Metal Complexes of Phosphoglucomutase *in Vivo*

ALTERATIONS INDUCED BY INSULIN

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SUMMARY

A procedure is presented for measuring the relative amounts of the Mg2+ and Zn2+ forms of phosphoglucomutase in rabbit muscle extracts. The results indicate that the ratio of these forms in *vivo* is the same as that observed in extracts *in vitro*, viz., about 0.5 for mature Purdue Dutch rabbits; other phosphoglucomutase-metal species do not appear to be present in appreciable amounts. The concentration of free Mg2+ in such extracts is saturating with respect to phosphoglucomutase. These observations, together with metal binding data, provide estimates of the relative concentrations of free Mg2+ and free Zn2+ in *vivo*, about 10^{-6} to 10^{-5} M, and of the actual concentration of free Zn2+, about 10^{-11} M.

The fraction of the total endogenous phosphoglucomutase in the Mg2+ form was essentially constant in animals treated alike; however, this fraction varied markedly in animals subjected to different treatments; viz., the fraction depended on the physiological state of the animal. Thus the fraction of the total enzyme in the Mg2+ form could be increased from about 0.35 ± 0.10 to about 0.83 ± 0.08 (S.D.) by treatment with insulin in fasting animals. By contrast, the total enzyme per ml of standard extract varied among animals by less than 8% (S.D.), regardless of treatment. Since the Zn2+ form of phosphoglucomutase is essentially inactive, these results indicate that the average catalytic efficiency of phosphoglucomutase is very much with the physiological state of the animals, while the total catalytic potential remains unchanged.

Phosphoglucomutase, EC 2.7.5.1, exhibits an obligatory requirement for a bivalent cation (1-3); in addition, the catalytic efficiency of the enzyme is a function of the identity of the bound metal (4). For example, at pH 7.4, if the catalytic efficiency of the enzyme-Mg-glucose phosphate complex, EP·M·glucose-P, is 100, the efficiency of EP-Zn-glucose-P is 0.3 and that of metal-free enzyme is < 10^{-4}. Thus variations in the concentrations of free metal ions in muscle might regulate the average catalytic efficiency of the endogenous enzyme. The present investigation was undertaken to determine the identities of metal bound to phosphoglucomutase in rabbit muscle and to examine the effect of physiological state on the ratio of bound metal ions.

Since the purification of phosphoglucomutase involves procedures such as heat treatment, ammonium sulfate fractionation, and column chromatography (6) and since the metal ions bound to phosphoglucomutase may exchange with the medium within a few minutes to a few hours, one cannot expect metal analyses of the purified enzyme to reflect the metal or metals which are present *in vivo*. However, in a mixture of the Mg2+ and Zn2+ complexes of phosphoglucomutase, for example, the fraction of the total catalytic efficiency due to the less efficient Zn2+ form is readily distinguished from that of the more efficient Mg2+ form because of a unique property of ternary EP·M·glucose-P complexes: the relatively efficient metal ions dissociate from the ternary complex much more rapidly than the less efficient ones in the presence of excess EDTA (4). We have used this property to deduce the identity of the metal ions bound to phosphoglucomutase and to assess the ratio of free Mg2+ to free Zn2+ in the muscle of rabbits fed ad libitum, starved, and/or insulin-shocked.

**EXPERIMENTAL PROCEDURE**

Materials—Glucose-1-P, glucose-6-P, and glucose-1, 6-diP were prepared as described previously (7, 8). All other reagents were analytical grade or the purest grade available. The chlorides of reagent grade metals and of Tris (Mann) buffer were used throughout. EDTA was recrystallized from water as the free acid.

The phospho form of phosphoglucomutase, EP, was prepared as described previously (6). “Metal-free” phosphoglucomutase was produced by dialysis against EDTA and Chelex-100 (4). A molecular weight of 62,000 (9) and an E280 of 7.7 (10) were employed in all calculations.

32P-Labeled glucose 1-phosphate was prepared from glycogen and [32P]orthophosphate via the muscle phosphorylase reaction (11). The product was purified via the ion exchange chromatography and isolation procedures of Ray and Roscelli (7).

Rabbits used in this study were of the Purdue Dutch strain and were maintained on Wayne Rabbit Ration (Allied Mills, Chicago, Illinois). Animals fed *ad libitum* weighed 2.3 to 2.6 kg at maturity. Some animals were starved for 14 to 16 days, after which time their weight had decreased to 1.2 to 1.4 kg. Crystalline bovine insulin, B grade (Calbiochem), was dis-
solved in 0.1 N HCl, and the pH of the solution was adjusted to 6.0 with 0.1 N NaOH. Rabbits were injected subcutaneously with levels of 40 to 250 units per kg.

Muscle Extracts—Rabbits were killed with a sharp blow to the back of the head and muscle tissue from the thigh (Biceps femoralis) was quickly excised. Muscle samples of about 1 g were homogenized immediately in cold distilled water (1 ml of water per g of muscle) for 1 min with a motor-driven Teflon tissue grinder (Thomas No. B33502). The operation was conducted at room temperature; however, the homogenate was subsequently centrifuged at 4° for 6 min at 18,000 × g. The clear supernatant was placed on ice in Parafilm cups and used immediately in the enzymatic assays which are described below. The total time between killing and initiation of the assay was about 10 min.

