BIOORGANIC CHEMISTRY 20, 62–66 (1992)

ATP Cofactor Regeneration via the Glycolytic Pathway[1]

LISA L. WEI AND WARREN J. GOUX[2]

Department of Chemistry, The University of Texas at Dallas, P.O. Box 830688, Richardson, Texas 75083-0688

Received May 7, 1991

In this communication the glycolytic pathway is shown to be a useful means of regenerating ATP *in situ*. The system has the rather attractive advantage that the two phosphoryl donor substrates, glucose and inorganic phosphate, are readily available at minimal cost. In addition, the free energy change for the ATP regeneration reaction is much more negative than other previously utilized ATP regeneration systems. Hence, the phosphorylation of compounds having high free energies of hydrolysis is thermodynamically more favorable using the glycolytic pathway as a means of ATP regeneration. The utility of the method is demonstrated by coupling the creatine kinase-catalyzed phosphorylation of creatine to the glycolytic conversion of glucose to lactic acid.

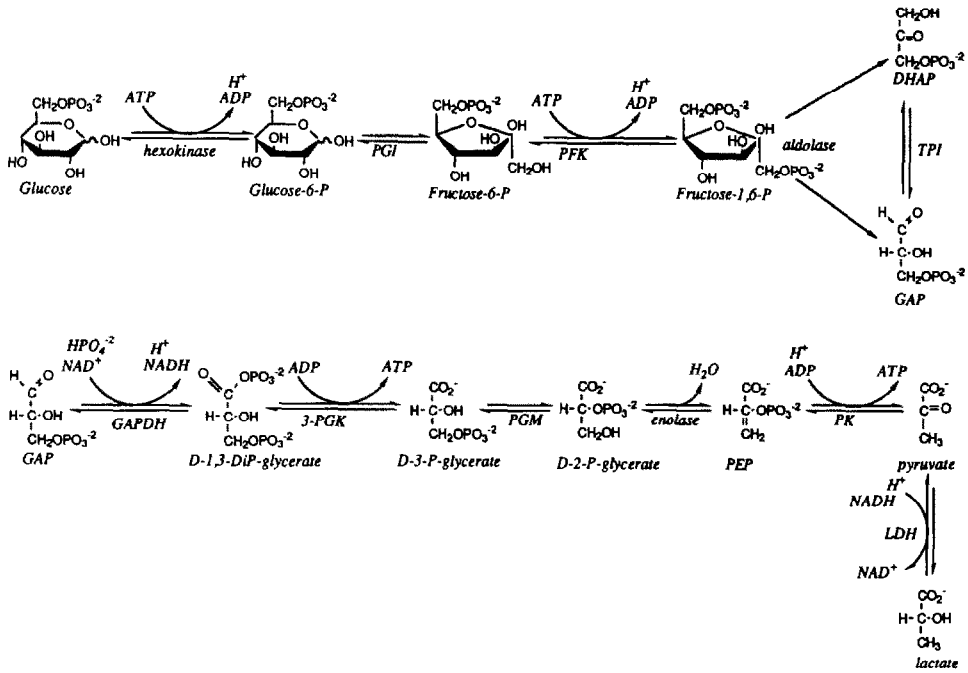
Most enzyme-catalyzed syntheses of phosphorylated products use ATP as a required cofactor. Products can be produced at relatively low ATP concentrations by coupling the reaction of interest to a second enzyme-catalyzed reaction which utilizes ADP and a phosphoryl donor as substrates and regenerates the needed ATP *in situ* (*1*). The overall yield of product is dependent upon the relative free energies of the reaction responsible for ATP regeneration and the reaction in which product is formed. By utilizing relatively low nucleotide concentrations, both cost and substrate inhibition of enzymes are minimized.

