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Activation of Phosphoglucomutase*

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ABSTRACT: The specific activity of phosphoglucomutase may be increased 2- to 6-fold by preincubation in Mg^{2+} and imidazole. Neither alone is effective. The rate of activation is a function of temperature reaching a maximum in about 5 minutes at 0° and in 30 seconds at 30°. The process is readily reversed upon lowering the concentration of Mg^{2+} and imidazole by dilution or by dialysis. The rate of deactivation is comparable to that of activation. Activation is immediately arrested but not reversed by the addition of substrate and is assumed to result from the association of a Mg^{2+}-imidazole complex with the enzyme which stabilizes an active configuration. The Mg^{2+}-imidazole-induced activation has an activation energy E of 4.8 kcal which contrasts with a value of 19.3 kcal for that of catalysis.

It has been reported from this laboratory that the observed specific activity of phosphoglucomutase (PGM)[1] may be considerably increased by preincubating the enzyme with Mg^{2+} and imidazole. This process will be referred to, henceforth, as activation and the enzyme so treated as activated enzyme (Robinson and Najjar, 1960, 1961). This induced activation does not obviate the necessity for the continuous presence of both Mg^{2+} and imidazole during the catalytic action of the enzyme. Thus it is clear that the phenomenon of activation differs from the usual type of catalytic stimulation of enzyme activity that has been observed with metal ions and metal binding agents (Cori *et al.*, 1937; Sutherland, 1949). A detailed study of the effect of Mg^{2+} and imidazole on the catalytic stimulation of PGM is the subject of an accompanying report (Robinson *et al.*, 1965). In this paper, a study of the factors affecting the time-dependent activation is reported. This includes the influence of temperature, hydrogen ion, Mg^{2+}, and imidazole concentrations, as well as the effect of imidazole derivatives and other complexing agents. The data reported here support the hypothesis that a specific complex composed of magnesium and imidazole is involved in this type of activation.

Experimental Procedure

Crystalline PGM, prepared either from fresh or frozen rabbit muscle (Pel-Freez, Rogers, Ark.) was used throughout this study. A slight but important modification of the earlier procedure (Najjar, 1948) was found to be useful in removing heat-denatured proteins from solution which are of gelatinous consistency and not readily sedimented by centrifugation. After the first heat step of the aqueous muscle extract, ammonium sulfate is added to 0.20 saturation and the precipitated protein is removed by centrifugation. The rest of the procedure remains unaltered.

The glucose 1-phosphate (glucose-1-P) (Nutritional Biochemicals Corp., Cleveland, Ohio) used in all these experiments contained a sufficient quantity of the coenzyme glucose 1,6-diphosphate (glucose-1,6-diP) (Leloir *et al.*, 1948) for optimal enzyme activity.

The imidazole (Aldrich Chemical Co., Milwaukee, Wis.) used throughout showed no activating effect unless Mg^{2+} was added. Occasional batches exhibited some activation. These were presumed to be contaminated with traces of activating metal ions and were not used further. Water was first distilled, then passed through a Barnstead ion-exchange demineralizer. This was found necessary to remove trace amounts of inhibitory metals. The same assay procedure was used throughout all these studies and was essentially similar to that described earlier (Najjar, 1948). Unless otherwise indicated, the standard reaction was carried out at 30° and *p*H 7.5 in a mixture that contained the following components expressed as μ moles: imidazole, 20; glucose-1-P, 2.0; $MgCl_2$, 0.5; in a final volume of 0.5 ml. The reaction was followed by estimation of the appearance of acid-stable phosphate. The specific

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[1] Abbreviation used in this work: PGM, phosphoglucomutase.

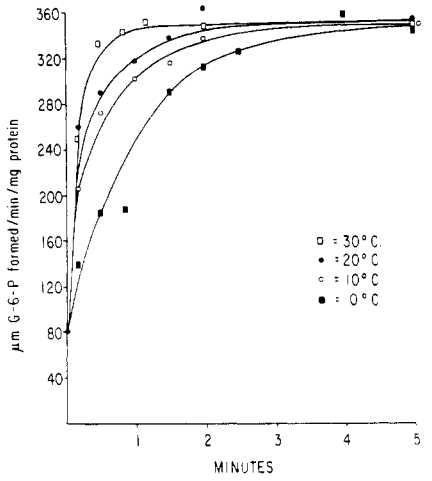

FIGURE 1: The effect of temperature on the rate of activation of PGM. The enzyme was preincubated at 0°, 10°, 20°, and 30° in a solution of 1×10^{-3} M $MgCl_2$ and 4×10^{-2} M imidazole, *p*H 7.5, at a final concentration of 40 μg/ml. At the times indicated, aliquots were transferred to the reaction mixture and assayed at 30° under standard conditions. G-6-P = glucose-6-P.

