The Inhibition of Phosphoglucomutase by Beryllium

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(Received 1 July 1965)

1. The inhibition of phosphoglucomutase by beryllium has been examined. 2. Inhibition by beryllium does not occur unless a complex-forming agent such as cysteine or imidazole is present. It is therefore similar to activation by magnesium. 3. The inhibition is progressive and the rate follows first-order kinetics, which may be defined by a bimolecular rate constant. 4. In the presence of magnesium the rate of inhibition is less. By using a fixed time for inhibition competition between beryllium and magnesium may be demonstrated. After inhibition has taken place, the addition of magnesium does not reverse it.

It has previously been reported that beryllium inhibits phosphoglucomutase (D-glucose 1,6-diphosphate-D-glucose 1-phosphate phosphotransferase, EC 2.7.5.1) and that this inhibition is less in the presence of magnesium (Cochran, Zerivic & DuBois, 1951). The inhibitory activity of beryllium against this enzyme has been confirmed (Thomas & Aldridge, 1966) and the present paper describes some novel features of the kinetics of the inhibitory process. A preliminary account of this work has been published (Aldridge & Thomas, 1964).

MATERIALS AND METHODS

Special chemicals. The following chemicals were obtained from the sources indicated: dipotassium glucosyl-phosphate, cysteine hydrochloride and $\text{BeSO}_4 \cdot 4\text{H}_2\text{O}$ from British Drug Houses Ltd., Poole, Dorset; imidazole from Koch-Light Laboratories Ltd., Colnbrook, Bucks.; crystalline rabbit-muscle phosphoglucomutase from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany.

Determination of phosphoglucomutase. The method used was essentially that of Najjar (1948). The final composition of the medium was: cysteine (25 mM), MgSO_4 (1.5 mM), glucose 1-phosphate (1–1.8 mM), pH 7.4; the total volume was 1.6–2.0 ml. The progress of the reaction was followed at 30° by the determination of the acid-labile phosphorus (3 min. at 100° in $\text{N-H}_2\text{SO}_4$) by the method of Fiske & Subbarow (1925). The reaction was zero-order until 75% of the substrate had been converted. For some experiments, and always for those experiments where the enzyme was incubated in the absence of cysteine (and was therefore largely unbuffered), a final concentration of 4.2 mM- KHCO_3 was added as 0.4 ml. of 21 mM- KHCO_3 saturated with $\text{CO}_2 + \text{N}_2$ (5:95). Such an addition does not influence the inhibition by beryllium in the presence of cysteine. The time allowed for activation of the enzyme by cysteine and magnesium was never less than 1 min. In the experiments described below the enzyme was often preincubated with

MgSO_4 or cysteine alone with or without BeSO_4 . In these cases a further 1 min. with both MgSO_4 and cysteine present was always allowed before the substrate was added. The concentrations of BeSO_4 stated were always those during the preincubation, which was at 30° except where otherwise stated.

Most of the experiments were carried out with a partially purified phosphoglucomutase from rabbit muscle prepared as described by Thomas & Aldridge (1966). The response of this preparation to cysteine and BeSO_4 was identical with that of the crystalline preparation.

RESULTS

Complex-forming agents and inhibition by beryllium. Phosphoglucomutase requires magnesium, a complex-forming agent and glucose 1,6-diphosphate as coenzyme for maximal activity (Najjar, 1948; Robinson & Najjar, 1961; Sutherland, 1949; Cori, Colowick & Cori, 1938; Leloir, Trucco, Cardini, Paladini & Caputto, 1948; Cardini, Paladini, Caputto, Leloir & Trucco, 1949; Milstein, 1961b). Since the enzyme as normally isolated is in the phosphoryl-enzyme form (Jagannathan & Luck, 1949; Najjar & Pullman, 1954), no glucose 1,6-diphosphate, other than that in the glucose 1-phosphate, has been added. For most of the experiments described below cysteine has been used as the complex-forming agent because with our enzyme preparation this has given a higher activity than imidazole. In the absence of magnesium or cysteine or both the enzyme has no activity. Although Robinson & Najjar (1961) demonstrated that the activation of phosphoglucomutase by imidazole and magnesium was a time-dependent process and for full activation several minutes were required, we have been unable to demonstrate such a time-dependent activation with cysteine

and magnesium. Full activity has always been obtained when cysteine and magnesium are present at 30°; even with lower concentrations of cysteine or at 0° maximal activity was obtained with no preincubation. We conclude from these observations that cysteine is a more efficient activator than imidazole and the activation process is rapid. Since the concentration of cysteine in tissues is much less than that of glutathione (Bhattacharya, Robson & Stewart, 1955; Malathi, Seshadri Sastry & Ganguly, 1961; Thomson & Martin, 1959), glutathione has been tested as an activator of phosphoglucumutase (cf. Lehmann, 1939). In the presence of glutathione an activity only 60% of that with cysteine can be obtained; the concentration for half-maximal activation is approx. 0.5 mM for each compound.