In the last decade a number of phosphoryl donors, including acetyl phosphate (*2, 3*), phospho(enol)pyruvate (PEP) (*4–6*), 3-D-phosphoglyceric acid (*7*), and methoxycarbonyl phosphate (*8*), have been prepared and shown to be of use for the *in situ* regeneration of ATP. Of these, the two which appear to show the greatest long term stability in aqueous solvents and possess relatively high free energies of hydrolysis are PEP and 3-phosphoglyceric acid. In this communication we show that ATP can be regenerated using commercially available soluble enzymes of the glycolytic pathway. The system is similar in principle to the use of immobilized yeast cells for ATP regeneration (*9*) but is more tractable for larger scale synthetic processes. Both systems have the rather attractive advantage that the two phosphoryl donor substrates, glucose and inorganic phosphate, are readily available at minimal cost. In addition, the free energy of the glycolysis/ATP regeneration

[1] Supported in part by Dallas Biomedical Corporation and The Robert A. Welch Foundation (AT-1162).

[2] To whom correspondence should be addressed.

0045-2068/92 $3.00



SCHEME 1

reaction is much more favorable than previously utilized systems.[3] Hence, the phosphorylation of compounds having high free energies of hydrolysis is thermodynamically more favorable using the glycolytic pathway as a source of ATP.

The essentials of the glycolytic pathway used in our ATP regeneration system are illustrated in Scheme I. The sequence of reactions comprising the system may be divided into two parts. In the first part glucose is phosphorlated by 2 mol ATP to yield fructose 1,6-diphosphate (FDP). FDP then undergoes a reverse aldol condensation to yield 1 mol of glyceraldehyde 3-phosphate (GAP) and 1 mol of dihydroxyacetone phosphate (DHAP). These intermediates are in equilibrium with one another via a triose phosphate isomerase-catalyzed reaction. A stochiometric balance of the reaction from glucose to GAP shows that 2 mol of ATP are utilized and 2 mol of acid are produced (Eq. [1]). Under conditions of slightly alkaline pH the equilibrium of the sequence of coupled reactions lies to the right.[4] The 2 mol of ATP which were initially invested in the first part of the reaction sequence are returned with a profit of 2 additional mol of ATP when the 2 mol of GAP are

[3] Glycolysis is able to convert ADP + P_i to ATP with a $\Delta G^{0'} = -14.73$ kcal. In comparison 1 mol of ATP is formed from PEP and ADP with a $\Delta G^{0'} = -7.5$ kcal (10).

[4] At neutral pH the equilibrium lies slightly in favor of glucose ($\Delta G^{0'} = 0.56$ kcal). Under slightly alkaline conditions, however, the equilibrium is shifted to the right due to the uptake of the product protons by base and because of the strong pH dependence of the equilibrium constant for the hexokinase-catalyzed reaction (11).

converted in the ensuing reactions to lactate (Eq. [2]). Although the cofactor NAD^+ is needed in converting GAP to 1,3-diphosphoglyceric acid, it is regenerated *in situ* in the final step of the reaction sequence. In addition, the two protons which were produced as products during the conversion of glucose to FDP are taken up in reactions which convert GAP to lactate, negating any direct effect of pH on equilibrium.

We demonstrate the practicality of the glycolytic/ATP regeneration system by using as an illustrative example the creatine kinase-catalyzed phosphorylation of creatine to form phosphocreatine (Scheme I). For each mole of glucose converted to lactate, 2 mol of inorganic phosphate are absorbed from the medium and 2 mol of lactic acid are produced.

$$C_6H_{12}O_6 + 2\ ATP^{4-} \rightleftharpoons 2GAP^{2-} + 2\ ADP^{3-} + 2\ H^+ \quad [1]$$

$$2\ GAP^{2-} + 2\ H^+ + 2\ PO4^{3-} + 4\ ADP^{3-} \rightleftharpoons 2\ H_2O + 4\ ATP^{4-} + 2\ lactate^{1-} \quad [2]$$

$$2\ creatine^{1-} + 2\ ATP^{4-} \rightleftharpoons 2\ P\text{-}creatine^{3-} + 2\ ADP^{3-} + 2\ H^+ \quad [3]$$

$$C_6H_{12}O_6 + 2\ H+ + 2\ PO_4^{3-} + 2\ creatine \rightleftharpoons 2\ P\text{-}creatine^{3-} + 2\ H_2O + 2\ H^+ + 2\ lactate^{1-}$$