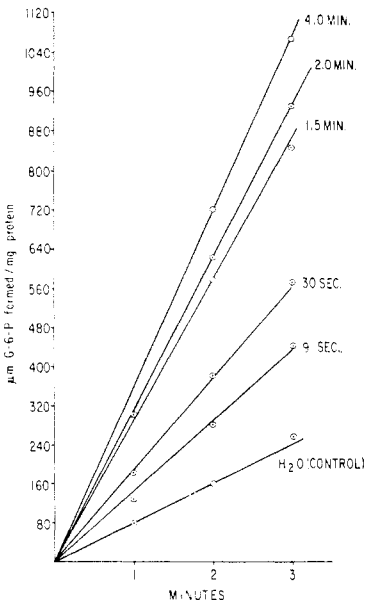

FIGURE 2: The linear rate of PGM activity at all levels of activation. The enzyme was activated by preincubation of $MgCl_2$ 1×10^{-3} M and imidazole 4×10^{-2} M at 0° and a concentration of 40 μg/ml. At the intervals shown after the onset of activation, the aliquots were assayed at 30° under standard conditions. G-6-P = glucose-6-P.

activity is here expressed in terms of μmoles of the acid-stable glucose 6-phosphate (glucose-6-P) formed per mg of protein per minute.

Results

Rate of Activation. Preincubation of PGM in imidazole buffer at *p*H 7.5 containing magnesium chloride increases the catalytic activity to a level two to six times that of the unincubated control. This is observed in freshly prepared extracts of fresh or frozen muscle as well as in crystalline preparations of the enzyme. The extent of activation varies with the enzyme preparation. It is time dependent and temperature sensitive. Activation is readily observed at 0° and reaches a near maximum level in approximately 4–6 minutes. The same level is, however, attained in about 30 seconds at 30° (Figure 1). Co^{2+} or Mn^{2+} are poor substitutes for Mg^{2+}. Their maximum activating effect is, respectively, 0.2 and 0.1 of that attained by comparable concentrations of Mg^{2+}.

Effect of Substrate. An interesting feature of the activation phenomenon is its extreme sensitivity to inhibition by the glucose monophosphates. Glucose-1-P or glucose-6-P in a concentration as small as 1×10^{-6} M caused 60–80% inhibition (Robinson and Najjar, 1961). However, no inhibition was observed with glucose diphosphate at concentrations as high as 1.6×10^{-7} to 2.0×10^{-5} M. Transfer of the enzyme to the reaction mixture at successive intervals after the onset of activation stops the activation process at the level attained at the moment of transfer. Thus the level of activation achieved during preincubation with the Mg^{2+} and imidazole is maintained after transfer to the assay solution. Figure 2 illustrates this point. Samples of enzyme were obtained at various intervals after the onset of activation and added to a reaction mixture, and the rate of catalysis was then measured at 1-minute intervals for 3 minutes. It is apparent that the rate is strictly linear, indicating that activation had been permanently arrested after exposure of the enzyme to the substrate. Had this not been the case an autocatalytic type of curve would have resulted.

Interdependence of Magnesium and Imidazole. A kinetic study of the requirement of Mg^{2+} and imidazole for catalytic activity indicated that the two moieties acted in consort as a Mg^{2+}-imidazole complex. Since the activation phenomenon also requires these two components, the question arose as to whether activation also depends on a specific complex of Mg^{2+} and imidazole. On this basis, the rate of activation would depend on the concentration of the complex. Therefore, it follows that this rate must relate directly to the product of the initial concentrations of Mg^{2+} and imidazole. To test this hypothesis two series of experiments were carried out at *p*H 7.5. In the first set Mg^{2+} concentration was held constant at 1×10^{-3} M and that of imidazole was varied from 4×10^{-5} to 8×10^{-1} M. In the second set the imidazole concentration was held constant at 4×10^{-2} M and the Mg^{2+} concentration varied from 1×10^{-5} to 1×10^{-1} M. Ionic strength and *p*H were held constant by appropriate additions of Tris. After 10 minutes at 0°, at which time

