Phosphorylation of Pyruvate by the Pyruvate Kinase Reaction and Reversal of Glycolysis in a Reconstructed System

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Lardy and Ziegler (1) obtained synthesis of phosphoenol pyruvate from pyruvate and adenosine triphosphate by reversal of the pyruvate kinase reaction. A possible mechanism was thus demonstrated for the initial step in the synthesis of glycogen from pyruvate. The amounts of phosphoenol pyruvate synthesized were, however, small, and it is rather widely held that reversal of the pyruvate kinase reaction leading to net synthesis of phosphoenol pyruvate in vivo is insignificant (2-5).

It is the purpose of this paper to report the synthesis of relatively large amounts of phosphoenol pyruvate in an enzymatic system similar to that of Lardy and Ziegler. They used muscle extracts to generate ATP by the oxidative phosphorylation of glyceraldehyde 3-phosphate; the enolase reaction was blocked with fluoride. It was found that substitution of the muscle extracts by purified glycolytic enzymes resulted in much higher steady state concentrations of PE.\(^1\) Synthesis of PE by reversal of the pyruvate kinase reaction was also obtained when respiring mitochondria were used as the ATP generator. Supplementation of this system with glycolytic enzymes led to formation of fructose-DP and other glycolytic intermediates.

**EXPERIMENTAL**

**Enzyme Preparations**—Phosphoglycerate mutase, phosphoglycerate kinase, enolase, and pyruvate kinase were prepared as described in the preceding paper. Glyceraldehyde-3-P dehydrogenase from muscle was prepared by the method of Beisenherz et al. (6) and from yeast by the method of Krebs et al. (7); the extracting medium used in preparing both dehydrogenases contained 2 mg. per ml. of ethylenediaminetetraacetate. Lactic dehydrogenase was prepared by the method of Beisenherz et al. (8); aldolase by the method of Taylor et al. (9); alcohol dehydrogenase by the method of Racker (10); and rabbit muscle a-glycerophosphate dehydrogenase was a gift from Dr. T. Bucher or was purchased from C. F. Boehringer and Sons, Mannheim, Germany.

**Reagents**—2,3-Diphosphoglycerate was prepared as described in the preceding paper; potassium pyruvate was prepared by the method of Korkes et al. (11).

**Analytical Methods**—PE and PE were determined as described in the preceding paper.

\(^1\) The abbreviations used are: PE, phosphoenol pyruvate; fructose-DP, fructose 1,6-diphosphate; Pi, inorganic orthophosphate.

3-Phosphoglycerate was measured by oxidation of DPNH in the presence of ATP, Mg++, phosphoglycerate kinase, and glyceraldehyde-3-P dehydrogenase (12); dihydroxyacetone phosphate by oxidation of DPNH with \(\alpha\)-glycerophosphate dehydrogenase (13); fructose-DP by oxidation of DPNH with aldolase and \(\alpha\)-glycerophosphate dehydrogenase (13); pyruvate by oxidation of DPNH with lactic dehydrogenase (13); and glycerophosphate by the method of Bublitz and Kennedy (14).

ATP and ADP were assayed by the method of Bowen and Kerwin (15) after removal of Mg++ from the trichloroacetic acid filtrates of reaction mixtures by passing them through a column of Dowex 50-X2, H+ form. Removal of Mg++ is necessary since it interferes with the subsequent assay.

**Mitochondrial Preparations**—These were made from rat liver by the method of Hogeboom (16) except that the suspending medium used was 0.25 M mannitol containing 1 mg. per ml. of ethylenediaminetetraacetate. Mannitol was used instead of sucrose because contamination of some yeast enzyme preparations with invertase (which catalyzed glucose formation) and hexokinase led to net esterification of Pi, i.e. glucose 6-phosphate formation, without the addition of pyruvate as a phosphate acceptor.

