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THE BINDING OF LITHIUM AND OF ANIONIC METABOLITES TO PHOSPHOGLUCOMUTASE

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Summary

Intercept inhibition of rabbit-muscle phosphoglucomutase (α-D-glucose-1,6-bisphosphate:α-D-glucose-1-phosphate phosphotransferase, EC 2.7.5.1) produced by several nucleotide diphosphates and compounds related to coenzyme A was re-examined in order to re-evaluate an earlier suggestion that this enzyme has an allosteric regulatory site. However, in all cases intercept inhibition constants were much larger than those previously reported, and in all but two cases were too large to assess in the assay system, i.e., were greater than 10 mM. Most of the intercept inhibition previously observed apparently was caused by the use of the Li^+ salts of inhibitors. Thus, Li^+ binds competitively with the natural activator, Mg^{2+}, and in the presence of glucose phosphates binds almost as well as Mg^{2+}: $K_d \approx 10$ μM. The observation that glucose phosphates bind to the Li^+ complex of phosphoglucomutase some 900 times more tenaciously than to the corresponding Mg^{2+} complex could provide a partial rationale for the lack of reactivity of the Li^+ form of the enzyme. Attempts to verify the dimeric structure of phosphoglucomutase that was previously reported also produced negative results.

Introduction

Although phosphoglucomutase (α-D-glucose-1,6-bisphosphate:α-D-glucose-1-phosphate phosphotransferase, EC 2.7.5.1) is not a branch-point enzyme and thus is not an attractive candidate for regulatory control, it has been suggested [1] that the results of inhibition studies with nucleotide di- and triphosphates and with CoA and several of its derivatives [1], and of structural studies [2] are consistent with the existence of at least a vestigial control mechanism in the rabbit-muscle enzyme. The present study represents an attempt to follow up this possibility. However, the results call into question the basis upon which the suggestion was made in the first place.

Materials and Methods

The phospho- form of phosphoglucomutase was prepared [3], activated [4], assayed [3,5*,6] and stored [7] by means of previously described procedures, or modifications thereof; its activity was 1050—1150 I.U./mg at 30°C and pH 7.5. Nucleotide diphosphates, CoA, and acetyl CoA were purchased from P.L. Biochemicals; Li^+ salts were converted to the corresponding Na^+ salt by using a short column of Chelex-100 resin, Na^+ form, or Dowex-50, H^+ form, followed by a pH adjustment with NaOH. Ultraviolet difference measurements were made according to previously described procedures [8], at enzyme concentrations between 1 and 1.5 mg per ml. High purity $LiNO_3$ was obtained from Ventron.

Results

Inhibition by metabolic intermediates. Enzymic activity and inhibition of this activity was measured by means of standard procedures involving either coupling with (excess) glucose-6-*P* dehydrogenase [3,5,9] or by direct measurement of the disappearance of acid-labile phosphate [6,9]. All nucleotide diphosphates and CoA derivatives that were examined (as their Na^+ salts) produced slope inhibition effects [10] with either Glc-1-*P* or Glc-P_2 as the varied substrate at a saturating concentration of the other: $K_{is(Glc\text{-}1\text{-}P)}$ and $K_{is(Glc\text{-}P_2)}$, respectively. The apparent values of these constants, which are shown in Table I, differ from the true values (more so in the case of $K_{is(Glc\text{-}P_2)}$ than for $K_{is(Glc\text{-}1\text{-}P)}$) since in order to insure saturation of phosphoglucomutase with the non-varied assay components, and to achieve a steady-state rate with the coupling enzyme, the non-varied assay components were used at concentrations that produced artificially high values of the apparent Michaelis constant of the varied substrate, although no effect on V was produced. (For example, the $NADP^+$ required for the glucose-6-*P* dehydrogenase reaction is a potent inhibitor of phosphoglucomutase competitive with glucose-1,6-P_2: $K_I = 50$ μM.) The apparent values of these constants are shown only for subsequent comparison with intercept inhibition constants [10].