An alternative procedure was sometimes employed in which 1- to 3-g portions of excised muscle were plunged into liquid nitrogen immediately after excision and were stored in liquid nitrogen for later analysis. After thawing at room temperature until the tissue softened, the muscle was extracted as above.

Enzymic Assays—Phosphoglucomutase activity was measured at 30° in the presence of saturating concentrations of glucose-1-P and glucose-1,6-diphosphate in a manner similar to that described by Ray and Roscelli (12). The decrease in acid-labile phosphate which accompanies the conversion of glucose-1-P to glucose-6-P was measured by using the procedure of Bartlett (13); conversion was routinely 20 to 35%, and never exceeded 40%. This limitation precludes any complications due to product inhibition (3).

To ensure that the decrease in acid-labile phosphate (glucose-1-P) was strictly parallel with the appearance of glucose 6-phosphate in assays involving both native and activated muscle extracts (see below), the products of typical assays conducted with 32P-labeled glucose 1-phosphate were subjected to ion exchange chromatography as described below.

The total catalytic potential of muscle was measured by first diluting muscle extracts 200-fold with a solution which contained 100 mM imidazole, 50 mM Tris, 1.5 mM EDTA, and 5 mM MgCl2, at pH 7.4; after 10 min phosphoglucomutase activity was measured by adding 0.2 ml of the mixture to 0.4 ml of a substrate mixture, 1.25 mM in glucose-1-P and 25 μM in glucose-1,6-diphosphate. Triplicate assays were quenched at 15 and 30 sec with 0.5 ml of 0.5% trichloroacetic acid, washed, the supernatant was centrifuged to remove precipitated protein, and 0.5-ml aliquots were analyzed for acid-labile phosphate according to Bartlett (13).

The average catalytic efficiency of muscle was measured by direct addition of 1 μl of muscle extract to 0.6 ml of a solution 16.7 mM m-tris, 0.5 mM in EDTA, 1.66 mM in MgCl2, 0.83 mM in glucose-1-P, and 16.7 μM in glucose-1,6-diphosphate. Triplicate assays were stopped at either 30 sec or 1 min by the addition of 0.5 ml of 80% trichloroacetic acid. Acid-labile phosphate was measured as above.

The phosphoglucomutase in muscle to which was bound a rapidly dissociating metal was assessed by adding 2- to 5-μl aliquots of muscle extract to 0.6 ml of a solution which contained 16.7 mM m-tris, 2 mM EDTA, 0.83 mM glucose-1-P, and 16.7 μM glucose-1,6-diphosphate, at pH 7.5 and 30°. No metal was added. The course of the reaction was followed by conducting separate assays for successively longer time intervals before quenching with 30% trichloroacetic acid. Subsequent steps were the same as given above for the measurement of total catalytic potential.

Product-time plots were constructed, and the linear portion of the plot at time intervals greater than 3 min was extrapolated to t = 0.

The possible presence of metal-free phosphoglucomutase in muscle extracts was assessed by adding 2 μl of extract to 0.1 ml of solution containing 1 mM MgCl2, 16.7 mM Tris, and 0.5 mM EDTA; immediately (<5 sec) after addition of the extract the assay was initiated by adding 0.5 ml of substrate (0.83 mM in glucose-1-P, 16.7 μM in glucose-1,6-diphosphate) containing excess EDTA (2 mM). No further addition of metal was made. The course of the reaction was followed as indicated above.

The concentration of protein in rabbit muscle extracts was measured by using the micro-Folin procedure as described by Lowry et al. (14).

Chromatography of glucose phosphates was performed on Dowex 1 (formate) resin, 8% cross-link, 200 to 400 mesh. Columns were eluted with a linear gradient of formic acid, 0 to 1 N as described by Bartlett (15).

RESULTS

Definitions—Since various metal ions elicit varying levels of activity in the phosphoglucomutase reaction (4), total catalytic potential is the glucose-1-P to glucose-6-P conversion per min per mg of soluble protein in a tissue extract prepared under standard conditions and treated to convert all of the phosphoglucomutase in the extract to its most efficient form, viz., the Mg2+ form. The procedure for converting the various metal forms of phosphoglucomutase to the Mg2+ form (see “Experimental Procedure”) has been adequately verified (4, 16). Moreover, the catalytic potential was essentially the same whether calculated per mg of soluble protein as defined, or per ml of the standard extract. In addition, a variety of minor changes in the extraction procedure such as altering the speed of the pestle or the length of the grinding time did not significantly alter the total catalytic potential.

The average catalytic efficiency is the observed efficiency in the phosphoglucomutase reaction per mg of soluble protein per min when the same extract is assayed under conditions designed to prevent the exchange of metal ions bound to phosphoglucomutase, viz., the enzyme is assayed during a very short time interval in the presence of high concentrations of glucose-1-P and saturating concentrations of Mg2+. Under such conditions no appreciable conversion of the Zn2+, Co2+, or Mn2+ forms of the enzyme to the Mg2+ form would occur; some conversion of the Ni2+ form to the Mg2+ complex could occur (if it were present), but this would have little effect on average catalytic efficiency since the Ni2+ form of the enzyme is 60% as active as the Mg2+ form. However, as demonstrated below, the actual results indicate that only the Mg2+ and Zn2+ forms of phosphoglucomutase need be considered as components of the muscle extract, and the lack of metal ion exchange under the above assay conditions has been adequately verified with artificial mixtures of these two forms of the enzyme (16).