In experiments in which mitochondria were used to generate ATP for pyruvate phosphorylation, incubation was for 30 minutes at 27°C in 20-ml. beakers shaken at about 2 cycles per second in a Dubnoff metabolic shaking incubator (Precision Scientific Company, Chicago, Illinois); deproteinization was by addition of 0.1 volume of 25 per cent trichloroacetic acid. Zero time controls were obtained by adding all the reagents directly to a solution of trichloroacetic acid.

**RESULTS**

Table I shows the enzyme requirements for accumulation of PE in a system in which ATP is generated by the glycolytic reactions leading from fructose-DP to phosphoglycerate. Pyruvate is phosphorylated by reversal of the pyruvate kinase reaction. It will be noted that PE accumulation is strongly dependent on the presence of lactate dehydrogenase; reoxidation of DPNH formed in the glyceraldehyde-3-P dehydrogenase reaction is necessary to maintain a high level of 1,3-diphosphoglycerate which in turn, via the phosphorylase kinase reaction, permits a high ATP:ADP ratio required for reversal of the pyruvate kinase reaction.

Table II records an experiment designed to show that the amount of PE present depends on the establishment of equilibrium mixtures of the various components through the linked
enzyme reactions. It is seen that the amount of PE present and of P, eстерified is substantially constant in the period between 15 and 35 minutes of incubation. The pH dependency of the over-all equilibrium mixture is also demonstrated.

The results given in Table III show reversal of the pyruvate kinase reaction when ATP is regenerated through mitochondrial rather than through glycolytic phosphorylation. Pyruvate, in the presence of pyruvate kinase, acts as the phosphate acceptor. Absence of PE formation when pyruvate kinase is omitted rules out the oxaloacetate carboxylase reaction as the source of PE in this system. The amount of PE formed indicates the ability of the mitochondria to maintain high ATP:ADP ratios.

Table IV records formation of various intermediate compounds of glycolysis when the system shown in Table III was supplemented with DPN, catalytic amounts of 2,3-diphosphoglycerate, and the enzymes required for conversion of PE to fructose-DP. Contamination with α-glycerophosphate dehydrogenase led to production of α-glycerophosphate. Small amounts of fructose-DP, dihydroxyacetone phosphate, and α-glycerophosphate were formed even in the absence of an added DPN-reducing system.

**Equilibrium Constant of Pyruvate Kinase Reaction**—Because of the absence of data on the H⁺ ion concentration in the report of Meyerhof and Oesper (17) on the equilibrium of the pyruvate kinase reaction, the relative concentrations of the reactants to be expected at a given pH are uncertain. The equilibrium mixtures at various H⁺ ion concentrations were therefore determined. The data conform to the equation:

\[
\frac{(ATP)(\text{pyruvate})}{(ADP)(\text{PE})(\text{H}^+)} = K
\]

Table V gives the results of a series of determinations of the equilibrium mixtures. The values for the equilibrium constant range between 0.98 × 10⁻⁸ and 1.51 × 10⁻⁸. The variation is largely due to inaccuracy in determining ADP concentrations. The latter are, in the method used, estimated as relatively small differences between two large numbers.

On the other hand, the measurements of PE are independent of differences between two large numbers.

**Table I**
Reversal of pyruvate kinase reaction dependent on ATP regeneration by phosphoglycerate kinase reaction

Incubation for 15 minutes at 37°C. The complete system contained in 1 ml: 100 μmoles of tris(hydroxymethyl)aminomethane, pH 8.3; 7.5 μmoles of K₂HPO₄; 5 μmoles of MgCl₂; 20 μmoles of KF; 1 μmole of DPN; 6 μmoles of ATP; 47 μmoles of potassium pyruvate; 18 μmoles of fructose-DP; 160 pg. of aldolase; 320 pg. of pyruvate kinase; 500 pg. of lactic dehydrogenase; 100 pg. of phosphoglycerate kinase. The pH of the incubation mixtures was 8.0. Omissions as indicated.

