Inhibition of Phosphoglucomutase by Trace Metals

By C. MILSTEIN*

Department of Biochemistry, University of Cambridge

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The interaction between phosphoglucomutase and metal ions is of great importance in the activation of the enzyme; the essential role of magnesium has already been discussed (Milstein, 1961*a*). In addition to magnesium, a chelating agent is also essential for activity (Najjar, 1948; Sutherland, 1949). The necessity for chelating agents is due to the removal of trace metals present in the assay mixture, which are inhibitory to the enzyme (Milstein, 1960, 1961*b*). Additional evidence for this view has now been obtained in a quantitative study of the inhibition of phosphoglucomutase by Zn^{2+} and Cu^{2+} ions.

Phosphoglucomutase is very sensitive to heavy metals. Copper and Hg^{2+} ions (Jagannathan & Luck, 1949), or Zn^{2+} , Cu^{2+} , Ag^+ and Hg^{2+} ions (Sutherland, 1949), inhibit the rabbit-muscle and yeast enzymes respectively. Recently, however, McCoy & Najjar (1959) reported that Zn^{2+} ions could replace Mg^{2+} ions as activator for the yeast enzyme.

Since phosphoglucomutase has been listed as a 'SH enzyme' (Barron, 1951) it was thought that the inhibition might be a result of blocking by the metal of the SH groups essential for activity. The evidence for the presence of active SH groups in the enzyme was, however, rather poor. Only scattered studies have been reported in the literature. Lehmann (1939) found inhibition of crude muscle preparations by oxidized glutathione. With crude yeast preparations incubation with 2 mM-iodoacetate for 10 min. produced 50% inactivation (Sutherland, 1949). Iodoacetate, however, is one of the most unreliable of SH inhibitors, both for its lack of specificity and for its failure to react at low concentrations (Dixon, 1948; Barron, 1951). Furthermore, the experiments of Lehmann were made on very crude preparations, for which the cofactor requirements were still unknown. It is shown in this paper that there are no grounds for considering phosphoglucomutase as an enzyme depending on its SH groups for activity. The high affinity of the enzyme for heavy metals at its active site is probably concerned with other groups.

* Present address: Instituto Nacional de Microbiología, Av. Vélez Sarsfield 563, Buenos Aires, Argentina.

MATERIALS AND METHODS

Abbreviations used are: tris, 2-amino-2-hydroxymethylpropane-1:3-diol; pCMB, *p*-chloromercuribenzoate; EDTA, ethylenediaminetetra-acetic acid.

Materials and methods were as described in previous papers (Milstein, 1961*a, b*), except for the following:

Thiol groups. These were titrated with pCMB by the method of Boyer as described by Fraenkel-Conrat (1957), modified for use with a 1 ml. microcell. Readings in the ultraviolet were made in a Unicam SP. 500 spectrophotometer.

Inhibition studies. Inhibition studies with metals were made with the complete system, and the inhibition values were referred to the concentration of free metal present. For a quantitative study of the inhibition, the 'metal-buffer' technique (Raaflaub, 1956) was used. By this technique the concentration of free metals is kept at known low values by the use of chelating agents. A histidine 'metal buffer' was used and the concentration of the free metal was altered by changing the concentration of either total metal or histidine. The enzyme activity was then determined with different concentrations of the free metal, and expressed as a percentage of the activity when no metal was added apart from Mg^{2+} ion, which was present in optimum concentration.

With the SH reagents, the buffered enzyme (30–80 μ g./ml.) was incubated with the inhibitor and samples (0.02–0.05 ml.) were taken at different times and assayed for activity. Protection by components of the assay system is thus avoided. The inhibition is expressed as

$$I (\%) = (1 - A/A_0) \times 100,$$

where A is the activity of the inhibited enzyme and A_0 the activity of a control treated in the same way but without inhibitor.

