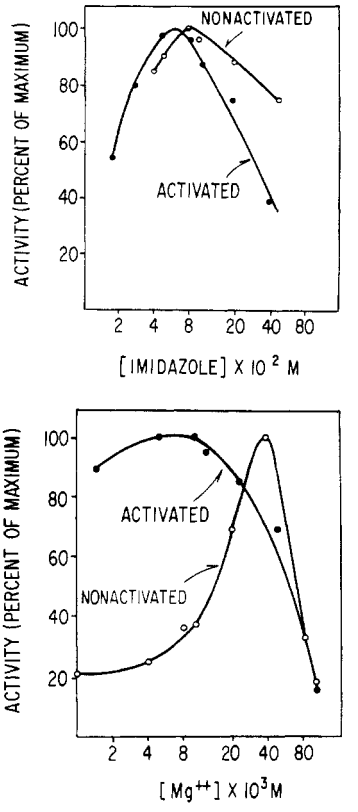

FIGURE 2: A comparison of the effect of varying Mg^{2+} and imidazole concentrations on the catalytic rate of activated and nonactivated enzyme. The enzyme was activated by preincubation for 10 minutes in $MgCl_2$ 1×10^{-3} M and imidazole 4×10^{-2} M at *p*H 7.5 and 0° and at a concentration of 10 μg of enzyme per ml. The nonactivated enzyme was diluted in water at 0°. The upper graph shows the effect of varying imidazole concentration while $MgCl_2$ was held constant at 4×10^{-2} M. The lower one shows the effect of varying $MgCl_2$ concentration in constant imidazole 4×10^{-2} M. The nonactivated enzyme in the amount of 1.0 μg, or 0.25 μg of the activated form, was added to the reaction mixture at zero time. The remaining components and characteristics of the reaction are otherwise as described in Figure 1. The activation process raised the activity level 5-fold to yield a specific activity of 90 or a formation of 580 μmoles of glucose-6-P/mg/min.

Results

Mg^{2+}-Imidazole-linked Effect on the Catalytic Parameters. EFFECT OF Mg^{2+} ON THE OPTIMAL REQUIREMENT OF IMIDAZOLE AND VICE VERSA. In this study, the concentration of Mg^{2+} and imidazole in the reaction mixture was varied within appropriate limits and a comparison was made of the optimal conditions required for the activated and nonactivated enzyme. It is apparent that both components are involved in the expression of enzyme activity. The results observed with the nonactivated enzyme are illustrated in Figure 1. The Mg^{2+} concentration required for maximum activity drops from 4×10^{-2} to 2.8×10^{-2} M when the concentration of imidazole is raised from 4×10^{-2} to 8×10^{-2} M, respectively. In a similar manner, the concentration of imidazole giving maximum activity is found to drop from 9×10^{-2} to 5×10^{-2} M when the Mg^{2+} concentration is raised from 4×10^{-2} to 8×10^{-2} M.

Similar effects were obtained qualitatively with the activated enzyme. However, there was a definite quantitative difference. It is clear from Figure 2 that the activated enzyme gives a broad range of Mg^{2+} concentration for catalytic stimulation with maximum activity at 6×10^{-3} M in 4×10^{-2} M imidazole. By contrast, the nonactivated enzyme has a sharp maximum at a markedly greater concentration of 4×10^{-2} M Mg^{2+} for the same imidazole concentration. Further differences are discernible in the high ranges of Mg^{2+} concentrations. The activated enzyme is strongly inhibited by the concentration of Mg^{2+} which gives maximum activity with the nonactivated enzyme. Figure 2 also shows that the requirements for imidazole vary quantitatively with the state of activation of the enzyme, both for stimulation as well as inhibition of activity. The activated enzyme in both respects responds to lower concentrations than are required for

the nonactivated form. In Mg^{2+} 4×10^{-2} M, the imidazole requirement for maximum activity of the activated enzyme is about 5×10^{-2} M, while under the same conditions that of the nonactivated enzyme is about 9×10^{-2} M. Furthermore, 4×10^{-2} M Mg^{2+} and 4×10^{-1} M imidazole cause 60% inhibition of the catalytic activity of the activated enzyme while the same concentrations inhibit the nonactivated enzyme only to the extent of 20%.