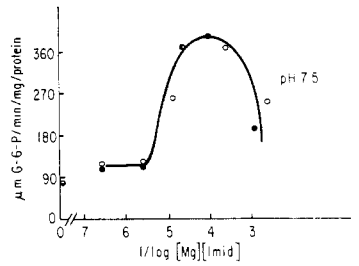

FIGURE 3: Activation as a function of the product of Mg^{2+} and imidazole concentrations. The enzyme, at a concentration of 40 μg/ml, was activated by preincubation for 5 minutes at 0° with varying concentrations of $MgCl_2$ 1×10^{-1} to 1×10^{-5} M in imidazole 4×10^{-2} M (—O—) and with varying imidazole concentrations 8×10^{-1} to 4×10^{-5} M in $MgCl_2$ 1×10^{-3} M (—●—). The *p*H in all cases was held at 7.5 and the ionic strength was balanced by the addition of appropriate amounts of Tris buffer. Aliquots of the enzyme were assayed under standard conditions. The product of the concentration of Mg^{2+} and imidazole are plotted against the specific activity obtained. G-6-P = glucose-6-P.

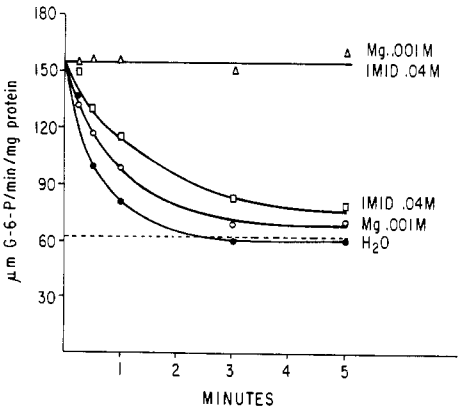

FIGURE 4: The dependence on both Mg^{2+} and imidazole for the maintenance of the activated state. PGM, at a concentration of 10 μg/ml, was maximally activated by preincubation for 5 minutes at 0° in a solution of 1×10^{-3} M $MgCl_2$ and 4×10^{-2} M imidazole, *p*H 7.5. Aliquots were then diluted 10-fold at 0° in one of the following diluents: (●) H_2O; (○) $MgCl_2$ 1×10^{-3} M; (□) imidazole 0.04 M, *p*H 7.5; (△) $MgCl_2$ 1×10^{-3} M, in imidazole 4×10^{-2} M, *p*H 7.5, as shown on the graph. At the intervals indicated following dilution, samples were assayed under standard conditions and the specific activity was recorded. The dashed line indicates the level of activity before activation. G-6-P = glucose-6-P.

activation reached maximum levels, the enzyme was transferred to the standard assay medium and the level of activity was assessed. The results of both sets of experiments are comparable and describe a common curve, showing that the level of activation increases with the product $[Mg^{2+}] \times$ [imidazole], reaches a maximum at about 4×10^{-4}, and is then followed by inhibition at higher values. Below 4×10^{-6} little or no stimulation is observed (Figure 3). These findings indicate that a Mg^{2+}-imidazole complex is responsible for the activation phenomenon. Since charge repulsion would exclude complex formation between Mg^{2+} and the imidazolium ion, it would be reasonable to suppose that the uncharged imidazole (imidazole°) is the complexing ligand.

For the maintenance of a maximally activated state, the continuous presence of optimal concentrations of Mg^{2+} and imidazole is required. This indicates that whatever the nature of the activated state, the phenomenon is highly reversible. In this connection, it has been possible to study the deactivation process by dialyzing or diluting the activated enzyme under conditions such that the concentration of either Mg^{2+}, imidazole, or both is lowered. Figure 4 illustrates one such experiment in which maximum enzyme activation was attained by preincubation in $MgCl_2$ 1×10^{-3} M and imidazole 4×10^{-2} M at *p*H 7.5. The enzyme was then diluted 10-fold in only imidazole 4×10^{-2} M, *p*H 7.5, $MgCl_2$ 1×10^{-3} M, or water. The control was diluted in the activating mixture. The rate of deactivation was then followed by activity assay at various times (Figure 4). A comparison of Figures 1 and 4 reveals in a striking fashion that the rate of deactivation is a mirror-image pattern of that of activation.

Effect of Hydrogen Ion Concentration on Mg^{2+}-*Imidazole Activation.* The process of activation was further investigated at various hydrogen ion concentrations. Identical mixtures of appropriate concentrations of Mg^{2+} and imidazole were adjusted to various *p*H values of 7.0, 7.3, and 7.5 and their activation effects were assessed. The concentration of imidazole^0 was calculated on the basis of a *pK* of 6.8. It is apparent from Figure 5 that the extent of activation exhibited a linear relationship with the product $[Mg^{2+}] \times$ [imidazole^0] at each given *p*H. However, at comparable product values, the enzyme nevertheless showed better activation with decreasing hydrogen ion concentrations. It is reasonable to attribute this to a direct effect on some ionizing group in the enzyme with a *pK* value not far from neutrality.