Attempts to estimate values for intercept inhibition constants (K_{ii}) from the reciprocal plots used to estimate apparent K_{is} values were unsuccessful since computer fits of the data did not indicate a significant different between values of V in the presence and absence of the inhibitor (plots not shown), although assay limitations precluded the use of inhibitors at concentrations exceeding 1 mM (either in the coupled or direct assay). Since it is difficult to assess an inhibitor-induced increase in the intercept of a double reciprocal plot when $K_{is} << K_{ii}$ unless the inhibitor concentration approaches K_{ii}, all of the inhibitors were re-tested by carefully comparing the activity of the enzyme at as high a concentration of all assay components as was practical, viz. at the 'assay concentration limit', in the presence and absence of 1 mM inhibitor. (The value of K^{app} for glucose 1,6-bisphosphate, for example, was equal to about $150 \times K_m$ in these assays in the absence of the tested inhibitor (see the conditions noted

* The imidazole used in the assays described in this reference was omitted.

TABLE I

INHIBITION OF PHOSPHOGLUCOMUTASE BY PHOSPHATE-CONTAINING METABOLITES

Assays were conducted at pH 7.4 and 30°C in the presence of 20 mM Tris chloride, and at 2 mM Mg^{2+} and 1 mM EDTA. The coupled assay with glucose-6-*P* dehydrogenase [5] was used except where indicated; the NADP concentration was 0.2 mM, and the Na^+ salts of all inhibitors were employed. The concentrations of inhibitors used in evaluating slope and intercept inhibition constants, respectively, are given in mM in parenthesis after each inhibitor. The apparent inhibition constant competitive with glucose-1,6-P_2, $K^{app}_{is(Glc\text{-}P_2)}$ was estimated from rate measurements at 1 mM glucose-1-*P*, in the presence and absence of the inhibitor; the apparent inhibition constant competitive with glucose-1-*P*, $K^{app}_{is(Glc\text{-}1\text{-}P)}$ was similarly estimated at 50 μM glucose-1,6-P_2. Values of the intercept inhibition constant, K_{ii}, were estimated at 10 mM glucose-1-*P* and 0.1 mM glucose-1,6-P_2^2 plus added Mg^{2+} to give a total concentration of 4 mM; the entire observed inhibition was taken as due to an intercept effect in the estimation of minimal values for K_{ii}.

Inhibitor	$K^{app}_{is(Glc\text{-}P_2)}$ (mM)	$K^{app}_{is(Glc\text{-}1\text{-}P)}$ (mM)	K_{ii} (mM)
ADP (1, 1)	1.0	≈2.0	⩾10
IDP (1, 1)	0.9	≈3.0	⩾10
GDP (0.8, 0.8)	0.2	⩾5.0	⩾10
CDP (0.85, 0.85)	0.5	≈2.0	⩾10
d-TDP (1, 1)	0.4	≈2.0	⩾10
UDP (0.8, 0.8)	0.6	≈2.0	⩾10
CoA (0.075, 0.5)	0.07	⩾0.3	2 *
3′,5′-ADP (0.05, 1)	0.02	⩾0.2	2
3′,5′-*d*-TDP (0.05, 1)	0.02	⩾0.3	⩾10
Acetyl CoA (—, 1)	—	—	⩾10

* Obtained instead by rate measurements based on the decrease with time of acid-labile phosphate during conversion of glucose-1-*P* to glucose-6-*P*.

in Table I plus reported competitive inhibition constants for assay components [11].) With 8 of the 10 inhibitors tested at this assay concentration limit, activities were 95% or more of the uninhibited velocity (1 mM inhibitor) and the activity difference could have been caused primarily by a slope-inhibition effect, viz. by an effect that, in theory, could be eliminated by a further increase in the concentrations of one or more of the assay components. No attempt was made to assess this possibility since if the entire effect were a true intercept effect, K_{ii} in all eight cases would be greater than 10 mM, and thus of marginal interest. The substantial inhibition by 3′,5′-adenosine bisphosphate at the assay limit was confirmed by the direct assay procedure [6] (no dehydrogenase or NADP present) and the direct procedure was used exclusively to assess inhibition by coenzyme A (since the effect of coenzyme A on the dehydrogenase could not be overcome without using unacceptable high levels of NADP). In neither case could an appreciable fraction of the observed inhibition be accounted for in terms of the respective K^{app} values (Table I) and the concentrations of assay components, although the possibility that the inhibition observed under these conditions might be produced by a contaminant of the inhibitor was not investigated.