EFFECT OF VARYING Mg^{2+} AND IMIDAZOLE CONCENTRATIONS ON *p*H OPTIMUM. It is clear thus far that the Mg^{2+} and imidazole-linked effect on the enzyme is both stimulatory and inhibitory in its expression. In this connection it was shown (Robinson and Najjar, 1961) that the extent of stimulation of catalysis varied directly with the product of Mg^{2+} and imidazole concentrations, indicating a Mg^{2+}-imidazole complex as the active agent. This was derived from the expression:

$$Mg^{2+}\text{-imidazole} \rightleftharpoons Mg^{2+}\text{-imidazole complex}$$

or

$$[Mg^{2+}] \times [\text{imidazole}] \times K = Mg^{2+}\text{-imidazole complex}$$

On the basis that the rate of catalysis, as expressed by the glucose 6-phosphate concentration formed per minute, is directly proportional to the concentration of Mg_1-$imidazole_1$ complex, the equation can then be expressed in terms of the enzymatic activity.

$$[Mg^{2+}] \times [\text{imidazole}] \times K = [\text{glucose-6-P}] \text{ formed per minute}$$

Using varied concentrations of Mg^{2+} and imidazole, within appropriate limits, a constant K was obtained only when one Mg^{2+} and one imidazole were considered (Najjar, 1962).

Only the $imidazole^0$ would participate in the formation of this type of complex since an association between Mg^{2+} and imidazolium is unlikely because of charge repulsion. On this basis, the concentration of Mg^{2+}-$imidazole^0$ complex would vary with *p*H. Accordingly, the *p*H optimum for enzyme activity, in turn, would depend on the concentrations of Mg^{2+} and imidazole. At low values, optimal concentrations of Mg^{2+}-imidazole complex would be approached only at higher levels of *p*H. On the other hand, with high concentrations of Mg^{2+} and imidazole, the appropriate Mg^{2+}-imidazole concentration is reached at lower *p*H values. It was such considerations that prompted an investigation of these effects.

Figure 3 shows representative data which demonstrate a clear dependence of the *p*H optimum on the concentrations of Mg^{2+} and imidazole in the reaction mixture. At Mg^{2+} 1×10^{-3} M and imidazole 4×10^{-2} M, the optimum *p*H for activity is about 8.0. However, when the imidazole concentration is increased to 1×10^{-1} M, the optimum *p*H shifts to about 7.5. With the same imidazole concentration of 1×10^{-1} M, an increase of Mg^{2+} to 4×10^{-2} M results in a further shift in the *p*H optimum down to about 7.3. Finally, at the highest concentrations used 1×10^{-1} M, for both Mg and imidazole, the lowest *p*H optimum of about 6.9 is obtained. It is immediately apparent from the figure that the *p*H optimum varies inversely with the products of the Mg^{2+} and imidazole concentrations and further supports the idea that a Mg^{2+}-imidazole complex is the active agent in catalysis. While this explanation is in harmony with the findings, similar effects on the enzyme itself should not be ignored as these may well play a role in determining the *p*H optimum of the enzyme. Phosphoserylhistidine peptide is indeed present at the active center of the enzyme (Milstein and Sanger, 1961; Harshman and Najjar, 1962; Harshman *et al.*, 1964). Should the *pK* of the imidazole moiety be close to the higher range of that shown for proteins, 5.6–7.0 (Cohn and Edsall, 1943), there is reasonable assurance then that the state of the histidine residue at the active site of the enzyme will also influence the catalytic activity in much the same manner as the added imidazole.