Because excess Mg2+ is included in the above assay, any metal-free phosphoglucomutase (inactive) would be converted to the Mg2+ form (maximally active) in the assay; hence, if a significant fraction of the phosphoglucomutase in muscle extracts were in the metal-free form, the observed average catalytic efficiency in the above assay would be too large. However, the presence of metal-free enzyme in muscle extracts is ruled out by assays in the presence of excess EDTA (see below).

Relating the average catalytic efficiency in extracts to the average catalytic efficiency in vivo depends on the assumption
that the ratio of the various forms of phosphoglucomutase present in the standard extract reflect the composition of phosphoglucomutase in intact muscle. This assumption cannot be rigorously tested by any techniques of which we are aware, however, several observations which serve to make the above assumption quite plausible are discussed in subsequent sections, e.g., the effect of the time between excision and homogenization or the effect of the time between homogenization and assay.

The process of cell rupture and extraction may not precisely preserve the balance of free metal ions originally present in the living cell and we have not assumed that this was the case, although we did operate in such a way as to optimize this possibility, viz. by minimizing the volume of water which was used during homogenization. Even if the original balance of free metal ions were altered during the 1-min homogenization the relative amounts of the various phosphoglucomutase-metal complexes would not be immediately altered because metal ion exchange processes which involve phosphoglucomutase usually require several minutes for completion; hence the occurrence of such an exchange process subsequent to homogenization should be subject to verification in the manner described below.

Glucose 6-Phosphate as Sole Product of Reaction with Crude Extracts—Fig. 1 compares column chromatograms of a labeled substrate mixture (a) and the products produced by incubation for 30 sec with a nonactivated (native) muscle extract (b) and an activated muscle extract (c). Open circles show the 32P label in both the starting material (glucose 1-phosphate) and the product (glucose 6-phosphate); the recovery of label was better than 98%. The closed circles represent the results of a colorimetric phosphate analysis for acid-labile phosphate conducted according to the method of Bartlett (13). These results indicate that essentially all of the decrease in acid-labile phosphate produced by incubation with muscle extracts appears in the glucose 6-phosphate, viz., is the result of phosphoglucomutase action.

Total Catalytic Potential and Average Catalytic Efficiency of Phosphoglucomutase System in Rabbit Muscle Extracts—The results of typical assays of muscle extract for total catalytic potential, i.e., phosphoglucomutase activity in the presence of saturating Mg2+ after a preassay treatment with EDTA and excess Mg2+ (see "Experimental Procedure"), are shown in Fig. 2, solid line. The results for average catalytic efficiency, i.e., phosphoglucomutase activity in the presence of saturating Mg2+, but with no preassay treatment, are also shown (dashed line). Note that there is no upward curvature to the plot for average catalytic efficiency. An upward curvature would be expected if a significant fraction of a less efficient metal ion were replaced by Mg2+ during the assay (16). In fact, the plot does not significantly deviate from linearity for at least 5 min (data not shown).

These results were obtained with an extract that was prepared by conducting the homogenization immediately after excision of the muscle; assays were initiated 10 min later. The effect on both types of assays of varying the time between excision of the muscle and homogenization, and between homogenization and initiation of the assay are described in the following sections.

Time Dependency of Measured Values for Average Catalytic Efficiency and Total Catalytic Potential in Muscle Extracts—Both average catalytic efficiency and total catalytic potential were determined in extracts of rabbit muscle prepared as described under "Experimental Procedure." The extract was made immediately after killing and was maintained at 0° prior to initiation of assays. Fig. 3 shows the variation in both assays as a function of time between homogenization and initiation of the assay. The average catalytic efficiency (○) decreases with time while the
FIG. 3. The average catalytic efficiency and total catalytic potential of muscle extracts as a function of the time elapsed between excision and homogenization of the enzyme. Average catalytic efficiency (O) and total catalytic potential (●) were determined in the manner described under “Experimental Procedure.” Samples of extract used for the former assay were held at 0°C with no further treatment after homogenization until assayed; extracts used for the latter assay were converted to the Mg2+ form (see “Experimental Procedure”) and held at 0°C until assayed. At 30 and 60 min (arrows), samples of untreated enzyme were converted to the Mg2+ form and assayed for total catalytic potential (O●). Assay results are expressed as micromoles of glucose-6-P produced per min per mg of soluble protein in the standard muscle extract.

The average catalytic efficiency remains constant, whether the potential was measured in the untreated extracts after conversion of the Mg2+ form of the enzyme at the times indicated by arrows (●), or in extracts treated to convert the enzyme to the Mg2+ form immediately after homogenization and subsequently maintained at 0°C until assayed (●). The results show that the decrease in average catalytic efficiency is not caused by an irreversible loss of enzyme activity, but by a slow replacement of one metal form of phosphoglucomutase by a less active form. This is probably caused by conversion of the Mg2+ form of the enzyme to the Zn2+ form (see next section). To standardize the assay, subsequent studies were performed by initiating assays 10 min after homogenization.