**Table II**
Dependence of PE concentration on pH of medium

Incubation at 37°C for periods indicated. System as in Table I except that pH of tris(hydroxymethyl)aminomethane buffer was adjusted to give pH of incubation mixtures as indicated.

**Table III**
Phosphorylation of pyruvate mediated by mitochondria and pyruvate kinase

The incubation mixtures contained, in 1 ml: 100 μmoles of tris(hydroxymethyl)aminomethane, pH 7.4; 20 μmoles of potassium glutamate; 5 μmoles of MgCl₂; 3 μmoles of ATP; 5 μmoles of potassium phosphate, pH 7.4; 45 μmoles of potassium pyruvate; 56 μg. of pyruvate kinase; 6 mg. of mitochondrial protein. Omissions as indicated.

**Table IV**
Formation of phosphorylated intermediates of glycolysis from pyruvate by mitochondria and glycolytic enzymes

The incubation mixtures contained, in 1 ml: 100 μmoles of tris(hydroxymethyl)aminomethane, pH 7.4; 20 μmoles of potassium glutamate; 5 μmoles of MgCl₂; 3 μmoles of ATP; 6 μmoles of potassium phosphate, pH 7.4; 1 μmole of DPN; 0.05 μmole of 2,3-diphosphoglycerate; 60 μg. of pyruvate kinase; 100 μg. of enolase; 75 μg. of phosphoglycerate mutase; 50 μg. of phosphoglycerate kinase; 300 μg. of glyceraldehyde-3-P dehydrogenase (muscle); 200 μg. of aldolase; 6 mg. of mitochondrial protein.
TABLE V
Equilibrium mixtures in pyruvate kinase reaction

Incubation at 30°. The incubation mixtures contained, in 6.0 ml.: 400 μmoles of tris(hydroxymethyl)aminomethane*; 4 μmoles of MgCl₂; 516 μmoles of potassium pyruvate; 30 μmoles of ATP; 200 μg. of pyruvate kinase. At 20 minutes and at 30 minutes, 2.2 ml. were removed from each incubation mixture and added to 0.22 ml. of 50 per cent trichloroacetic acid. Concentrations below are given as μmoles per ml.

Time | pH | Pyruvate | PE | ATP | ADP | K | K × (H+)  
--- | --- | -------- | --- | --- | --- | --- | ---  
20 min. | 8.70 | 84.8 | 1.19 | 4.02 | 1.19 | 1.21 × 10⁸ | 2.42 × 10⁸  
30 min. | 8.70 | 84.8 | 1.18 | 3.87 | 1.37 | 1.01 × 10⁸ | 2.02 × 10⁸  
20 min. | 8.31 | 85.2 | 0.80 | 4.30 | 0.71 | 1.34 × 10⁸ | 6.58 × 10⁸  
30 min. | 8.31 | 85.2 | 0.80 | 4.20 | 0.93 | 0.98 × 10⁸ | 4.81 × 10⁸  
20 min. | 7.94 | 85.5 | 0.53 | 4.58 | 0.41 | 1.51 × 10⁸ | 1.81 × 10⁸  
30 min. | 7.94 | 85.5 | 0.54 | 4.45 | 0.48 | 1.22 × 10⁸ | 1.46 × 10⁸  
20 min. | 7.72 | 85.6 | 0.42 | 4.60 | 0.37 | 1.36 × 10⁸ | 2.59 × 10⁸  
30 min. | 7.72 | 85.6 | 0.42 | 4.50 | 0.45 | 1.07 × 10⁸ | 2.04 × 10⁸  
Average | | | | | | 1.21 × 10⁸  
* The pH of tris(hydroxymethyl)aminomethane buffer was varied to give the final H⁺ concentrations shown in the table.
† Pyruvate concentration is calculated as the initial concentration (86 μmoles per ml.) minus the PE concentration.