RESULTS

Inhibition by Zn^{2+} and Cu^{2+} ions

Zinc and Cu^{2+} ions are powerful inhibitors of muscle phosphoglucomutase. An experiment with Zn^{2+} ions is shown in Fig. 1. The activities in the presence of different concentrations of Zn^{2+} ions and histidine are plotted against the concentration of free Zn^{2+} ions, calculated by the method of Raaflaub (1956), the dissociation constant of the Zn-histidine complex and the pK of the groups involved being obtained from tables. Inhibition of 50% is obtained at a concentration of free Zn^{2+} ion 1.7×10^{-12} M.

A similar experiment with Cu^{2+} ions is shown in Fig. 2, in which the relative activity is plotted against concentration of free Cu^{2+} ions, determined by the same method. The curve in this case is much steeper at the mid-point, and in fact suggests that more than one complex is involved. Theoretical calculations for complexes of 2 or 3 atoms of copper/molecule of enzyme, each inactive complex being formed independently of the other(s), did not fit the experimental points exactly, although they were much closer than that for only one inactive complex. The best fit is obtained for between 2 and 3 atoms/molecule of enzyme. The concentration of free Cu^{2+} ions giving 50% inhibition is $3.8 \times 10^{-16} \text{ M}$.

Since Mg^{2+} ion is an essential activator of the enzyme reaction, it was present in a concentration of 1.5 mM in the experiments mentioned above. The relationship between Mg^{2+} ion activation and heavy-metal inhibition was investigated by studying reciprocal plots of activity at different Mg^{2+} ion concentrations in the presence and the absence of inhibitory metal. The effect of Zn^{2+} ion follows the pattern of a purely competitive inhibition (Fig. 3). The dissociation constant for the inhibitor can be calculated, since the effect of the inhibitor will be to produce an increase in the apparent Michaelis constant for the activator (K_{app}) by a factor of $1 + i/K_i$ (i being the concentration of inhibitor and

K_i its dissociation constant), as with inhibition due to competition with the substrate (Malmström, 1955). From Fig. 3:

$$\frac{\text{Slope } A}{\text{Slope } B} = 1 + \frac{[\text{Free Zn}^{2+} \text{ ions}]}{K_{\text{Zn}}} \quad (1)$$

Fig. 2. Inhibition of phosphoglucomutase reaction by Cu^{2+} ions. The activity of 6 μg . of 'metal-free' phosphoglucomutase was determined under standard conditions (incubation period 2.5 min.), except that histidine was used in concentrations of 25 mM ($\square$) or 2.5 mM ($\circ$), and CuSO_4 added in concentrations from 3.2 mM to 0.32 mM. Activity is expressed as percentage of the activity in the absence of added Cu^{2+} ions. The value $\log K_{\text{CuHis}}^{\text{Cu}} = -18.3$ (Maley & Mellor, 1950) was used for the calculation of [free Cu^{2+} ions].

Fig. 1. Inhibition of phosphoglucomutase reaction by Zn^{2+} ions. The activity of 7 μg . (open symbols) or 3 μg . (solid symbols) of metal-free phosphoglucomutase was determined under standard conditions (incubation periods of 4 and 2 min. respectively), except that histidine was used in concentrations from 35 mM to 1.2 mM, and ZnSO_4 was present in concentrations of 0.175 mM ($\circ$, $\bullet$) and 0.087 mM ($\square$, $\blacksquare$).

Relative activity is expressed as percentage of the activity in the absence of added Zn^{2+} ions. In the calculation of the amount of free Zn^{2+} ions, $\log K_{\text{ZnHis}}^{\text{Zn}} = 12.0$.

Fig. 3. Effect of Zn^{2+} ions on Mg^{2+} ion activation. Conditions were as in the standard assay. Histidine 25 mM; 4.1 μg . of phosphoglucomutase. A ($\circ$), [Free Zn^{2+} ions] = $3.2 \times 10^{-13} \text{ M}$, ZnSO_4 , $1.3 \times 10^{-4} \text{ M}$; B ($\bullet$), no addition.