Time Dependency of Measured Values for Average Catalytic Efficiency and Total Catalytic Potential in Excised Muscle—After excision (see “Experimental Procedure”), muscle was stored on ice prior to homogenization; assays of extracts were initiated 10 min after each homogenization. Hence only a minor decrease in average catalytic efficiency and no decrease in total catalytic potential occurred between homogenization and initiation of the assay, as indicated in the previous section. The results (Fig. 4) indicate that average catalytic efficiency decreases with time while total catalytic potential remains unchanged. In fact, the change in average catalytic efficiency is similar in excised muscle and in homogenates, although it is actually about 1.5-fold faster in homogenates than in intact muscle. This suggests that the homogenization step does not itself appreciably alter the phosphoglucomutase forms in vivo or affect to a large extent the process responsible for converting one metal form of phosphoglucomutase to another.

In order to standardize the assay, in subsequent studies only muscle homogenized or frozen in liquid nitrogen (see below) immediately after excision was used.

Average Catalytic Efficiency and Total Catalytic Potential in Samples of Frozen Rabbit Muscle—Muscle tissue frozen as described under “Experimental Procedure” was later thawed at room temperature and homogenized as soon as softening occurred, and the extracts were assayed as above. No changes in either average catalytic efficiency or total catalytic potential were observed for storage periods of up to 2 weeks in liquid nitrogen. Moreover, both values were identical with those determined from material homogenized without freezing and assayed in the same manner. Hence, when tissue from several rabbits was to be examined, assays were frequently performed with frozen tissue.

Identification of Mg2+ as Rapidly Dissociating Metal Ion Bound to Phosphoglucomutase in Muscle Extracts—Since different metal ions dissociate from the phosphoglucomutase-metal-substrate complex at different rates, it is possible, at least in theory, to identify a metal ion bound to phosphoglucomutase by the rate at which it dissociates in the presence of saturating substrate and excess EDTA; such rates can be determined from the product-time curves produced under these conditions (4, 17). Fig. 5 shows a typical product-time curve produced by addition of muscle extract to an assay solution that contained saturating glucose-1-P and excess EDTA. The curve is obviously biphasic, and the dissociation of metals from at least two different phosphoglucomutase-metal complexes must therefore be involved. If only two are involved (see below), one metal ion must dissociate rapidly and thus be responsible for the initial curvature of the plot; the other must dissociate slowly and to an inappreciable extent during the interval studied in order to account for the linear phase of the curve at times greater than about 3 min. Note that the metal-free enzyme is completely inactive in this assay (4).

Since Mg2+, a rapidly dissociating metal, and Zn2+, a slowly dissociating metal (4), are the most likely metals bound to phos-
phosphoglucomutase in muscle extracts, attempts were first made to verify that Mg\(^{2+}\) is the rapidly dissociating metal ion. As a control, an aliquot of muscle extract was treated with excess Mg\(^{2+}\) and EDTA as described under “Experimental Procedure” to convert all of the phosphoglucomutase present to the Mg\(^{2+}\) form; the enzyme was then added to an assay solution containing excess EDTA and saturating substrate, and product-time plots were constructed. As expected for the Mg\(^{2+}\) form of the enzyme, after about 3 min no further increase in product was observed; also as expected, when the results were plotted as described by Ray and Roscelli (17), \(\log (P_e - P_l)/P_e\) versus \(t\), the plot was linear as shown in Fig. 6 (○). Next, the data in Fig. 5 were analyzed in a similar manner. Thus the linear portion of the plot was extrapolated to \(t = 0\) and the approach of the initial phase of the reaction to the extrapolated line was plotted as above, i.e., the \(y\) intercept was taken as equal to \(P_e\) and the difference between the observed product at time, \(t\), and the product at the same time along the extrapolated line was taken as \(P_l\). The results were again linear as shown in Fig. 6 (●). The first order rate constant for the rapidly dissociating metal ion, 1.8 \(\text{min}^{-1}\) (slope of the Fig. 6 plot when the ordinate values are expressed in terms of natural logarithms), was thus the same when all the phosphoglucomutase in muscle extracts was in the Mg\(^{2+}\) form and when untreated extract was used, and was identical with the value reported earlier by Ray and Roscelli (17), for the purified enzyme-Mg complex. Hence, in all probability the rapidly dissociating species in muscle extracts is Mg\(^{2+}\).

A variation of the above procedure can be used to validate further the assay for average catalytic efficiency, irrespective of the identity of the rapidly dissociating metal ion. Thus the \(y\) intercept in the Fig. 5 plot is the total product produced during the time required for complete dissociation of the rapidly dissociating metal ion and is related by the following equation to the catalytic constant for that metal ion in the phosphoglucomutase reaction, \(k_{\text{cat}}^M\), the amount of that form present, enzyme\(\cdot M\), and the rate constant for dissociation of the metal ion from the enzyme under the conditions of the assay, \(k_d^M\) (18):

\[
y \text{ intercept} = (\text{enzyme} \cdot M)k_{\text{cat}}^M/k_d^M.
\]

The \(y\) intercept and the \(k_d^M\) value have been measured above; hence, \((\text{enzyme} \cdot M)k_{\text{cat}}^M/k_d^M\) can be determined.

