ON THE "PHOSPHORYL-ENZYME" OF PHOSPHOGLYCERATE KINASE

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The 'phosphoryl-enzyme' prepared from phosphoglycerate kinase and ATP in the presence of an ADP trap, is shown to be a complex between the enzyme and 1,3-diphosphoglycerate. The capricious variation in the extent of phosphorylation is due to varying amounts of contaminating 3-phosphoglycerate. Each of the partial exchange reactions may be artefactual due to the presence of very small amounts of contaminating cosubstrate. The balance of evidence favours a sequential pathway in which the phosphoryl group is transferred between substrates in a ternary complex.

The mechanistic pathways of enzymes that catalyse the intermolecular transfer of phosphoryl groups are customarily divided into two groups[1]: sequential pathways that involve phosphoryl transfer between substrates within ternary complexes, and ping-pong pathways that require a free phosphoryl-enzyme intermediate, E ~ P. The evidence relating to one of the simplest such enzymes, 3-phosphoglycerate kinase (EC 2.7.2.3) is conflicting, and is enumerated below.

Favouring the sequential pathway are the results from steady-state kinetic work, which show convergent double-reciprocal plots.[2] This behaviour may be diagnostic of a sequential pathway, though the method is complicated by the non-linearity of the double-reciprocal plots[3], the problem of the Mg^{++} concentration[1], by the possibility of multiple substrate binding sites[2] and by the orderdness of substrate addition.[1] For other kinases, serious conflicts have

arisen; thus acetate kinase shows convergent plots in one direction and parallel plots in the other[4], and in the absence of other data, the results of steady-state kinetics need cautious interpretation.

Favouring the ping-pong pathway, which for the present reaction is represented by:

$$\begin{aligned} E + ATP &\rightleftharpoons E \sim P + ADP \\ E \sim P + PGA &\rightleftharpoons E + DPGA \end{aligned} \qquad (1)$$

(where PGA is 3-phosphoglycerate and DPGA is 1,3-diphosphoglycerate), is the isolation of $E \sim P$ from incubations of the enzyme with ATP in the presence of an ADP trap.[5] The chemical competence of the $E \sim P$ has been demonstrated;[5] the phosphoryl group can be transferred either to ADP or to PGA. Further evidence in favour of the ping-pong pathway is the observation of each of the partial reactions of equation (1), by isotope exchange (between ATP and [^{3}H]-ADP in the absence of PGA,[5,6] or between [^{14}C]-PGA and glyceraldehyde 3-phosphate in the absence of ADP,[7] when the chemical instability of DPGA is circumvented by equilibrating DPGA with glyceraldehyde phosphate using glyceraldehyde phosphate dehydrogenase/NADH).

In examining the evidence for the mechanistic pathway of phosphoglycerate kinase we have found a number of inconsistencies in the data, and have been forced to reassess the significance of the experiments summarized above. Using the method of Walsh and Spector[5] in which the enzyme is phosphorylated by γ [^{32}P]-ATP and the product ADP is rephosphorylated by coupling to phosphoenol pyruvate and pyruvate kinase, there is no reason why consistent extents of phosphorylated enzyme should not be obtained. Yet, even with enzyme preparations of maximal specific

activity that are homogeneous on polyacrylamide gel and isoelectric focussing, the yeast enzyme was phosphorylated to varying degrees (from 1 to 50%) and the horse enzyme[9] was never more than 1.5% phosphorylated. The preparation of E ~ P has also proved a fickle process in other laboratories.[10] The lack of reproducibility of E~P formation caused us to reinvestigate the chemical and kinetic competence of this intermediate.

The chemical competence of 'E~P' has been demonstrated[5], and we have confirmed the complete transfer of ^{32}P from radioactive 'E ~ P' both to PGA and to ADP. Kinetically, however, the catalysis of the partial reactions by phosphoglycerate kinase (see equation 1) is very inefficient, and each partial exchange reaction is 10^3 to 10^4 times slower than is observed in the presence of mM levels of the second substrate. As has been pointed out[11], a slow partial reaction need not invalidate the ping-pong pathway, if one invokes 'substrate synergism', such that partial exchange is accelerated markedly in the presence of the second substrate. We have investigated a large number of PGA analogues and some ADP analogues, in the hope of finding compounds that could not act as phosphoryl acceptors (i.e., could not be substrates) but nevertheless accelerated the partial exchange. This search has been in vain. Further, it is obvious that ADP-ATP exchange can be catalysed by a large number of kinases, and that the exchange observed with phosphoglycerate kinase could be due to very small amounts ($<0.1\%$) of a contaminating kinase. A contaminating enzyme is an unlikely explanation for the specific PGA-DPGA exchange, but this partial reaction is also very slow, and we have found that a contaminating concentration of ADP even less than that of the phosphoglycerate kinase itself, suffices to produce the observed reaction rate. In summary,

then, both the isolation of 'E ~ P', and the existence of each of the partial exchange reactions, seemed to rest upon shaky ground.