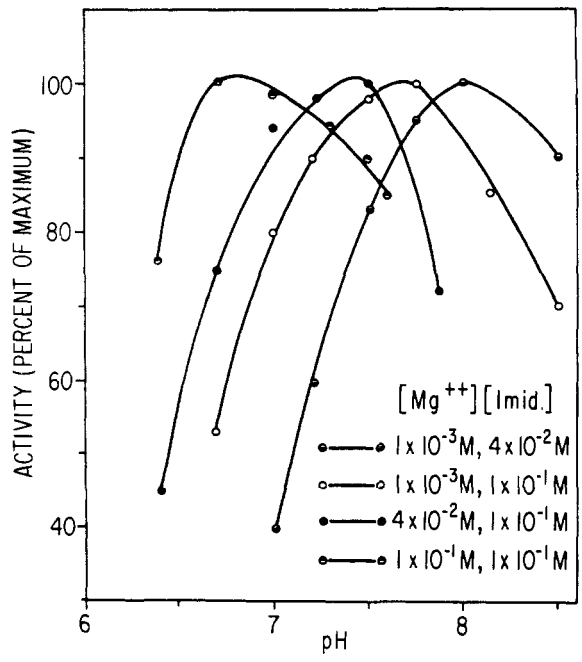

FIGURE 3: The effect of varying Mg^{2+} and imidazole on the *p*H optimum. The reaction mixture was composed of $MgCl_2$ and imidazole as shown in the graph. The *p*H was adjusted with NaOH or HCl to the required value. The nonactivated enzyme (1 μg) with a specific activity of 22 catalyzing the formation of 142 μmoles of glucose-6-P/mg/min at standard conditions is described in the text. The remaining components and characteristics of the reaction are as described in Figure 1.

The pH Optimum of Activated and Nonactivated Enzyme. We have seen earlier that the optimal concentrations for Mg^{2+} and imidazole obtained for the activated enzyme were substantially lower than those

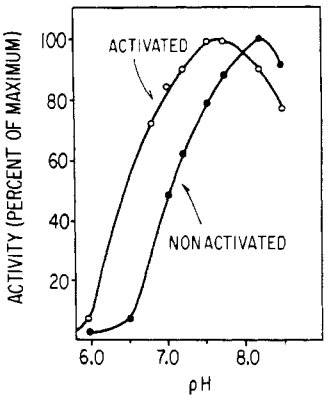

FIGURE 4: The pH optimum of activated and nonactivated enzyme. The enzyme was activated in Mg^{2+} 1×10^{-3} M and imidazole 4×10^{-2} M at 0° for 10 minutes (0.25 μg of the activated or 1 μg of the nonactivated enzyme was used to start the reaction). The *p*H was adjusted with NaOH and HCl. The remaining components and characteristics of the reaction are as described in Figure 1.

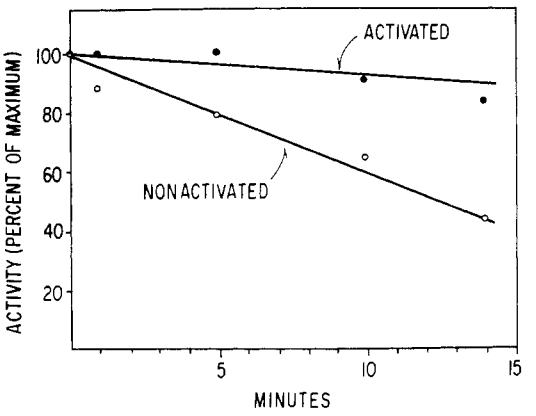

FIGURE 5: Thermal stability of activated and nonactivated enzyme. The activated enzyme, 0.9 mg/ml in $MgCl_2$ 1×10^{-3} M, and imidazole 4×10^{-2} M, final *p*H 7.5, and the nonactivated enzyme, also 0.9 mg/ml in NaCl 3×10^{-3} M, and imidazole 4×10^{-2} M, final *p*H 7.5, were placed in a water bath at 50°. Samples were removed at the times indicated and diluted appropriately in their respective media. Nonactivated (1 μg) or activated (0.25 μg) enzyme was used for the activity assay. The remaining components and characteristics of the reaction are as described in Figure 1. Control experiments, not in the graph, show no change in the effect on the nonactivated enzyme when NaCl was omitted from the medium.

needed for optimal activity of the nonactivated enzyme. This and other considerations (Harshman, *et al.*, 1965) indicated that the activated enzyme was essentially a Mg^{2+}-imidazole-protein complex and in this state requires less Mg^{2+}-imidazole concentration for maximal activity. On this basis, and because of the foregoing effect of Mg^{2+}-imidazole on the *p*H optimum, it would be expected that the activated enzyme would show a lower *p*H optimum than the nonactivated form at appropriate concentrations of Mg^{2+} and imidazole. The results shown in Figure 4 so indicate.

The activated enzyme shows maximum activity at *p*H 7.6, a substantially lower value than that obtained for the nonactivated enzyme of *p*H 8.2. In fact, the whole *p*H curve of the activated form is shifted to lower *p*H values by about the same amount of 0.6 unit shown at the respective maxima. At *p*H 6.8 where 50% of the imidazole is in the charged form, the activated enzyme functions at about 70% of the maximum rate while the nonactivated enzyme shows about 30% of the maximum rate.