In early experiments with phosphoglucumutase and beryllium it was found that little or no inhibition was obtained if cysteine was not present during the incubation with beryllium (Table 1). Preincubation in the absence of cysteine and magnesium leads to only 7% inhibition, whereas in the presence of 25 mM-cysteine 80% inhibition is obtained. Beryllium also inhibits with imidazole in the place of cysteine. If magnesium sulphate (1.5 mM) is present with cysteine during the preincubation a 15-fold increase in the concentration of beryllium sulphate is required to give the same degree of inhibition.

Competition between beryllium and magnesium. The following reactions take place in the activation by magnesium and inhibition by beryllium of phosphoglucumutase:

By using the steady-state kinetic analysis outlined in Dixon & Webb (1964, p. 429), with the assumption that the activation by magnesium and the reaction of the enzyme with the substrate are independent of one another, it can be shown that:

$$v = \frac{V_{\text{Mg}}}{1 + \frac{K_{\text{Mg}}}{[\text{Mg}]}}$$

where v is the velocity of reaction, V_{Mg} is the maximum velocity of reaction at a particular substrate concentration in the presence of magnesium and K_{Mg} , i.e. k_{-1}/k_{+1} , is the dissociation constant of the enzyme-magnesium complex. In the presence

Table 1. *Inhibition of phosphoglucumutase by beryllium*

The medium before the addition of glucose 1-phosphate contained MgSO_4 (1.5 mM), cysteine (25 mM), KHCO_3 saturated with $\text{CO}_2 + \text{N}_2$ (5:95) (see the Materials and Methods section). The pH was 7.4 and the incubation with or without BeSO_4 was at 30° for 10 min. Controls were carried out in the absence of BeSO_4 and enzyme activity was the same for all three conditions of incubation.

Incubation conditions	Activation for 1 min.	Inhibition (%)
Enzyme + BeSO_4 (2.7 μM)	MgSO_4 + cysteine	7
Enzyme + cysteine + BeSO_4 (2.7 μM)	MgSO_4	80
Enzyme + MgSO_4 + cysteine + BeSO_4 (40 μM)	—	80

of another metal, I, competing with magnesium but forming an inactive enzyme complex:

$$v = \frac{V_{\text{Mg}}}{1 + \frac{K_{\text{Mg}}}{[\text{Mg}]}(1 + A[\text{I}])} \quad (1)$$

where the combination of enzyme with metal is reversible A equals k_{+2}/k_{-2} , i.e. $1/K_i$, the reciprocal of the dissociation constant of enzyme-inhibitor complex. As shown below, the reaction of phosphoglucumutase with beryllium is progressive (i.e. k_{-2} tends towards 0) and is described by a velocity constant rather than an equilibrium constant. In these circumstances A equals k_2 , the bimolecular rate constant for the reaction of enzyme with beryllium. As shown in Fig. 1, a graph of $1/v$ against $1/[\text{Mg}]$ indicates that competition between magnesium and beryllium takes place. However, as pointed out by Wilkinson (1961), from a statistical point of view, calculation of the constants for the reactions involved (K_{Mg} , V_{Mg} and k_{+2}) are better derived from the regression lines relating $[\text{Mg}]/v$ and $[\text{Mg}]$. The results of these calculations are given in Table 2, the concentration of magnesium sulphate giving half maximum activity being 75 μM and the bimolecular rate constant for the reaction of enzyme with beryllium is 6.1×10^4 l. mole⁻¹ min.⁻¹. A value for K_{Mg} of approx. 50 μM has been obtained by Milstein (1961a).

Progressive inhibition. When phosphoglucumutase is incubated with beryllium sulphate and cysteine for various times, the inhibition increases with time and shows the characteristics of a first-order reaction (Fig. 1). The bimolecular rate constants derived from these results are 7.2×10^4 , 6.1×10^4 and 8.4×10^4 (mean $7.2 \pm 1.1 \times 10^4$) l. mole⁻¹ min.⁻¹.

In other experiments a similar rate constant was

obtained with a crystalline enzyme preparation. The calculation of such rate constants is only valid if the concentration of one component (beryllium) of the system is effectively constant. Calculation from a molecular activity of $12400 \text{ mole}^{-1} \text{ min.}^{-1}$ of enzyme (Najjar, 1962) indicates an enzyme concentration of $0.013 \mu\text{M}$, at least 200–300 times less than the concentration of beryllium sulphate.