However, the assay for average catalytic efficiency provides an alternative procedure for determining \((\text{enzyme} \cdot M)k_{\text{cat}}^M/k_d^M\). Thus, if only two forms of phosphoglucomutase are present in muscle extracts, only the rapidly dissociating form makes a significant contribution to the average catalytic efficiency since (a) the slowly dissociating form could not be converted into the Mg\(^{2+}\) form during the above assay (because the dissociation step is too slow) and (b) the ratio of the final slope (which is a measure of the catalytic efficiency of the slowly dissociating species) to the initial slope (which is actually a rather inaccurate measure of the average catalytic efficiency) is only 0.005. Hence the average catalytic efficiency measured in Fig. 2 should be approximately equal to \((\text{enzyme} \cdot M)k_{\text{cat}}^M/k_d^M\) which should in turn be equal to the \(y\) intercept/\(k_d^M\) ratio from Figs. 5 and 6 as shown above. This is indeed the case; the respective values are 0.050 and 0.046 m mole per min per mg. The agreement of these results strongly suggests that the Mg\(^{2+}\) form of the enzyme is solely responsible for the activity measured in the assay for average catalytic efficiency.

Identification of Zn\(^{2+}\) as Slowly Dissociating Metal Ion Bound to Phosphoglucomutase in Muscle Extracts—Whether or not the Zn\(^{2+}\) form of the enzyme is the slowly dissociating species in muscle extracts is more difficult to demonstrate. Under the assay conditions used the Zn\(^{2+}\) enzyme does indeed give linear product-time plots for up to 40 min, as observed in Fig. 5. However, it is technically difficult to measure the rate constant for dissociation of Zn\(^{2+}\) and compare it with the rate constant for dissociation of the slowly dissociating metal bound in muscle extracts because of slow inactivation of the enzyme during the long time intervals required for such a study (4, 16). However, if only the Zn\(^{2+}\) and Mg\(^{2+}\) forms of phosphoglucomutase are present in muscle ex-
The amount of the Zn\(^{2+}\) form in a given extract calculated in these two ways is 5.3, and 4.8 to 5.7 μg per mg of soluble protein, respectively.

Hence, not only is the general shape of the Fig. 5 plot consistent with the presence of only the Zn\(^{2+}\) and Mg\(^{2+}\) forms of phosphoglucomutase in muscle extracts, but the amounts of the two different forms are quite comparable when calculated in two different ways by means of two different assay methods.

Finally, the solid line in Fig. 5 was calculated by using the following values, some of which have been noted above: \(k_{\text{Zn}} = 1.8 \text{ min}^{-1}\) (this paper); \(k_{\text{Zn}} = 4.8 \times 10^{-4} \text{ min}^{-1}\) (11); \(k_{\text{Zn}} = 144 \text{ min}^{-1}\) (4). An enzyme-Mg/enzyme-Zn ratio of 0.35 was also used with a total enzyme of 1.15 \(\times 10^{-5}\) μmol. The appropriate equation is

\[
P = k_{\text{Zn}}(\text{enzyme-Mg})/(1 - \exp -k_{\text{Zn}}/k_{\text{Mg}}) + k_{\text{Zn}}(\text{enzyme-Zn})t
\]

The fit of the experimental data to the calculated line is sufficiently good to add additional weight to the identification of the important metal ions bound to phosphoglucomutase in muscle extracts as Mg\(^{2+}\) and Zn\(^{2+}\).

Absence of Metal-free Phosphoglucomutase in Muscle Extracts—The absence of a metal-free form of phosphoglucomutase in muscle extracts can be verified by comparing the y intercept values for an assay conducted and plotted as in Fig. 5 and a comparable assay in which the muscle extract was briefly exposed to Mg\(^{2+}\) before initiation of the assay; the difference in y intercept would reflect the amount of metal-free enzyme present. However, in no case was a measurable increase detected in the value of the y intercept due to prior treatment with Mg\(^{2+}\). Hence, no metal-free phosphoglucomutase is present in muscle extracts, and such extracts must therefore contain a saturating concentration of free Mg\(^{2+}\).

Effect of Insulin Treatment on Average Catalytic Efficiency—The total catalytic potential of rabbit muscle extracts was quite constant among different rabbits and was unchanged by fasting, administration of insulin, or a combination of the two. Thus, in 42 rabbits, half of which had been subjected to one or both of the above treatments, the total catalytic potential was 5.4 ± 0.4 (S.D.) μmoles of glucose-6-P per min per mg of soluble protein (see Table I). By contrast, the average catalytic efficiency varied according to treatment but was quite constant for a given treatment. Thus the average catalytic efficiency for 21 rabbits fed ad libitum was about 50% of their total catalytic potential, viz., 1.9 ± 0.5 μmoles per min per mg.

Glycogen depletion induced by starvation produced no significant change in the average catalytic efficiency of phosphoglucomutase (Table I), even though total body weight decreased by about 50% during starvation. Insulin shock produced a small but significant increase in the average catalytic efficiency of rabbits fed ad libitum: from 1.9 ± 0.5 to 3.0 ± 0.4 μmoles per min per mg (p < 0.01). By contrast, insulin shock produced a dramatic increase in average catalytic efficiency of phosphoglucomutase in starved rabbits: from 1.9 ± 0.3 to 4.3 ± 0.4 μmoles per min per mg (p = 0.0001). These results indicate that hormones may be involved in the regulation of the concentrations of free metal ions in muscle.