Stability of Activated and Nonactivated Enzyme. In the course of these comparative studies on the activated and nonactivated enzyme, it was observed that the activated enzyme appeared to be more stable than the nonactivated form. It seemed quite reasonaole that complex formation, in the activated state, between the enzyme and Mg^{2+}-imidazole might impart some measure of increased rigidity to the molecule. A more detailed study of thermal stability of the two forms was therefore undertaken. The enzyme was dialyzed at 4° for 2 days against two changes, 1 liter each, of 4×10^{-2} M imidazole at *p*H 7.5. An aliquot was activated as usual by preincubation for 10 minutes at 0° in 1×10^{-3} M $MgCl_2$ and 4×10^{-2} M imidazole at *p*H 7.5 and a protein concentration of 0.9 mg/ml. The nonactivated enzyme was prepared from the same dialyzed enzyme in 4×10^{-2} M imidazole. However, no Mg^{2+} was added and instead 3×10^{-3} M NaCl was used to maintain a comparable ionic strength for both enzyme forms. Both preparations were placed in a 50° water bath and 0.1-ml samples were removed at 0-, 1-, 5-, 10-, and 15-minute intervals. Appropriate dilutions were made in 1×10^{-3} M Mg^{2+} and 4×10^{-2} M imidazole for the activated sample and in 4×10^{-2} M imidazole alone for the nonactivated control. Activity measurements were then made in the standard reaction mixture.

It can be seen in Figure 5 that the enzyme under conditions of activation is more stable than the nonactivated enzyme. The former lost only about 10% of its activity in 15 minutes while the latter lost almost 60%.

Discussion

Earlier findings (Robinson and Najjar, 1961) strongly suggested that a complex composed of Mg^{2+}-imidazole[0] was an active component of the catalytic reaction. Neither Mg^{2+} nor imidazole alone was active. The catalytic rate also varied directly with the product of the individual concentrations of Mg^{2+} and imidazole yielding a constant K at a given *p*H value. The additional

findings reported here support this view. At appropriate concentrations, a change in *p*H which alters the concentration of the un-ionized imidazole affects the catalytic rate accordingly; an increase in *p*H increases the stimulation and a decrease in *p*H produces the opposite effect. On the basis that the activated enzyme is a Mg^{2+}-imidazole-protein complex, the differences in the kinetic parameters obtained between the activated and the nonactivated forms are understandable. Thus the requirement for lower concentrations of Mg^{2+} and imidazole for the activated form is predictable on the basis that the enzyme already is complexed with Mg^{2+} and imidazole and in this manner the conformation of the active site is stabilized. Furthermore, because less Mg^{2+} and imidazole are required for optimal activity of the activated enzyme, the optimal value would therefore be reached at a lower *p*H than that required for the nonactivated enzyme. The latter would need a higher *p*H value to obtain higher concentrations of nonionized imidazole to arrive at the necessarily higher concentrations of Mg^{2+}-imidazole complex needed for optimal activity.

It has been proposed (Najjar, 1951) that the substrate for phosphoglucomutase is the metallosubstrate Mg^{2+}-glucose-1-P complex in view of the specific inhibition by $Mg^{2+}F_2$-glucose-1-P. Since Mg^{2+} is active only as an imidazole complex, the metallosubstrate was assumed to be imidazole-Mg^{2+}-glucose-1-P complex. This would anchor on the enzyme through Mg^{2+} where it is further bonded to the phosphate of serine and the imidazole group of the adjacent histidine (Robinson and Najjar, 1961). At the time, the active site was known to include Thr-Ala-Phosphoser-His-Asp (Milstein and Sanger, 1961). The active site has now been extended to include another phosphoserine adjacent to a glutamic residue (Harshman and Najjar, 1962; Harshman, *et al.*, 1964). In the latter case, the metallosubstrate complexi midazole-Mg^{2+}-glucose-1-P can be pictured as being bonded through Mg^{2+} on the γ-carboxyl of the glutamic acid and the phosphate of the adjacent serine residue. The presence of the added imidazole ligand may well facilitate the transfer of phosphate from one serine to the other to arrive at a state of equilibrium between the two serines of the peptide site which would be a necessary additional step for the mechanism of action of phosphoglucomutase.

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