Inhibition of enzymes by metal ions has usually been described by an equilibrium process (Dixon & Webb, 1964, p. 346). In view of the lack of equilibrium with beryllium and phosphoglucumutase, the effect of the other constituents of the medium on the inhibitory process was determined. Milstein (1961b)

has shown that the activation of phosphoglucumutase is a complex process and is partly due to removal of heavy-metal impurities. It was therefore essential to examine the influence of cysteine added at various times. If the enzyme is incubated with beryllium, the progressive inhibition still occurs (Fig. 2); if the enzyme is incubated with beryllium in the absence of cysteine, little or no inhibition occurs. In Figs. 1, 2 and 3 the lines sometimes do not pass through 2.0 ($\log 100\%$). This has been found to be somewhat variable between experiments; we have no explanation for it.

Since it has been shown above that the inhibition by beryllium is competitive with respect to magnesium, the effect of magnesium on the rate of

Fig. 1. Competition between beryllium and magnesium. The conditions are as given in Table 2. Concn. of BeSO_4 : $\circ$, none; $\blacktriangle$, $1.0 \mu\text{M}$; $\triangle$, $3.2 \mu\text{M}$; $\bullet$, $16 \mu\text{M}$. Owing to overlapping of points not all the results used for the calculations in Table 2 are included in this Figure.

Fig. 2. Progressive inhibition of phosphoglucumutase by beryllium. The enzyme was incubated with BeSO_4 in the presence of cysteine (31.2 mM) for 0–10 min. (which is the time plotted); MgSO_4 was added and, after a further 1 min., $2 \mu\text{moles}$ of glucose 1-phosphate. The unchanged substrate was determined at 16 min. Concn. of BeSO_4 : $\bullet$, $3.1 \mu\text{M}$; $\circ$, $8.3 \mu\text{M}$; $\blacktriangle$, $31.3 \mu\text{M}$.

Table 2. Competition between beryllium and magnesium

The enzyme was added to the medium containing BeSO_4 , MgSO_4 and cysteine. The mixture was incubated for 10 min. at 30° before adding glucose 1-phosphate (1.8 mM , total vol. 1.6 ml.). The concentration of MgSO_4 was in the range 0.02 – 2.5 mM . The values were calculated from regression lines relating $[\text{Mg}]/v$ and $[\text{Mg}]$, where v is the velocity of the reaction in μmoles of substrate used in 10 min. (see the text for explanation of constants).

Concn. of BeSO_4 (μM)	No. of observations	V_{Mg} (μmoles of substrate/5 min.)	Concn. of MgSO_4 giving $V_{\text{Mg}}/2$ (μM)	$10^{-4} k_{+2}$ ($1. \text{ mole}^{-1} \text{ min.}^{-1}$)
0.0	15	1.96	75.5	—
0.64	8	2.10	105	4.7
1.0	7	1.88	130	6.0
3.2	8	2.19	219	7.3
16	6	1.62	627	6.3
		Mean		Mean
		1.95		6.1 ± 1.1

inhibition has been examined. Again no inhibition is obtained if cysteine is omitted from the medium and the enzyme incubated with beryllium in the presence of magnesium (Fig. 3). If the enzyme is preincubated with cysteine or (cysteine plus magnesium) before beryllium the progressive inhibition still occurs. The rate of inhibition in the presence of 1.8 mM-magnesium sulphate and 40 μ M-beryllium sulphate is approximately the same as that with 2.7 μ M-beryllium sulphate in the absence of added magnesium (bimolecular rate constant $3.2 \times 10^3 \text{ l.mole}^{-1} \text{ min.}^{-1}$ in the presence of 1.8 mM-magnesium sulphate). Magnesium therefore slows down the rate of progressive inhibition. However, if the enzyme is inhibited by incubation with beryllium sulphate (2.7 μ M) and cysteine and then magnesium sulphate are added, no further inhibition occurs. This agrees with the slight inhibition expected by 2.7 μ M-beryllium sulphate and 1.8 mM-magnesium sulphate (calculated from a rate constant of $3.2 \times 10^3 \text{ l.mole}^{-1} \text{ min.}^{-1}$ this is only 8%). More

important is the observation that the enzyme is not reactivated by the addition of magnesium sulphate.

From a few experiments at different temperatures (10°, 20°, 30°, 37°) the apparent energy of activation of the progressive inhibition by beryllium is 13300 ± 3200 (4) cal./mole. This is similar to the value of 14200 ± 780 (8) cal./mole for the enzymic process.

DISCUSSION

There are therefore two phenomena to be discussed. First, inhibition of phosphoglucomutase after incubation with beryllium requires the presence of a complex-forming agent (cysteine and imidazole); secondly, in the presence of a complex-forming agent the inhibition is progressive, follows first-order kinetics and shows the characteristics of a bimolecular reaction with one component in excess.