**DISCUSSION AND CONCLUSIONS**

The present study indicates that phosphoglucomutase in rabbit muscle extracts exists as a mixture of Mg\(^{2+}\) and Zn\(^{2+}\) complexes. Moreover, the controls described under "Results" suggest that the ratio of Mg\(^{2+}\) and Zn\(^{2+}\) forms in the standard extract accurately reflects the corresponding ratio in vivo, although possible changes induced by the homogenization step cannot be directly evaluated. However, substantial changes in the identity of bound metal ions during homogenization appear to be unlikely unless an appreciable fraction of the enzyme exists as the metal-free form in vivo. In such a case, if a large change in the concentration of Mg\(^{2+}\) or Zn\(^{2+}\) (or both) was produced by homogenization, the ratio of enzyme forms would certainly be altered. Although no metal-free enzyme could be detected in extracts 10 min after homogenization, the rate of binding of metals to the enzyme is quite rapid so that this observation is inconclusive. However, since the dissociation constant for the phosphoglucomutase-Mg complex is about 2.5 \(\times 10^{-5}\) in vitro at pH 7.4 (18) and since most estimates of intracellular concentrations of free Mg\(^{2+}\) are appreciably higher than this value (see below), it appears unlikely that substantial amounts of metal-free enzyme are present in vivo.

The certainty with which the present results preclude the existence of other phosphoglucomutase-metal complexes is difficult to assess. If the concentration of free metals such as Mg\(^{2+}\), Mn\(^{2+}\), Zn\(^{2+}\), and Ca\(^{2+}\) were known in advance, the relative concentrations of the various metal forms could be approximated from the known values of the enzyme-metal dissociation constants (4, 16, 18). Unfortunately, the concentration of a free metal ion is difficult to assess in a complex mixture such as the milieu of a cell. By contrast, substantial data exist on the total intracellular metal concentration (19). If total concentrations reflected free concentrations of free metal ions.

3 If the value of 0.13 mm for free Mg\(^{2+}\) is accepted (see below), about 7% of phosphoglucomutase initially would be in the metal-free form.

4 J. S. Multani, W. J. Ray, Jr., and E. J. Peck, Jr., manuscript in preparation.
The presence of bound substrate is known to exert only a minor effect on the dissociation constants can be neglected. Fortunately, the relative concentrations of free Mg$^{2+}$ to free Zn$^{2+}$. Thus the ratio, the level of free Zn$^{2+}$ can be estimated as about $10^{-10}$.

The distribution of Mg$^{2+}$ has been re-examined by Rose England et al. (23), using the aconitase system of rat heart. Both of these studies indicate that free Mg$^{2+}$ must be much lower than supposed by Nanninga, perhaps as low as 0.13 mM (22).

Mg$^{2+}$ and principal known ligands, and the dissociation constants of the various metal forms of phosphoglucomutase in muscle extracts, both in a qualitative and in a quantitative manner.

To the extent that the present results reflect the true composition of endogenous phosphoglucomutase, the result reflects the relative concentrations of free Mg$^{2+}$ to free Zn$^{2+}$. Thus $[\text{Mg}^{2+}]/[\text{Zn}^{2+}] = [E_{Mg}K_{E_{Mg}}(pZn)K_{E_{Zn}}(pMg)]$ in the absence of metal-free enzyme, if the effect of substrate on the dissociation constants can be neglected. Fortunately, the presence of bound substrate is known to exert only a minor effect on the $K_d$ values for both Mg$^{2+}$ and Zn$^{2+}$ under in vivo conditions (pH 7.4 and 30°C) (6, 10), for this effect should be similar to the situation in vivo. In the present case this means that $[\text{Mg}^{2+}]/[\text{Zn}^{2+}]$ is about $10^{10}$.

Nanninga (20) has calculated that the free Mg$^{2+}$ ion concentration of frog muscle is in excess of 1 mM by using the total content of Mg$^{2+}$ and principal known ligands, and the dissociation constant of the various Mg$^{2+}$ ligand complexes. However, with the use of the distribution equations of Fenn and Haege (21), the concentration of free Mg$^{2+}$ in rabbit muscle should be about 0.4 mM. The distribution of Mg$^{2+}$ has been re-examined by Rose (22), using the adenylate kinase system of intact red cells, and by England et al. (23), using the aconitase system of rat heart. Both of these studies indicate that free Mg$^{2+}$ must be much lower than supposed by Nanninga, perhaps as low as 0.13 mM (22).

By using this value for free Mg$^{2+}$ and the above [Mg$^{2+}$]/[Zn$^{2+}$] ratio, the level of free Zn$^{2+}$ can be estimated as about $10^{-10}$ M. Thus, while about 1% of the total magnesium is in the form of the free ion, less than 0.0001% of the total Zn$^{2+}$ in muscle is in the free state.