TABLE VI
Equilibrium constant of pyruvate kinase reaction based on PE measurements

| pH | Pyruvate | PE | ATP | ADP | K | K × (H⁺)  
--- | --- | --- | --- | --- | --- | ---  
8.70 | 84.8 | 3.82 | 1.18 | 1.16 × 10⁸ | 2.33 × 10⁸  
8.31 | 85.2 | 4.20 | 0.80 | 1.14 × 10⁸ | 5.59 × 10⁸  
7.94 | 85.5 | 4.47 | 0.53 | 1.13 × 10⁸ | 1.36 × 10⁸  
7.72 | 85.6 | 4.58 | 0.42 | 1.17 × 10⁸ | 2.22 × 10⁸  

Therefore,

\[
\frac{(\text{MgATP}^2-)}{(\text{Mg}^{++})(\text{ATP}^4-)} = 3000 \tag{2}
\]

\[
\frac{(\text{MgADP}^2-)}{(\text{Mg}^{++})(\text{ADP}^4-)} = 1000 \tag{3}
\]

Under the conditions of the experiment, where the concentration of ATP greatly exceeded that of MgCl₂, substantially all of the magnesium in the system, 0.67 μmoles per ml., was in the form of nucleotide complexes (20). Binding of magnesium by PE is insignificant compared with binding by ATP and ADP (21, 22). Therefore,

\[
\text{MgATP}^2- = 0.67 - \text{MgADP}^2- \tag{5}
\]

Also,

\[
\text{ADP}^4- = \text{ADP}^2- - \text{MgADP}^2- \tag{6}
\]

\[
\text{ATP}^4- = \text{ATP}^2- - \text{MgATP}^2- \tag{7}
\]

Substitution of Equation 5 into Equation 7 gives

\[
\text{ATP}^2- = \text{ATP}^2- - 0.67 + \text{MgADP}^2- \tag{8}
\]

Substitution of Equations 5, 6, and 8 into Equation 4 gives

\[
\frac{(0.67 - \text{MgADP}^2-)(\text{ADP}^2- - \text{MgADP}^2-)}{(\text{MgATP}^2-)(\text{ATP}^2- - 0.67 + \text{MgADP}^2-)} = 3 \tag{9}
\]

MgADP²⁻ can be computed from Equation 9 by use of the experimentally found values for ADP²⁻ and ATP²⁻. From Equations 5, 6, and 7, MgATP²⁻, ATP⁴⁻, and ADP⁴⁻ can be computed. Assuming that the magnesium complexes of the nucleotides

the other constituents of the mixtures; they are very reproducible and are in excellent agreement with PE determinations made by measuring DPNH oxidation in the coupled pyruvate kinase and lactic dehydrogenase reactions. The pyruvate kinase used in this study was free of myokinase and enolase. In view of these considerations, calculations of the equilibrium constant based on the PE determinations are presented in Table VI. Pyruvate concentration is taken as initial pyruvate minus PE; ATP as initial ATP minus PE; ADP as equal to PE. $K$ equals $1.15 \times 10^8$.

It should be noted that the concentration of MgCl₂ used (Table V) was 0.67 μmoles per ml. This concentration was chosen because with higher concentrations there was a lower steady state concentration of PE (possibly due to preferential binding of ATP by Mg++) and with lower concentrations equilibrium was reached too slowly (Table VII).

At first sight the constancy of the equilibrium constant calculated without regard to pH effects on ionic species and to magnesium complexes may be surprising. However in the pH range covered in this study (7.72 to 8.70) ATP, ADP, and PE are in their completely dissociated forms since the respective relevant pK's of these compounds are 6.6, 6.6, and 6.4 (18). As to the effect of magnesium complexes, the following calculations show that, under the experimental conditions, they would produce relatively small changes in the equilibrium mixtures. From the data of Smith and Alberty (19),

\[
(MgATP²⁻)(ADP²⁻) = 3000 \tag{2}
\]

\[
(MgADP²⁻)(ATP²⁻) = 1000 \tag{3}
\]