Contrast between Imidazole and EDTA Effects. Following the reported activation of PGM by Mg^{2+} and imidazole at 0° (Robinson and Najjar, 1960, 1961), a time-dependent activating effect of EDTA at 37° was observed (Milstein, 1961). Since the EDTA effect differed in the extent and rate of activation from that of the magnesium-imidazole, it was deemed desirable to compare the two systems. Preincubation of PGM with equimolar concentrations of magnesium and EDTA (Mg^{2+}-EDTA) of 5×10^{-4}, 5×10^{-3}, and 5×10^{-2} M for 5 minutes at 0° did not activate the enzyme but yielded activities comparable to the controls. The addition of magnesium chloride in excess of EDTA also did not result in any measurable activa-

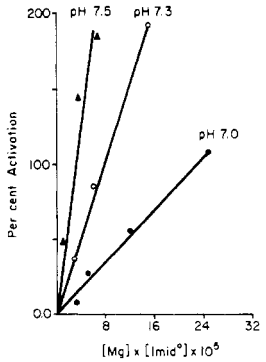

FIGURE 5: The effect of *p*H on the activation of PGM. The enzyme, at a concentration of 40 μg/ml, was maximally activated as before by preincubation for 5 minutes at 0° in the following Mg^{2+} and imidazole mixtures: $MgCl_2$ 1×10^{-3} and 5×10^{-3} M each in imidazole 4×10^{-2} and 8×10^{-2} M. The *p*H of the mixtures was adjusted to 6.5, 6.8, 7.0, 7.3, 7.5, 7.8, and 8.0. Nonactivated controls were incubated simultaneously in water at 0° for 5 minutes. Catalytic rates were measured under standard conditions. The extent of activation is plotted against the product of the concentrations of Mg^{2+} and un-ionized imidazole for *p*H 7.0, 7.3, and 7.5. Below *p*H 7.0 little or no activation was observed and at *p*H 7.5 and above activation was maximal.

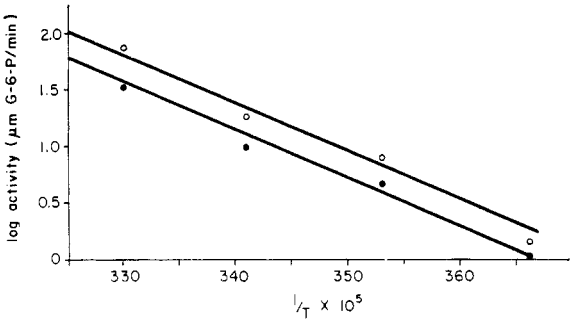

FIGURE 6: Arrhenius plot for the phosphoglucomutase reaction showing the effect of temperature on the rate of catalysis by activated (○) and nonactivated (●) enzyme. E = 19,300. Enzyme (10 μg) was used to determine the rate of the reaction at 0°, 10°, and 20°, and 4 μg for the rate at 30°. The enzyme was maximally activated by preincubation at 0° for 5 minutes in $MgCl_2$ 1×10^{-3} M and imidazole 4×10^{-2} M, *p*H 7.5, at a protein concentration of 100 μg/ml. Specific activities were then determined under standard conditions. G-6-P = glucose-6-P.

tion. However, when Mg^{2+}-EDTA-treated preparations were further incubated with Mg^{2+} and imidazole, the usual activation rates were observed. Furthermore, the addition of Mg^{2+}-EDTA at 5×10^{-4} or 5×10^{-3} M to an activation mixture containing 1×10^{-3} M magnesium chloride and 4×10^{-2} M imidazole resulted in no stimulation above that obtained with magnesium chloride and imidazole alone. EDTA at 5×10^{-2} M inhibited the Mg-imidazole activation of the enzyme. It appears therefore that EDTA cannot replace imidazole in the preincubation mixture. These observations, coupled with the obvious reversibility of the Mg^{2+}-imidazole activation (Figure 4), indicate that the mechanism of the activation achieved by Mg^{2+}-imidazole is other than simple heavy metal binding. There is little doubt that when the enzyme is contaminated with inhibitory heavy metal ions, EDTA would be expected to show an apparent activation in a nonreversible manner.