Since so little of the total zinc in rabbit muscle is present as free Zn$^{2+}$, most of the total zinc must be bound by complex-forming agents. There are, of course, many groups such as phosphates which might bind Zn$^{2+}$; however, most such groups would not bind a significant amount of Zn$^{2+}$ at a free Zn$^{2+}$ concentration of $10^{-5}$ M. In fact, the present results preclude the possibility that an appreciable fraction of any simple phosphate in the cellular milieu is present as the Zn$^{2+}$ complex unless the intracellular milieu is not at equilibrium relative to free and bound Zn$^{2+}$.

On the other hand, proteins may bind Zn$^{2+}$ quite tenaciously, e.g., carbonic anhydrase, $K_d \approx 10^{-12}$ M (24), and phosphoglucomutase, $K_d \approx 10^{-11}$ M (16). Undoubtedly the binding of metals at specifically structured sites is responsible for such small dissociation constants. However, a concentration of 200 mg of protein per ml with a specific site for Zn$^{2+}$ would in fact be required to bind all the Zn$^{2+}$ in muscle cells if binding were limited to a single site per 30,000 molecular weight. Since this seems unreasonable, the Zn$^{2+}$ binding unit must have a molecular weight substantially less than 30,000, and may not be a protein at all. It might also be pointed out that at least the commonly known enzymes with catalytic activity usually associated with Zn$^{2+}$ binding do not occur to any significant extent in muscle tissue e.g., carbonic anhydrase, alkaline phosphatase, carboxypeptidase, and alcohol dehydrogenase (25).

The hormonal alteration of catalytic efficiency in vivo that is demonstrated by the present results extends and clarifies the observation of Joshi et al. (26) and Hashimoto et al. (27), that the activity of endogenous phosphoglucomutase is increased by administration of insulin. However, conditions necessary for a realistic appraisal of the ratio, average catalytic efficiency to total catalytic potential, have not been met in the past, and this precludes any lengthy comparison of published data on the effect of hormones on the activated state of phosphoglucomutase. We can only state that all alterations in efficiency of muscle phosphoglucomutase action induced by insulin treatment that we observed can be rationalized in terms of a change in the fraction of endogenous phosphoglucomutase in the enzyme-Mg complex; an increased efficiency is produced by an increase in the fraction of enzyme in the Mg$^{2+}$ complex which is produced by administration of insulin.

Either of two possibilities or a combination of both could explain the increase in the fraction of phosphoglucomutase in the Mg$^{2+}$ form induced by insulin: an increase in the level of free Mg$^{2+}$ or a decrease in the level of free Zn$^{2+}$. The former seems more likely in view of the work of Aikawa on the effect of insulin and alloxan diabetes on Mg$^{2+}$ flux in rabbit muscle (28, 29). Aikawa found that insulin treatment increases the exchangeability of Mg$^{2+}$ within the muscle (28) whereas Mg$^{2+}$ exchangeability decreases with alloxan diabetes (29). Perhaps the increase in exchangeability on insulin treatment is a reflection of the release of Mg$^{2+}$ from an intracellular depository. If the binding of Mg$^{2+}$ in such a depository is reduced by insulin, the total pool of free Mg$^{2+}$ for competition with free Zn$^{2+}$ would increase, and thus increase the enzyme-Mg/enzyme-Zn ratio as was observed. This increase in phosphoglucomutase activity could be important in the increased deposition of glucose as glycogen which is observed on insulin treatment.

It should not be inferred from the above suggestion that the authors feel that the primary site of insulin action involves the activity of phosphoglucomutase. Instead, it seems much more probable that the same changes in the [Mg$^{2+}$]/[Zn$^{2+}$] ratio which alter the efficiency of phosphoglucomutase action might also alter the efficiency of other metal-requiring enzymes.

The present study presents a method for studying alterations in the ionic milieu of the cell by using endogenous phosphoglucomutase to detect alterations in the balance of free divalent cations within tissues.

The present results also provide some suggestions as to a primary site for insulin action. Stadie et al. (30) first proposed that insulin binds specifically to the plasma membrane of muscle cells before exerting an effect on glucose metabolism. Recently Rodbell (31) has suggested the same site of action and has described a sequence of events which leads to structural alterations of the plasma membrane, which in turn alter the affinity or permeability of the membrane for ions, glucose, etc. If the plasma membrane is a depository for a large bulk of the intracellular Mg$^{2+}$, and certainly a high proportion of Mg$^{2+}$ may be bound to phospholipids (32), the action of insulin in altering the structure of the...
membrane might decrease the binding affinity of such sites for Mg$^{2+}$ and thus increase the free pool of Mg$^{2+}$ as described above.

The metabolic consequences of insulin action include increases in glycolysis (33), glycolysis (34), and protein synthesis (35). These processes involve a large number of steps in which ATP is utilized or phosphate moieties are transferred (or both). Magnesium is known to be an activator of most systems which utilize ATP or transfer phosphate (36). It seems possible that Mg$^{2+}$ is a positive effector of protein synthesis (36). The metabolism of hormone effects may involve alterations in levels of free Mg$^{2+}$ which act to modulate the efficiency of some intracellular enzymes. An additional criterion necessary for operation of such a mechanism is the existence of critical enzymes in states of submaximal efficiency in the native state of the cell, as has been shown for phosphoglucomutase in the present study.