Phosphoglucomutase requires for activation both magnesium and a complex-forming agent. It seems likely that the main function of the complex-forming agent (cysteine) is to remove heavy-metal inhibitors from the enzyme (Milstein, 1961*a,b*) so that magnesium can gain access. It is therefore probable that the requirement for cysteine to obtain inhibition by beryllium (Table 1 and Figs. 3 and 4) is the same. The inhibition by beryllium increases with time and it is important to know that this apparent slow inhibition is not due to a slow removal

Fig. 3. Progressive inhibition of phosphoglucomutase by beryllium. The conditions before the addition of substrate were: ●, BeSO_4 (2.7 μM) plus cysteine (0–10 min.), MgSO_4 (10–11 min.); ○, cysteine (0–5 min.), BeSO_4 (2.7 μM , 5–15 min.), MgSO_4 (15–16 min.); ▲, BeSO_4 (2.7 μM , 0–10 min.), MgSO_4 plus cysteine (10–11 min.). During the above incubations the medium contained MgSO_4 (1.8 mM) and cysteine (31.2 mM). Enzyme activity was obtained by the addition of glucose 1-phosphate (2 μmoles) and the residual substrate determined after 5 min. Parallel controls were carried out in the absence of BeSO_4 for all times and conditions of incubation. In no instance did the activity of the enzyme change during the experiment. For the purpose of plotting the results the 1 min. incubations with MgSO_4 or cysteine or both to activate the enzyme have been ignored.

Fig. 4. Progressive inhibition of phosphoglucomutase by beryllium. The conditions before the addition of substrate were: ▲, BeSO_4 (40 μM) plus MgSO_4 (0–10 min.), cysteine (10–11 min.); ●, cysteine (0–5 min.), BeSO_4 (40 μM) plus MgSO_4 (5–15 min.); ○, cysteine plus MgSO_4 (0–5 min.), BeSO_4 (40 μM , 5–15 min.); △, BeSO_4 (2.7 μM) plus cysteine (0–5 min.), MgSO_4 (5–15 min.). Other conditions and controls are as stated in Fig. 3.

of heavy metals from the enzyme. This cannot be so, since the activation by cysteine is rapid (cf. Table 1) and in other experiments has been shown to be complete in 15 sec. at 0°. Further, incubation of the enzyme with cysteine for 5 min. at 30° (Figs. 3 and 4), whether with or without magnesium, followed by the addition of beryllium sulphate, does not lead to an immediate rapid inhibition but to the progressive inhibition obtained without a preliminary incubation with cysteine. Therefore the progressive inhibition is due to a reaction between the active enzyme and beryllium and is not due to a progressive reaction induced by the other reagents present.

It seems probable that the initial attachment of beryllium to the enzyme is in principle the same process as the attachment of magnesium during the activation process; this conclusion is reinforced by the demonstration of competition between magnesium and beryllium. However, such a similarity between activation and inhibition gives no clue of the processes involved at a molecular level. It is unlikely that the processes of activation by magnesium and the progressive inhibition by beryllium are formally the same. The kinetics of the inhibitory process are identical with those of the inhibition of esterases by organophosphorus compounds (Aldridge, 1956). By analogy with the reaction of enzyme with substrate it might be expected that there would be an intermediate 'esterase-organophosphate' complex before the formation of the phosphorylated enzyme. There is no direct evidence for such a complex with erythrocyte cholinesterase (Aldridge & Davison, 1952), but for human serum cholinesterase and one organophosphorus compound an 'inhibitor-enzyme intermediate' has been claimed to have been detected (Main, 1964). The inhibition of phosphoglucosomutase by beryllium is analogous and there is no direct evidence of a two-or-more-stage process. For inhibition of esterases by organophosphorus compounds it is as though collision of inhibitor and enzyme in the right way leads directly to reaction to give a phosphorylated enzyme; it is possible that the inhibition of phosphoglucosomutase by beryllium is similar and that the presence of magnesium merely lowers the probability of a 'fruitful' collision. Although the breakage and formation of the covalent bonds involved in the phosphorylation of esterases is compatible with an apparent energy of activation of 10–11 kcal./mole (Aldridge, 1953), a value of 13–14 kcal./mole for the inhibition of phosphoglucosomutase by beryllium is difficult to reconcile with a simple ionic interaction of enzyme and beryllium. If no other changes occur in the protein, then the beryllium must be attached

by a covalent bond to the protein. There is evidence that beryllium can behave chemically in a somewhat anomalous manner (Pauling, 1948; Van Uitert, Fernelius & Douglas, 1953); it is therefore at present not possible to eliminate a combination of beryllium with enzyme by covalent bonds. However, temperature-dependent changes in the protein initiated by the primary attachment of beryllium (instead of magnesium), leading to an inactive enzyme, must be considered. Theoretically this change could be catalytic, for we have no conclusive evidence that beryllium is attached to the inhibited enzyme.

We thank Mr B. W. Street and Mr D. J. Moore for valuable technical assistance.

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