Inhibition experiments conducted under the conditions described by previous investogators [1], either with the Li^+ salts of nucleotide inhibitors, or with

the sodium salts plus an equal number of equivalents of Li^+, produced a much larger apparent non-competitive inhibition similar to that reported for those inhibitors that are obtainable commercially as their Li^+ salts (data not shown).

Inhibition by monovalent lithium. The Li^+ complex of phosphoglucomutase, prepared by treating the metal-free enzyme with 100 mM Li^+ (a concentration about 10 times as large its dissociation constant, see below), is inactive in the direct enzymic assay [6]. In fact, when the assay contains 10 mM Li^+, the activity observed via the coupled assay is less than $2 \cdot 10^{-8}$ of that for the Mg^{2+} enzyme. However, the inhibition by Li^+ is reversible, since dilution of the Li^+-enzyme mixture into 1 mM Mg^{2+} restores full activity in the normal enzymic assay. In addition, at an assay concentration of 10^{-4} M Mg^{2+}, 10^{-4} M Li produces nearly 50% inhibition, but less than 2% inhibition at 10 mM Mg^{2+}.

Inhibition by Li^+ does not appear instantaneously. Thus, when the Mg^{2+} form of phosphoglucomutase is added to an assay mixture (30°C) that contains 10 mM Li^+, the fall-off in activity obtained from product-time plots (cf. ref. 12) occurs in a first-order manner with a time-constant of about 0.030 s^{-1}. This rate constant correlates well with that for dissociation of Mg^{2+} in the presence of excess EDTA [13], and the lag phenomenon is quantitatively analogous to the onset of inhibition by the competitive inhibitor, Zn^{2+} [12], under similar conditions. Thus, dissociation of Mg^{2+} from the enzyme probably occurs before Li^+ binds and almost certainly occurs before inhibition by Li^+ is elicited.

The binding of Li^+ to the inhibition site was assessed by establishing a competition between Mg^{2+} and Li^+ for the enzyme and measuring the fraction of the Mg^{2+} complex present by adding an aliquot of the equilibrated solution to an assay mixture that contained excess EDTA, to prevent the binding of substrate in the assay from altering the ratio of bound Mg^{2+} to bound Li^+ that was present in the equilibrium mixture (see refs. 6 and 9 for a description of this assay and its use). A linear plot (0, Fig, 1a) was obtained for the reciprocal fraction of phosphoglucomutase (PGM) present in such mixtures as the Mg^{2+}-form and the concentration of added Li^+, according to the expected relationship for competitive binding

$$\frac{[E_T]}{[C_P \cdot Mg]} = 1 + \frac{[K_{Mg}]}{[Mg^{2+}]} + \frac{K_{Mg}[Li^+]}{[Mg^{2+}]K_{Li}}$$

where K refers to a dissociation constant. (The Mg^{2+} complex of EDTA was used in the equilibration step of these studies, see figure legend, to reduce the variability observed for replicate equilibration mixtures. Presumably this variability arose from traces of the tightly bound inhibitor, Zn^{2+} [9,13]. Thus, the equilibrium, Zn · PGM + Mg · EDTA ⇌ Mg · PGM + Zn · EDTA lies to the right under the conditions employed [13]. No significant change in $[Mg^{2+}]$ was produced by an ionic strength-induced change in the dissociation constant for Mg · EDTA during the titration, as calculated from published constants [14].) A dissociation constant for the $E_P \cdot Li$ complex, K_{Li}, of 9 ± 1 mM was obtained (see figure legend). This value is about 900-fold larger than the corresponding constant for Mg^{2+}, 10 μM. (A value of 29 μM for K_{Mg} was previously reported by this laboratory [15]. Although the previous procedure was repeated several times, each time the same results were obtained: K_d = 10 μM. The only expla-