The value of the inhibitor constant, K_{zn} , calculated in this way is $3.9 \times 10^{-14} M$. With yeast phosphoglucomutase, Zn^{2+} ion can partially replace Mg^{2+} ion as an activator (McCoy & Najjar, 1959). Since the coenzyme and substrate requirements and the equilibrium constant of the two steps of the reaction are the same for both enzymes, this difference between the yeast and muscle enzymes in the effect of Zn^{2+} ions may perhaps be ascribed to different enzymic configurations. This again points to the importance of the enzyme-metal complex in the activation, as discussed by Milstein (1961a).

Copper ion, on the other hand, is not a purely competitive inhibitor. This is shown in Fig. 4, which again suggests a participation of more than one atom of copper/enzyme molecule at the range of concentration used. As can be seen, both K_{app} (apparent Michaelis constant of the activator) and V are affected by the inhibition. Malmström (1955) derived an equation for the enolase system, assuming that one molecule of inhibitor I (with a dissociation constant K_i) competes with the activating metal (m) and that another molecule of I (with a dissociation constant K'_i) binds the enzyme, giving an independent non-competitive inhibition.

$$v = \frac{VK'_im}{[K_{app}(1+i/K_i)+m](K'_i+i)} \quad (2)$$

Then
$$vc = \frac{V}{1+K_{app}/m[1+(i/K_i)]} \quad (3)$$

where
$$c = 1+i/K' \quad (4)$$

Fig. 4. Effect of Cu^{2+} ions on Mg^{2+} ion activation. Conditions were as in the standard assay. Histidine, 25 mM; 4.1 μg . of phosphoglucomutase. A, [Free Cu^{2+} ions] = $2.5 \times 10^{-17} M$, $CuSO_4$, 1.2 mM; B, no addition; C, as A but the ordinate is $(cv)^{-1}$, with $c = 1.1$. (For explanation see text.)

c may be calculated from the values of V , obtained by plotting $1/v$ against $1/m$ in the presence and the absence of inhibitor [V_B and V_A respectively (see Fig. 4)]. At infinite concentration of m , $V_B = V_A c$ (see equation 3). Now, replacing the ordinate $1/v$ by $1/cv$ the new line should resemble that produced by a competitive inhibition (Fig. 4, line C). The two dissociation constants can thus be calculated: K_i , from the slopes of lines B and C (Fig. 4), with a value of 2.3×10^{-17} , and K'_i , from equation (4), with a value of 2.5×10^{-16} . These values suggest that, at low concentrations of Mg^{2+} ion, Cu^{2+} ion inhibition is mainly due to the formation of a complex with the Cu^{2+} ion at the same site as the active Mg^{2+} ion, but as the concentration of Mg^{2+} ion increases, higher concentrations of Cu^{2+} ion are necessary for inhibition and Cu^{2+} ion then begins to combine non-competitively with other sites. The agreement between the experimental data in Fig. 4 and the assumption of two complexes must be treated with some caution, mainly because of the fact that the inhibition by formation of the second complex is very small and a more complicated effect might be present, although not evident under these conditions.

Thiol groups in phosphoglucomutase

A number of reagents that give an irreversible inhibition with enzymes containing essential SH groups for activity were tested, including an oxidizing reagent (*o*-iodosobenzoate), two alkylating reagents (chloroacetophenone and iodoacetate) and an unsaturated imide (*N*-ethylmaleimide).

Iodosobenzoate in low concentrations usually requires only a few minutes to react with SH groups. To obtain inhibition with phosphoglucomutase, rather long incubation periods and high concentrations of the reagents were necessary. Thus as is shown in Table 1, Expt. A, 0.4 mM-iodosobenzoate (which represents a 1000-fold excess compared with the amount of enzyme) requires 10 min. incubation for a detectable inhibition. It may then be concluded that the results do not support the idea of specific oxidation of SH groups. 'Metal-free' phosphoglucomutase reacts very much like phosphoglucomutase (Table 1, Expt. B).