This has the effect of removing the buffering effect of the inorganic phosphate from the medium and lowering the pH of the reaction mixture. In practice commercially available purified enzymes needed to carry out the phosphorylation of creatine and the regeneration of ATP are added to a buffered solution of substrates and cofactors. In order that the synthesis of FDP from glucose and of phosphocreatine from creatine be as favorable as possible, the pH of the reaction mixture was kept slightly alkaline using a pH controller. These conditions also prevent the reversible inactivation of phosphofructokinase known to occur much below neutral pH (12). Sodium pyruvate was also added to the reaction mixture in order to facilitate the initial regeneration of NAD^+ via the lactate dehydrogenase-catalyzed reaction. After 2 days at room temperature the final yield of phosphocreatine from inorganic phosphate was shown to be 90% by ^{31}P NMR. The enzymes were removed by ultrafiltration and shown by enzymatic assay to have the following residual activities: pyruvate kinase, 85%; creatine phosphokinase, 80%; enolase, 72%; phosphoglycerate mutase, 50%; 3-phosphoglycerate phosphokinase, 76%; glyceraldehyde-3-phosphate dehydrogenase, 53%; aldolase, 35%; phosphoglucose isomerase, 25%; hexokinase, 8%; phosphofructokinase, 100%; and lactate dehydrogenase, 68%.

One of the factors which will ultimately determine the utility of the glycolytic/ATP regeneration system described is a comparison of the cost of the system compared with other ATP regeneration systems used in large scale preparations of phosphorylated products. A cost comparison between our system and the PEP/ATP regeneration system shows that the two are comparable,[5] with the cost of the preparation of PEP offset by the cost of additional enzymes in the glycolytic/ATP regeneration system. In theory one should be able to minimize enzyme cost by simply adding less enzyme. However, from a practical aspect it must be realized that enzyme activity, particularly of rabbit muscle aldolase, phosphoglycerate

[5] The enzyme cost per gram of crystallized sodium phosphocreatine is about $0.60 using the glycolytic/ATP regeneration system. It is about one-tenth this amount using the PEP/ATP regeneration system. The cost of preparing PEP is about $0.30.

mutase, and yeast hexokinase, falls over a period of days. Thus attempts to maximize cost efficiency by cutting the amounts of enzyme added may ultimately fail due to the decrease in the activity of at least some of the enzymes.

There is potentially a much more serious problem with respect to the general utility of the glycolytic/ATP regeneration system as shown in Scheme I. Using the published equilibrium constant for hexokinase extrapolated to pH 8, we estimate an initial concentration of ATP of about 10 μM (12). This is less than the K_m of phosphofructokinase (about 25 μM at pH 8). Hence, we would expect the phosphofructokinase reaction to be rate limiting. This situation will persist unless the rate of ATP regeneration in the ensuing reactions of the glycolytic pathway are faster than the rate of ATP depletion by the hexokinase reaction and the reaction between ATP and the phosphoryl acceptor. The phosphorylated product will still be formed but at a rate dependent upon the ATP concentration and the amount of phosphofructokinase present. In practice, enzyme activity is generally lost before a reasonably good yield of product is attained. Rabbit muscle aldolase and yeast hexokinase appear to be the first enzymes to lose significant activity over a 2-day period. Hence, our reaction mixtures where the glycolytic enzymes have been reduced in an effort to maximize cost efficiency tend to have high FDP concentrations with little or no product formed.

In summary, using the glycolytic pathway as a means of ATP regeneration may offer a convenient alternative to other means of ATP regeneration. The system can be operated at a cost eficiency comparable to those of other ATP regeneration systems. At the same time the preparation of a phosphoryl donor can be avoided. However, care must be taken to maximizing the ratio of the rate of ATP generation to ATP utilization by the hexokinase and the phosphoryl acceptor reactions.