Activation of the Enzyme by Biologically Functional Compounds. The presence of an activation mechanism for PGM *in vivo*, akin to that of Mg^{2+}-imidazole *in vitro*, is an attractive possibility. Such a process could operate within the framework of a control mechanism. It is recognized however that imidazole is not a likely candidate for such a role under physiological conditions. Consequently, a series of physiologically functional compounds were tested for their capacity to substitute for imidazole in the activation process. Of the compounds investigated, cysteine 2.5×10^{-2} to 5×10^{-2} M, histidine 4×10^{-3} to 8×10^{-1} M, histidylhistidine 4×10^{-2} M, glutathione 1.25×10^{-2} M, and histamine 1×10^{-2} to 4×10^{-2} M showed an activation effect comparable to that shown by imidazole. The histidine effect was comparable to that obtained with imidazole. Acetylation of histidine at the amino group or esterification with a methyl group abolished its activity. 2,4-Diaminobutyric acid 2×10^{-5} to 2×10^{-3} M, carnosine 2×10^{-5} to 2×10^{-3} M, agmatine 2×10^{-5} to 2×10^{-3} M, adenosine 3×10^{-3} M, urocanic acid 4×10^{-2} M, EDTA 1×10^{-1} to 1×10^{-3} M, benzimidazole 4×10^{-2} M, and 3-amino-1,2,4-triazole 4×10^{-2} M were not effective. A further point of interest is that all compounds tested were either effective both in the activation process described here and in augmenting the catalytic rate as shown in the accompanying paper (Robinson *et al.*, 1965) or not effective in either. Furthermore, the relative order of their effectiveness was maintained in both processes. Again, this emphasizes the intimate relationship between both processes and the probability that both effects are concerned with the catalytic site.

Activation Energy of Activated and Nonactivated Enzyme. In exploring further the nature of the activated state a study of the catalytic rate of PGM at various temperatures, both before and after activation, was done. This permitted the calculation of energies of activation for the catalytic process. An Arrhenius plot of the data is given in Figure 6. The absence of any measurable difference between the two states suggests that no significant alteration of the catalytic activity with respect to temperature was brought about by activation. However, such findings would be consistent with the conversion of an inactive to a catalytically active enzyme. The calculated energy of activation E,

about 19.3 kcal, is in the upper ranges of the usual values reported for enzyme reactions (Sizer, 1943).

In contrast to the high E value for catalysis, the E value for activation by Mg^{2+}-imidazole calculated from Figure 1 is about 4.8 kcal.

Discussion

It is clear from Figures 1 and 2 that preincubation of PGM with magnesium and imidazole increases the specific activity up to 6-fold depending on the preparation. It has not been determined why enzyme preparations can be activated to a variable degree. It may relate merely to subtle effects during the preparation or to the physiological state of the animal. The need for the continuous presence of both magnesium and imidazole for the maintenance of the activated state indicates that the process of activation is highly reversible. Activation is immediately lost upon reduction of the Mg^{2+}-imidazole concentration by dilution. This reversibility, coupled with the finding that the substrate (glucose-1-P) is an effective inhibitor of the process of activation, might well qualify the system for the possible regulation of glucose-1-P concentration. In such a system the function of the ligand or complexing agent could be assumed by other biologically active compounds. Cysteine, histamine, histidine, and histidylhistidine probably are unlikely candidates because of their low tissue concentration.

Although the basic phenomenon that is occurring during the process of activation is not yet defined, it would appear to involve an association between a Mg^{2+}-imidazole complex and the enzyme, thereby stabilizing an active configuration. Studies at various hydrogen ion, magnesium, and imidazole concentrations indicate that a complex composed of one magnesium ion and one imidazole^0 is the effective activator. It is also apparent that the enzyme molecule is influenced by high pH values. This may indeed be related to the ionization of some group on the enzyme.

Recent preliminary work in this laboratory (Bocchini *et al.*, 1964) has shown that a state of activation comparable in extent to that obtained with Mg^{2+}-imidazole and also time dependent can be obtained by exposure of the enzyme to either low or high pH. In the lower range, the maximum effect was shown at pH 3.5 and in the high range at pH 10.5. The pH-dependent activation requires neither magnesium nor imidazole. These observations led to the suggestion that this type of activation results from breaking a hydrogen or an ionic bond between two residues possessing functional groups with pK values of about 3.5 and 10.5, respectively. This, in turn, provides the proper functional configuration for enzyme activity. The activation by Mg-imidazole complex would have logical relevance to the pH type of activation if it is assumed that by associating with the enzyme surface the complex stabilizes this functional configuration.

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