The effect of *N*-ethylmaleimide on free SH groups is very rapid (Gregory, 1955). Concentrations of 1 μM have an antimitotic effect (Friedman, Marrian & Simon-Reuss, 1949), and unequivocal SH inhibitions at low enzyme concentrations are usually complete with 10 μM -*N*-ethylmaleimide in less than 5 min. This is not the case with phosphoglucomutase. The reaction is very slow indeed, and even high concentrations fail to inactivate the enzyme significantly after short periods of incubation.

Table 1. *Effect of sulphhydryl inhibitors on activity of phosphoglucomutase*

'Metal-free' phosphoglucomutase was used in Expts. B and C. Phosphoglucomutase dialysed overnight against 1 mM-EDTA was used in Expt. E. Other conditions were as indicated in the Materials and Methods section.

Expt.	Addition	Buffer	Time (min.)	Inhibition (%)
A	Iodosobenzoate 0.02 mM	0.1M-Sodium acetate	10	1.0
	0.4 mM		10	11.8
	2.0 mM		10	21.5
B	Iodosobenzoate 1.7 mM	58 mM-Histidine, pH 7.5	25	27.3
C	<i>N</i> -Ethylmaleimide 5.0 mM	58 mM-Histidine, pH 7.5	25	48.3
D	Chloroacetophenone 2.0 mM	40 mM-Sodium acetate	10	7.5
	2.0 mM		70	29.0
	2.0 mM		150	68.0
E	Chloroacetophenone 2.0 mM	40 mM-Sodium acetate	10	-3.0
	2.0 mM		55	-9.0
	2.0 mM		125	9.0
	2.0 mM		220	33.4
F	Iodoacetate 2.0 mM	10 mM-Sodium bicarbonate	25	33.3
	2.0 mM	20 mM-Acetate buffer, pH 5.7	25	0.3
G	Oxidized glutathione 20.0 mM	0.1M-Tris-HCl, pH 7.5	15	25.0

tion. However, a slow reaction takes place, which in 2 hr. produces a complete inactivation. Table 1 (Expt. C) shows an experiment in which about 50 % inhibition was obtained with 5 mM-*N*-ethylmaleimide and 'metal-free' enzyme after 25 min. incubation.

Alkylating agents are in general unreliable for the detection of SH groups (Barron, 1951). Chloroacetophenone, however, seems to be a reasonable one to choose since it has been shown to react slowly with native ovalbumin, and very fast (when slight excess is present) with urea-treated ovalbumin (Dixon, 1948). 2 mM-Chloroacetophenone, representing a 2000-fold excess of the reagent over the enzyme, produces 50 % inhibition after 2 hr. incubation (Table 1, Expt. D). The reaction with an EDTA-dialysed enzyme is even less pronounced.

As mentioned above, iodoacetate is the most unreliable of all the so-called SH reagents. Even in cases in which other SH reagents are potent inhibitors its reaction is often very slow, and it is unspecific since it also reacts with histidine, lysine and methionine (Gundlach, Stein & Moore, 1959). The effect of this reagent on phosphoglucomutase follows a pattern similar to that of other SH inhibitors. High concentrations and very long incubation periods are necessary for inhibition. The results may indicate a reaction with an amino group of lysine since inhibition is not observed at slightly acidic pH values. The fact that no inactivation is observed at pH 5.7 seems to indicate that if a histidine residue is essential for catalysis, it does not react with iodoacetate under these conditions.

Oxidized glutathione has also some inhibitory effect (Table 1, Expt. G).

Fig. 5. Titration of SH groups with pCMB. Phosphoglucomutase ($6.5 \mu\text{M}$) in: Δ , 0.01 M-phosphate buffer, pH 7.0; $\circ$, 12 mM-histidine buffer, pH 7.5; $\square$, 12 mM-histidine buffer, pH 7.5, + $44 \mu\text{M}$ - ZnSO_4 . pCMB was added with a micropipette to both blank and sample. The lines indicate the theoretical titration curve for 1 and 2 SH groups/molecule. Point (B) is a second reading of point (A) after 15 min. incubation.