EXPERIMENTAL PROCEDURES

Preparation of phosphocreatine. A solution of 90 mM creatine, 10 mM sodium pyruvate, 50 mM potassium phosphate, 25 mM glucose, 2 mM ATP, and 2 mM NAD^+ were prepared in 200 ml of 0.1 M Tris buffer, 10 mM $MgCl_2$, 2 mM ethylenediaminetetraacetic acid (EDTA), 2 mM mercaptoethanol, and 0.05% sodium azide. The reaction mixture was brought to pH 8.0 and the solution was purged for 15 min with argon. To this mixture was added 35 units of yeast hexokinase, 25 units of phosphoglucose isomerase (yeast), 25 units of phosphofructokinase (rabbit muscle), 27 units of rabbit muscle aldolase, 71 units of 3-phosphoglycerate phosphokinase (yeast), 31 units of phosphoglycerate mutase (rabbit muscle), 120 units of L-lactate dehydrogenase (rabbit muscle), 50 units of pyruvate kinase (rabbit muscle), 90 units of enolase (yeast), 175 units of creatine phosphokinase (rabbit muscle), and 90 units of glyceraldehyde 3-phosphate dehydrogenase (rabbit muscle). All enzymes were obtained from commercial sources (Sigma Chemical Co., St. Louis, MO) and were dialyzed against 500 ml of the same buffered salt solution as contained in the reaction mixture prior to use. Following addition of the enzymes, the reaction mixture was allowed to stir at room temperature for 2 days under an argon atmosphere. The pH of the mixture was controlled with a pH

monitor/controller which kept the pH between 7.9 and 8.1 by addition of 1 M NaOH. Phosphocreatine was precipitated as its barium salt and recrystallized as its sodium salt (13). The final yield of crystallized product was 70%.

REFERENCES

1. CHENAULT, H. K., SIMON, E. S., AND WHITESIDES, G. M. (1988) Biotechnology and Genetic Engineering Reviews (Russell, G. E., Ed.), Vol. 6, Ch. 6, Intercept, Wimborne, Dorset
2. SHIH, Y.-S., AND WHITESIDES, G. M. (1977) *J. Org. Chem.* **42,** 4165–4166.
3. CRANS, D. C., AND WHITESIDES, G. M. (1983) *J. Org. Chem.* **48,** 3130–3132.
4. WONG, C.-H., HAYNIE, S. L., AND WHITESIDES, G. M. (1983) *J. Am. Chem. Soc.* **105,** 115–117.
5. HIRSCHBEIN, B. L., MAZENOD, F. P., AND WHITESIDES, G. M. (1982) *J. Org. Chem.* **47,** 3765–3766.
6. BOLTE, J., AND WHITESIDES, G. M. (1984) *Bioorg. Chem.* **12,** 170–175.
7. SIMON, E. S., GRABOWSKI, S., AND WHITESIDES, G. M. (1989) *J. Am. Chem. Soc.* **111,** 8920–8921.
8. KAZLAUSKAS, R. J., AND WHITESIDES, G. M. (1985) *J. Org. Chem.* **50,** 1069–1076.
9. MURATA, K., TANI, K., KATO, J., AND CHIBATA, I. (1981) *Enzyme Microb. Technol.* **3,** 233–242.
10. LEHNINGER, A. L. (1970) Biochemistry, Ch. 15, Worth Publishers, New York
11. CRANE, R. K. (1962) The Enzymes (Boyer, P. D., Lardy, H. and Myrback, K., Eds.), 2nd ed., Vol. 6, pp. 47–66, Academic Press, New York
12. BLOXHAM, D. P., AND LARDY, H. A. (1973) *in* The Enzymes (Boyer, P. D., Ed.), 3rd ed., Vol. 8, pp. 239–278, Academic Press, New York
13. ENNOR, A. H., AND STOCKEN, L. A. (1957) Biochem. Prep. **5,** 9–12.