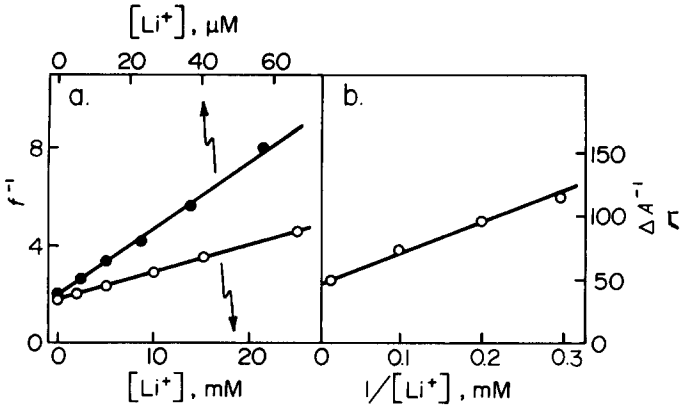


Fig. 1. The binding of Li^+ to phosphoglucomutase. a, Competition of $Mg^{2+}$and Li^+ for the metal-ion binding site. Metal-free phosphoglucomutase, 0.2 μM, was equilibrated woth 20 μM Mg^{2+} and 10 μM EDTA in the presence of 20 mM Tris/chloride, pH 7.4 and 24°C; estimated concentration of free Mg^{2+}: 12 μM (cf. ref. 14). The mixture was titrated by addition of small amounts of an LiCl solution and the titration was followed by removing 10 μl aliquots of the mixture and assaying in the presence of glucose-1-*P* and excess EDTA for the Mg · enzyme that was present (cf., Results and ref. 16). The assay results were compared with those from an identical solution of enzyme made up with a large excess of Mg^{2+} and no added Li^+ and f^{-1}, the reciprocal fraction of the enzyme present as the Mg^{2+} complex, plotted (○) against the Li^+ concentration (lower scale). K_d for the Li^+ complex of the enzyme, about 9 mM was calculated from the product of the intercept/slope ratio and $(1-f_i)$, where f_i is the value of f prior to the first addition of Li^+: 0.44. The results of an analogous experiment conducted in the presence of 1 mM glucose phosphates are shown: • and upper scale; $f_i = 0.50$; $K_d = 10$ μM. b, A spectral assessment of the binding of Li^+. To solutions of metal-free phosphoglucomutase, 2 mg per ml, in 20 mM Tris chloride, pH 7.4, in the sample and reference cells of a double-beam spectrophotometer were added 10-μl aliquots of either LiCl or water and the absorbance difference produced at 292 nm was assessed from spectral scans over the range of 340—280 (for details see ref. 8). The reciprocal of the absorbance change and the Li^+ concentration are plotted.

nation we can offer for this discrepancy is that the enzyme used in the present study is purified in a substantially different manner and is at least 50% more active than that used in the previous study.)

An estimate for K_{Li} similar to that estimated in the above manner was obtained by measuring the change in the ultraviolet spectrum of the enzyme at 292 nm as a function of the concentration of Li^+ that was present, by use of spectral difference techniques analogous to those used previously for the binding of bivalent metal ions to the enzyme [8]. At the lower concentrations of Li^+ (10 mM or less) no spectral change was observed when the active site was blocked by the tightly-bound metal ion inhibitor, Zn^{2+}, or by Mg^{2+}, although at 50 mM Li^+, only about 85% of the overall spectral change produced with metal-free enzyme could be eliminated by Zn^{2+}-blocking, presumably because of the binding of Li^+ at ancillary sites (cf., the ancillary binding of Mn^{2+} in ref. 16). Since any attempt to correct for ancillary binding effects would involve assumptions of some kind, such binding was ignored in obtaining a rough estimate of K_{Li}: approx 6 mM, see Fig. 1b. Although a quantitative correlation of this value with that obtained by the above competition experiments with Mg^{2+} thus cannot be made, a qualitative comparison indicates that Li^+ binds to phosphoglucomutase and that its dissociation constant is in the 10 mM range.