Under the conditions of the experiment, where the concentration of ATP greatly exceeded that of MgCl₂, substantially all of the magnesium in the system, 0.67 μmoles per ml., was in the form of nucleotide complexes (20). Binding of magnesium by PE is insignificant compared with binding by ATP and ADP (21, 22). Therefore,

\[
\text{MgATP}²⁻ = 0.67 - \text{MgADP}²⁻ \tag{5}
\]

Also,

\[
\text{ADP}^4⁻ = \text{ADP}²⁻ - \text{MgADP}²⁻ \tag{6}
\]

\[
\text{ATP}^4⁻ = \text{ATP}²⁻ - \text{MgATP}²⁻ \tag{7}
\]

Substitution of Equation 5 into Equation 7 gives

\[
\text{ATP}²⁻ = \text{ATP}²⁻ - 0.67 + \text{MgADP}²⁻ \tag{8}
\]

Substitution of Equations 5, 6, and 8 into Equation 4 gives

\[
\frac{(0.67 - \text{MgADP}²⁻)(\text{ADP}²⁻ - \text{MgADP}²⁻)}{(\text{MgATP}²⁻)(\text{ATP}²⁻ - 0.67 + \text{MgADP}²⁻)} = 3 \tag{9}
\]
are not substrates for pyruvate kinase (see Table VII), the equation for the equilibrium constant is

\[
\frac{(ATP^-)(pyruvate^-)}{(ADP^-)(PE^-)(H^+)} = K
\]

(10)

Table VIII lists the values for ADP\(^-\) and ATP\(^-\) and the equilibrium constants calculated on this basis. The magnitude of \(K\) is about 10 per cent less than it is when calculated, as in Table V, on the basis of total ATP and ADP. This follows from the fact that proportionately more ATP than ADP has formed a complex with Mg\(^{++}\).

**Discussion**

At present it is not known whether reversal of the pyruvate kinase reaction is quantitatively significant in the synthesis of fructose-DP from pyruvate in vivo. The two glycolytic reactions of this series most unfavorable for reversal are those catalyzed by phosphoglycerate kinase (\(K = 3 \times 10^3\) (12)) and by pyruvate kinase (\(K = 2 \times 10^4\) (17) presumably at pH 7.6 (21)). However, the former reaction is often described as "readily reversible" and is generally regarded as obligatory in schemes of hexose formation from pyruvate, whereas the latter is considered to be incapable of significant reversal (2-5). The demonstrated reversibility of both reactions does not justify such a distinction between them. Reversal of glycolysis in vivo by direct phosphorylation of pyruvate is therefore a strong possibility.

Kalkar (23) observed that oxidation of malate by kidney extracts gave rise to PE. He proposed, in view of the apparent irreversibility of the pyruvate kinase reaction (24), that the system studied by him might be involved in providing PE for glycogen synthesis. Bartley (25) with the use of kidney particulate, obtained PE synthesis during the oxidation of various citric acid cycle intermediates and also when pyruvate was the substrate; PE was formed from pyruvate only in the presence of bicarbonate. Conversion of pyruvate to PE was therefore considered to involve a preliminary carboxylation to a C\(_5\) compound. These findings together with isotope distribution in liver glycogen after administration of C\(^4\)-labeled pyruvate or C\(^4\)O\(_2\) (cf. review by Weinman et al. (5)) support the possibility that pyruvate incorporation into glycogen proceeds by condensation of pyruvate with CO\(_2\) formation of a symmetrical dicarboxylic acid, and subsequent phosphorylative decarboxylation (26, 27).

These data do not rule out glycogen synthesis by way of the pyruvate kinase reaction. Randomization of the isotopic C of pyruvate can be as readily explained by "shuttling" as by a predominantly "one-way" reaction; net synthesis of glycogen may still depend upon the pyruvate kinase reaction (28).

**References**

12. **Büchler, T., Biochim. et Biophys. Acta, 1, 292 (1947).**
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