In a large number of investigations of hormonal alterations of metabolic pathways (patterns) the enzymes under scrutiny have been examined under conditions which measure maximal activity or total catalytic potential. Such conditions are not native to the state of the cell and as such the activities measured are artifacts of systems in vitro. In view of the present results, where possible investigations should be conducted to determine the native state of the enzyme so that the average catalytic efficiency of the system, as well as the maximal activity or total catalytic potential of the cell, can be evaluated.

REFERENCES

1. Milstein, C., Biochem. J., 79, 574 (1961).
2. Robinson, J. P., Harshman, S., and Najjar, V. A., Biochemistry, 4, 401 (1965).
3. Ray, W. J., Jr., Roscelli, G. A., and Kirkpatrick, D. S., J. Biol. Chem., 241, 2933 (1966).
4. Ray, W. J., Jr., J. Biol. Chem., 244, 3740 (1969).
5. Peck, E. J., Jr., and Ray, W. J., Jr., J. Biol. Chem., 244, 3754 (1969).
6. Ray, W. J., Jr., and Koshland, D. E., Jr., J. Biol. Chem., 237, 9463 (1962).
7. Ray, W. J., Jr., and Roscelli, G. A., J. Biol. Chem., 239, 1228 (1964).
8. Ray, W. J., Jr., and Roscelli, G. A., J. Biol. Chem., 239, 3053 (1964).
9. Filmer, D. L., and Koshland, D. E., Jr., Biochim. Biophys. Acta, 77, 334 (1963).

10. Najjar, V. A., in P. D. Boyer, H. Lardy, and K. Myrbäck (Editors), The enzymes, Vol. 6, Ed. 2, Academic Press, New York, 1962, p. 161.
11. Cori, G. T., Illingsworth, B., and Keller, P. J., in S. P. Colowick and N. O. Kaplan (Editors), Methods in enzymology, Vol. 1, Academic Press, New York, 1955, p. 200.
12. Ray, W. J., Jr., and Roscelli, G. A., J. Biol. Chem., 241, 2596 (1966).
13. Bartlett, G. R., J. Biol. Chem., 234, 466 (1959).
14. Loewy, O. H., Roseborough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem., 193, 265 (1951).
15. Bartlett, G. R., J. Biol. Chem., 234, 449 (1959).
16. Ray, W. J., Jr., J. Biol. Chem., 242, 2737 (1967).
17. Ray, W. J., Jr., and Roscelli, G. A., J. Biol. Chem., 241, 3499 (1966).
18. Ray, W. J., Jr., and Roscelli, G. A., J. Biol. Chem., 241, 1012 (1966).
19. Long, C. (Editor), Biochemist handbook, D. Van Nostrand Company, Princeton, N. J., 1961, p. 70.
20. Nanninga, L. B., Biochim. Biophys. Acta, 54, 338 (1961).
21. Penn, W. O., and Haerge, L., J. Cell. Comp. Physiol., 19, 37 (1942).
22. Rose, L. A., Proc. Nat. Acad. Sci. U. S. A., 61, 1079 (1968).
23. England, P. J., Denton, R. M., and Randle, P. J., Biochem. J., 105, 32C (1967).
24. Lindberg, S., and Malmoström, B. G., J. Biol. Chem., 287, 1190 (1962).
25. Dixon, M., and Webb, E. C., Enzymes, Academic Press, New York, 1955, p. 638.
26. Joshi, J. G., Hooper, L., Kuwaki, T., Sakurada, T., Swan- son, J. R., and Handler, P., Proc. Nat. Acad. Sci. U. S. A., 67, 1452 (1967).
27. Hashimoto, T., Sasaki, H., and Yoshikawa, H., Biochem. Biophys. Res. Commun., 27, 368 (1967).
28. Aikawa, J. K., Proc. Soc. Exp. Biol. Med., 103, 363 (1960).
29. Aikawa, J. K., Amer. J. Physiol., 199, 1084 (1960).
30. Stadie, W. C., Hausgard, N., Marsh, J. B., and Hills, A. G., Amer. J. Med. Sci., 285, 265 (1949).
31. Rodbell, M., in M. Margoulies (Editor), Protein and polypeptide hormones, Proceedings of an International Symposium, 1968, Excerpta Medica Foundation, Amsterdam, 1969, p. 277.
32. Pocchi, J. A., Rueda, M., and Slaga-Stanley, C. H., in D. Richter (Editor), Metabolism of the nervous system, Pergamon Press, London, 1957, p. 174.
33. Villar-Palasi, C., and Laranje, J., Ann. Rev. Biochem., 39, 651 (1970).
34. Özand, P., and Narahara, H. T., J. Biol. Chem., 233, 3146 (1964).
35. Wool, I. G., Stetwell, W. S., Kurihara, K., Low, R. B., Bailey, P., and Oyter, D., Recent Progr. Hormone Res., 24, 139 (1968).
36. Wacker, W. E. C., Ann. N. Y. Acad. Sci., 152, 717 (1969).
37. Hooper, V. H., and Pette, D., Hoppe-Seyler's Z. Physiol. Chem., 346, 1376 (1968).
38. Manchester, E. K., Fed. Eur. Biochem. Soc. Lett., 5, 279 (1969).
39. Chihala, C. A., and Torbey, H. N., Biochem. Biophys. Acta, 198, 504 (1970).
40. Drummond, G. I., and Duncan, L., J. Biol. Chem., 245, 976 (1970).
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