When an equilibration of the enzyme with Mg^{2+} and Li^+, analogous to that

decribed above, was conducted in the presence of an equilibrium mixture of 1 mM (saturating) glucose phosphates, the binding of Li^+, relative to Mg^{2+}, was greatly increased, as was indicated by assaying (in the presence of excess EDTA, see above) for the glucose phosphate complexes of the Mg^{2+} enzyme viz., the catalytically active forms of the enzyme. (Sufficiently small aliquots of the equilibrium mixture were used in the assay so that the correction for the glucose-6-*P* that was added to the assay mixture, along with the enzyme, amounted to less that 5% of the glucose-6-*P* produced during the effective assay time.) In fact, the dissociation constant for Li^+ decreases by a factor about 0.001 (i.e., from 9 mM to 10 μM) in the presence of bound glucose phosphate (see ●, Fig. 1a), in contrast with the dissociation constant for Mg^{2+}, which is not appreciably decreased by bound glucose-1-*P* [17]. (In view of the discrepancy in K_d values reported for Mg^{2+} in the absence of glucose-1-*P* noted above, the conclusion that glucose phosphates do not substantially affect the binding of Mg^{2+} was re-checked and found to be correct, data not shown.) A similar increased binding of Li^+ in the presence of glucose-1-*P* also was observed under initial velocity, as opposed to equilibrium condition; viz., 1.3 mM Li produces 50% inhibition in the presence of 1 mM (saturating) Mg^{2+}, but displaces an insignificant fraction of the bound Mg^{2+} under the same conditions but in the absence of glucose phosphates. In the presence of 10 mM (nearly saturating) inorganic phosphate, the dissociation constant for Li^+ decreases from 9 mM to about 1 mM (data not shown).

The monomer molecular weight of phosphoglucomutase. When phosphoglucomutase was denatured for 10 min in boiling, 6 M guanidine hydrochloride which contained 1% mercaptoethanol, exhaustively alkylated with iodoacetate, and passed through 9.2 M urea into 0.1% sodium dodecyl sulfate (SDS) via dialysis, according to the procedure of Pringle [18], only one band was observed after SDS gel electrophoresis and staining. This band appeared at the position expected for a monomer of molecular weight of about 67 000. Neither overloading the gel with a 30 μg sample nor extensive dialysis against SDS (for 5 additional days) produced any change in these results.

Discussion

All of the large number of anions that have been tested inhibit phosphoglucomutase by binding competitively with glucose phosphates (cf., ref. 9) and some phosphates are quite efficient inhibitors (see Results), especially vis-a-vis glucose bisphosphate (which binds to the dephospho-enzyme). Only two of these anions also exhibit a measurable uncompetitive effect, viz., produce an overall noncompetitive inhibition, apparently by also binding to the enzyme-substrate complexes: coenzyme A and adenosine 3′,5′-bisphosphate. In one of these cases, a comparison of the competitive (slope) and uncompetitive (intercept) inhibition constants (Table I) indicates that binding to the (free) dephospho-enzyme is at least 1000 times more tenacious than any interaction with the enzyme · substrate complexes, and in both cases, binding to the substrate complexes is relatively weak, at best. (See Table I.)

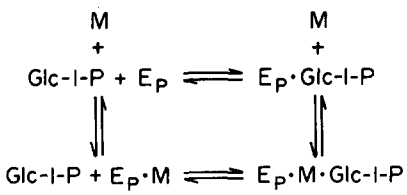
A previous report [1] suggested that phosphoglucomutase might possess a vestigal allosteric regulatory site partly on the basis of intercept inhibition con-

stants in the sub-millimolar concentration range for two nucleotide diphosphates and for acetyl coenzyme A. The present study shows that the true intercept inhibition constants for these three compounds are larger than 10 mM, and that the discrepancy between these and the earlier results probably arose from the use of Li^+ salts of inhibitors in the previous study plus a failure to recognize the potent inhibitory action of Li^+ on phosphoglucomutase. (All of the metabolic intermediates that previously were reported to be efficient inhibitors of phosphoglucomutase are sold commercially as their Li^+ salts.) Although the previously reported data no longer can be used to support the existence of an allosteric effector site, two inhibitors, 3′,5′-adenosine bisphosphate and coenzyme A, were found in the present study that do elicit a weak intercept inhibition effect. However, the observed inhibition (K_{ii} values of approx. 2 mM) was not large enough to provide an attractive basis for follow-up experiments to define its origin and thus should not be used to support the suggestion that phosphoglucomutase possesses an allosteric control site.