When 'E ~ P' is made from the DPGA direction using $^{32}P_i$, [^{14}C]-glyceraldehyde phosphate, glyceraldehyde phosphate dehydrogenase and NAD^+, equivalent amounts of ^{32}P and ^{14}C are bound to the kinase on gel filtration. This indicates formation of E·DPGA, and agrees with the finding of other workers[12] that the dissociation constant of DPGA from phosphoglycerate kinase is very small. Small enough, that is, for the E·DPGA complex to survive gel filtration. This suggests that the 'E ~ P' formed from kinase and ATP is, in fact, E·DPGA. Three lines of evidence support this. First, using different batches of yeast and of horse enzyme, there is a close correlation between the achievable 'E ~ P' formation from γ[^{32}P]-ATP, and the PGA content of this 'E ~ P' measured after denaturation of the enzyme and hydrolysis of the putative DPGA by boiling the solution (see Table 1). Secondly, the rate constants for the hydrolysis of 'E ~ P' (prepared from γ[^{32}P]-ATP) and of <u>bona fide</u> E·DPGA (prepared from $^{32}P_i$ and [^{14}C]-glyceraldehyde-3-phosphate) are the same within experimental error at two different pH-values (Table 2). [The hydrolytic half-life of DPGA on the enzyme is more than an order of magnitude longer than that of free DPGA, and this, together with the tight binding of DPGA to the kinase, is just what is expected for the protection of a chemically labile intermediate in a metabolic pathway.] Thirdly, the reaction of hydroxylamine (0.15 M) with 'E ~ P' and with E·DPGA releases $^{32}P_i$ at the same rate, and even with 3 M hydroxylamine the enzyme is not inactivated. If 'E ~ P' were—as has been proposed[5]—an

Table 1. The content of 3-phosphoglycerate in various preparations of 'E ~ P'

Enzyme	Ratio of bound ^{32}P (as 'E ~ P') to the 3-PGA-content of 'E ~ P'[a]	% Phosphorylation[b]
Horse muscle[c]	-	<1.5
Yeast[d]	-	<1
Yeast[e], lot 1	-	<1
Yeast[e], lot 2	1.02	17.0
Yeast[e], lot 3	0.95	12.7
Yeast[e], lot 3	1.01	16.5
Yeast[e,f], lot 3	0.96	42.4
Yeast[e,g], lot 2	1.14	53.0

[a] Ratio of μmoles of ^{32}P bound to the enzyme after gel filtration (after correction for the fall in specific radioactivity of the ATP during the incubation), to the μ moles of 3-phosphoglycerate assayed after denaturation and hydrolysis.

[b] μmoles of bound ^{32}P per μmole enzyme (%).

[c] Crystalline enzyme of specific activity 700 units/mg, prepared according to Johnson, P. E., Maister, S. G., Sémériva, M., Young, J. M. and Knowles, J. R. (unpublished).

[d] Enzyme of specific activity 740 units/mg, prepared according to Scopes[8].

[e] Different commercial preparations.

[f] Incubation solution contained added 3-PGA in 1.5-fold molar excess over enzyme.

[g] Incubation solution contained added 3-PGA in 10-fold molar excess over enzyme.

acyl phosphate involving an enzyme carboxyl group, inactive enzyme hydroxamate would be expected.[5,14]

The capriciously variable proportion of enzyme that can be phosphorylated is therefore a consequence of varying amounts of

Table 2. Hydrolysis rates of 'E ~ P' and of E·DPGA (30° C)

	$k_{hydrolysis}$ at pH 7.5 (min^{-1})	$k_{hydrolysis}$ at pH 2.7 (min^{-1})
'E ~ P'[a]	0.00052	0.0089
E·DPGA[b]	0.00044	0.0081

[a] 'E ~ P' was prepared by the method of ref. 5, from γ[^{32}P]-ATP and enzyme in the presence of an ADP trap.

[b] Bona fide E·DPGA was prepared from $^{32}P_i$ and [^{14}C]-glyceraldehyde-3-phosphate in the presence of catalytic amounts of glyceraldehyde-3-phosphate dehydrogenase. The ^{32}P:^{14}C ratio was 0.92:1.0.

PGA that contaminate the kinase (a heat-treatment in the presence of PGA is frequently included in the enzyme isolation described in the literature) or any of the other substrates, cofactors and enzymes used in the preparation of 'E ~ P'. The chemical competence of 'E ~ P' is also readily explained. Clearly E·DPGA plus excess ADP will yield ATP by the normal kinase reaction. Moreover, since we know that a slow PGA-DPGA exchange occurs even with highly purified kinase[7], we expect the synthesis of [^{14}C]-DPGA from [^{14}C]-PGA in the presence of E·DPGA. Any ADP or ATP contamination remaining from the preparation of 'E ~ P' would obviously accelerate this exchange.

The question of whether the slow partial exchange reactions are an intrinsic property of phosphoglycerate kinase, must remain open. As mentioned above, a small amount of a contaminating kinase could produce the observed ADP-ATP exchange, and it has recently been suggested[13] that adenylate kinase is responsible for the observed exchange with the yeast enzyme. However, the adenylate kinase content of our

enzyme preparation could only account for <1% of the observed ADP-ATP exchange. While the adventitious presence of some other kinase cannot be excluded, after isoelectric focussing of phosphoglycerate kinase the peak of ADP-ATP exchange activity and that of the overall catalytic activity coincide with the single protein peak. The same is true for the PGA-DPGA exchange activity. But the present results do not allow a definitive statement about the ability or otherwise of phosphoglycerate kinase to catalyse genuine partial exchange processes.

The reported isolation of diaminobutyric acid from yeast 'E ~ P' prepared from ATP, after hydroxylamine treatment and Lossen rearrangement[14], is unexplained. Brevet et al. found that the amount of diaminobutyrate correlated with the extent of enzyme phosphorylation, but this finding conflicts with the fact that hydroxylaminolysis of 'E ~ P' does not lead to loss of catalytic activity. Further investigation of this problem is necessary.

In summary, it has been shown that both the partial exchange reactions catalysed by phosphoglycerate kinase may be artefactual, and that the 'phosphoryl-enzyme' that has been previously isolated is in fact a tight complex between enzyme and 1,3-diphosphoglycerate (or, of course, such a complex in rapid equilibrium with E ~ P· PGA). This work (and see also ref. 15) illustrates some of the dangers involved in experiments where very small contaminating amounts of substrate may give rise to large kinetic effects, and to the mistaken identity of enzyme intermediates.

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