When phosphoglucomutase is titrated for SH groups with pCMB, about two SH groups/molecule are detected, and these react quite fast with the reagent, producing the characteristic shift in the absorption at $250 m\mu$ at pH 7.0. The titration is the same when histidine buffer is used instead of phosphate. The presence of $44 \mu\text{M}$ - ZnSO_4 together with the histidine does not introduce any significant change (Fig. 5). The addition of 1.7 mM- Zn^{2+} ions to the cuvette containing phosphate buffer (see Fig. 5) after two SH groups have been titrated does not produce any decrease in extinction. The same is true after addition of 0.14 mM- ZnSO_4 to the cuvette containing histidine.

On leaving excess of pCMB in contact with the enzyme in phosphate buffer (with or without 1.7 mM-ZnSO_4) for 2 hr., an increase in ΔE_{250} is produced which corresponds to a titration of three SH groups in all. Higher values have also been obtained on longer incubations.

The material after exposure to pCMB for varying times was assayed for activity by incubating a sample of the solution with the standard assay mixture containing histidine as activator. After titration of two SH groups, 75% of the enzyme activity remains, and after three SH groups have reacted the activity falls to about 50% of its original value. The activity is restored by incubating the enzyme with 0.1 M-cysteine , but incubation for 15 min. is required.

The result shows that a reaction takes place with the enzyme which partially inactivates the enzyme and is not immediately reversed by cysteine, as would be expected if the loss of activity were due to a simple reaction of pCMB with SH.

DISCUSSION

The mechanism of activation of phosphoglucumutase by chelating agents proposed in the preceding paper (Milstein, 1961b) is based on the breaking down of highly stable inactive metal-enzyme complexes. The complexes described here have these properties. The reason for using Zn^{2+} and Cu^{2+} ions in the studies is that they are common contaminants, especially in distilled water. The concentrations of these metals even in glass-distilled water may easily reach the values necessary for complete inactivation of phosphoglucumutase, especially if the still is not carefully designed (Thiers, 1957).

The extremely low value of the dissociation constants for the inhibitory Cu^{2+} and Zn^{2+} ions poses the question whether they have any physiological significance. This will in fact depend on the actual concentration of free metal ions in the tissue. If these concentrations are higher than the concentrations required for inhibition (10^{-18} M for Cu^{2+} ions), the activation by chelating agents may be a mechanism of control of glycolysis by hormones or other natural chelating agents. It is possible, however, that the activation is one of the artifacts of testing of activity *in vitro* arising from the extraction of the enzyme from its natural environment.

The effect of SH inhibitors on phosphoglucumutase gives some indication that destruction of SH groups may produce a partial loss of activity. This is suggested by the fact that inhibitors such as *N*-ethylmaleimide, iodosobenzoate and oxidized glutathione can partially inactivate the enzyme. However, the participation of SH groups in these

reactions is very uncertain. Under the conditions in which inhibition occurs, secondary reactions are likely to take place. With high concentrations of iodosobenzoate breaking of $-\text{S}-\text{S}-$ bonds and reaction with other groups such as hydroxyl or amino groups would be expected (Rabinovitch & Barron, 1955). A good example is the inhibition of ribonuclease by iodosobenzoate, interpreted by Ledoux (1954) as an effect on SH groups; it is now known that ribonuclease does not contain free SH groups. With excess of *o*-iodosobenzoate protein denaturation may be produced, as shown by Hellerman, Chinard & Deitz (1943) for urease. Lack of reaction of *N*-ethylmaleimide with $-\text{S}-\text{S}-$ has been reported by Gregory (1955). However, recently a reaction of *N*-ethylmaleimide with amino groups and with imidazole has been described by Smyth, Nagamatsu & Fruton (1960). The inhibition obtained with pCMB is not instantaneously reversed by high concentrations of cysteine; incubation for 15 min. is required for full recovery. The results suggest that the rate-limiting step is not the reaction between the inhibitor and a SH group but perhaps some type of unfolding of the protein molecule. Unspecific reactions are, however, possible with any of the reagents. The results do not support the idea that a SH group is directly involved in the catalytic action of the enzyme.