Supporting evidence for an allosteric effector site on phosphoglucomutase, viz., the apparent existance of two (dissimilar) polypeptide chains with approximately one half the molecular weight of the native enzyme (Duckworth and Sanwal [2]) also appears to be the result of an artifact, since a carefully purified sample of the enzyme with approximately twice the specific activity as the commercial sample previously used [2] fails to dissociate under analogous conditions: standing for several days in SDS electrophoresis buffer. Since Pringle [18] has pointed out that proteolytic digestion may occur under such conditions, in the present study Pringle's suggestions on how to obviate such problems were taken (see Results). Because the failure of a more highly purified protein to "dissociate" under a given set of conditions, except for a much more vigorous pre-treatment step (boiling for 10 min in 6 M guanidine · HCl with 1% mercaptoethanol) appears to be a more meaningful result, we conclude that phosphoglucomutase is monomeric, not dimeric, and that the earlier results cannot be used in support of the existance of an allosteric effector site.

That Li^+ should exhibit such a powerful inhibitory effect on phosphoglucomutase (see Results) is surprising, even though Li^+ resembles Mg^{2+} [19], the normal metal-ion activator, in terms of size, hydration, and propensity for interacting with oxyanions (which are thought to play an important role in metal-ion binding in phosphoglucomutase [20]). In fact, Li^+ does not bind particularly well to the free enzyme: $K_d \approx 9$ mM. However, the binding of Li^+ increases by nearly three orders of magnitude in the presence of an equilibrium mixture of glucose phosphates. Since serum concentrations of Li^+ frequently are maintained at 1—3 mM in the treatment of psychotic patients (cf., ref. 21), a substantial inhibition of phosphoglucomutase action could occur under such conditions. Although few if any specific enzymes, other than phosphoglucomutase, are known to be inhibited in vitro at these concentrations of Li^+, it is doubtful that inhibition of phosphoglucomutase constitutes the primary function of Li^+ in such treatment.

By using the "thermodynamic box" in Scheme I, one can show that if Li^+ binds to the E_P · Glc-1-*P* complex 1000 times more tenaciously than to the free phospho-enzyme, E_P, then glucose-1-*P* must bind to the E_P · Li complex 1000 times more tenaciously than to the metal-free enzyme. Moreover, since Mg^{2+},



Scheme 1. A thermodynamic box showing the binding of metal ion, M, and glucose mono-phosphates, Glc-*P*, to the phospho-enzyme, Ep, to produce mixtures of the enzyme · glucose phosphate complexes.

the normal activator, remains attached to the enzyme for many catalytic cycles [9], it appears reasonable, from a mechanistic standpoint, to consider increased binding of glucose-1-*P* to the E_P · Li complex, rather than increased binding of Li^+ to the E_P · Glc-1-*P* complex. The constant for dissociation of glucose-1-*P* from the E_P · Mg · Glc-1-*P* complex is approx. 8 μM and the same value can be approximated for the dissociation constant of glucose-1-*P* from E_P · Glc-1-*P* [17]. Since glucose-1-*P* binds to E_P · Li some 1000 times more tenaciously than to E_P, K_d for E_P · Li · Glc-1-*P* must be on the order of 10 nM. However, this unusually tight binding produces an inactive complex. The much tighter binding of glucose-1-*P* in the formation of an inactive complex might be rationalized in terms of a decreased utilization of the intrinsic binding energy of the substrate to produce an energetically unfavorable change [22] during binding to E_P · Li, as opposed to E_P · Mg, especially in view of the huge substrate-induced rate effect that is known to accompany substrate binding to E_P · Mg [23], and this possibility will be considered in a subsequent paper.

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