The lack of specific inhibition of the enzyme by SH reagents is an indication that its sensitivity towards heavy metals is not due to combination of the metals with SH groups. Furthermore, the fact that Zn^{2+} ion did not affect either the titration of SH groups or the partial inactivation of the enzyme by pCMB is a further indication that the high affinity of the enzyme for Zn^{2+} ion involves groups other than SH. This high affinity can be explained by the assumption that more than one side chain of the protein is involved in the chelation (Barnard & Stein, 1958; Malmström & Rosenberg, 1959). An alternative possibility would be combination with a *N*-terminal histidine residue, which is known to have a very high affinity for metals (Barnard & Stein, 1958), but even this affinity would not be high enough to explain the fact that a 10^4 – 10^5 excess of histidine with respect to enzyme is necessary for full activity (Milstein, 1961b).

Since SH groups may be excluded, a chelating site including amino, imidazolyl, carboxyl or phosphate groups may be involved. A pentapeptide with the sequence threonylalanylphosphoserylhistidyl-aspartic (or asparagine) (Thr. Ala. SerP. His. Asp) has recently been shown to be present in the active centre of phosphoglucumutase by Milstein & Sanger (1961). In a three-dimensional model of the peptide, arranged as in an α -helix, the imidazole ring is free to move so as to occupy a position very close to the phosphate or to the carboxyl

group of aspartic acid, giving two configurations, both of which could be expected to have strong metal-chelating properties.

SUMMARY

1. A quantitative study of the inhibition of phosphoglucumutase provides additional evidence that the necessity for a chelating agent is due to the presence of minute traces of inhibitory metals.

2. Zinc is an inhibitor of phosphoglucumutase, competing with Mg^{2+} ions, with dissociation constant $K_{Zn} 3.9 \times 10^{-14} M$. Copper also competes with Mg^{2+} ions, but the inhibition is not purely competitive. The inhibition by Cu^{2+} ions can be explained by competition with the Mg^{2+} ions ($K_{Cu} 2.3 \times 10^{-17} M$), together with non-competitive combination at a second site ($K'_{Cu} 2.5 \times 10^{-16} M$).

3. Three to four SH groups are present in phosphoglucumutase. Although some inhibition is observed with high concentrations of several SH reagents, phosphoglucumutase does not appear to be an enzyme requiring these groups for activity.

4. The effects are discussed in the light of the amino acid sequence in the active centre previously determined.

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The Effect of Insulin on Amino Acid Metabolism

By T. HERNANDEZ AND R. A. COULSON

Department of Biochemistry, Louisiana State University School of Medicine, New Orleans, La., U.S.A.

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In the course of studies on nitrogen metabolism in the alligator, it was observed that glycine, alanine, glutamine and leucine were the main amino acids utilized for the renal synthesis of ammonia (Coulson & Hernandez, 1959). The injection of insulin caused a dramatic decrease in the plasma concentrations of these compounds and as a consequence the renal synthesis of ammonia was virtually abolished.

The knowledge that plasma amino acids will decrease after the injection of insulin is almost as old as the observation that insulin lowers blood glucose (Luck, Morrison & Wilbur, 1928). For

several reasons, however, there have been few studies of the effect of insulin on individual amino acids of either configuration. Only recently have the pure isomers been available at a reasonable cost, and even now the amount required for experiments on large mammals such as the dog makes such studies exceedingly expensive. When an amino acid is given to a mammal by any route the compound is so quickly absorbed and metabolized or excreted that studies *in vivo* are often impractical. In spite of these limitations Hier (1947) compared the 'tolerances' of several amino acids in the dog. No plasma rise occurred after the oral admini-