THE USE OF HEXOSE PHOSPHATES TO SUPPORT PROTEIN SYNTHESIS AND GENERATE [γ-^{32}P]ATP IN RETICULOCYTE LYSATES

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1. Introduction

Baglioni and Weber have recently reported [1] that the creatine phosphate and creatine phosphokinase, customarily used as an ATP-generating system in cell-free protein synthesis assays, can be replaced, with somewhat better results, by fructose 1,6-bisphosphate in the case of extracts of HeLa, ascites and L cells. On the other hand, reticulocyte lysates did not utilise fructose 1,6-bisphosphate efficiently, and it was suggested [1] that this difference reflected the relative importance of the glycolytic pathway in the overall metabolism of different cell types.

For some years we have been using fructose 1,6-bisphosphate as the ATP-generating system when we wished to study the phosphorylation of proteins in reticulocyte lysates incubated under protein synthesis conditions, since ATP generation through glycolysis effects a rapid equilibration of label between the phosphate and nucleotide pools when [^{32}P]phosphate is present. As we show in this paper, reticulocyte lysates are perfectly capable of utilising fructose 1,6-bisphosphate as an ATP-generating system provided NAD is added.

2. Methods

Rabbit reticulocyte lysates and gel-filtered (Sephadex G-50) lysates were prepared and used in protein synthesis assays as in [2,3]. In assays using gel-filtered lysates, final spermidine was 0.4 mM, and 2 mM glucose was added to prevent the early reduction in the rate of initiation which often occurs with gel-filtered lysates [3]. When fructose 1,6-bisphosphate was used as ATP-generating system, both creatine phosphate and creatine phosphokinase were omitted unless otherwise stated.

To monitor the incorporation of ^{32}P into nucleotides in lysates incubated with [^{32}P]phosphate, samples (1 μl) were taken at various times and spotted onto a PEI-cellulose thin layer plate which was subjected to ascending chromatography in 0.85 M potassium phosphate buffer (pH 3.5). After drying, the thin layer plate was autoradiographed to locate the spots of radioactive nucleotides which were then cut out and placed in 2 ml water for Cerenkov counting. The spots were identified by reference to unlabelled markers detected under ultraviolet light.

3. Results and discussion

The generation of ATP through the glycolytic pathway requires sufficient NAD to potentiate the glyceraldehyde 3-phosphate dehydrogenase step. Our previous work suggested that NAD pools in reticulocyte lysates are very small, since the sensitivity of protein synthesis to inhibition by diphtheria toxin is rather low unless NAD, the cofactor for toxin action [4], is added. In attempts to establish a system in which glycolysis is the means of ATP-generation we therefore tested various combinations of added NAD and either fructose 1,6-bisphosphate or glucose 6-phosphate. Sodium pyruvate was added at 0.5 mM on the basis that it might accelerate the regeneration of NAD from reduced NAD. In fact it made no more than a marginal difference, but since it is not inhibitory it is routinely included.

Efficient protein synthesis in normal (i.e., not gel-filtered) lysates was almost completely dependent on the addition of NAD. If fructose 1,6-bisphosphate is added without NAD, the rate of protein synthesis is scarcely higher than when no ATP-generating system is present (fig.1). The addition of NAD alone, without added hexose phosphates, allowed a short burst of protein synthesis at a rate almost as high as that obtainable using creatine phosphate and creatine phosphokinase (fig.1). This short burst is presumably driven by the metabolism of endogenous glycolytic intermediates, since gel-filtered lysates showed no such burst (fig.2). The addition of fructose 1,6-bis-

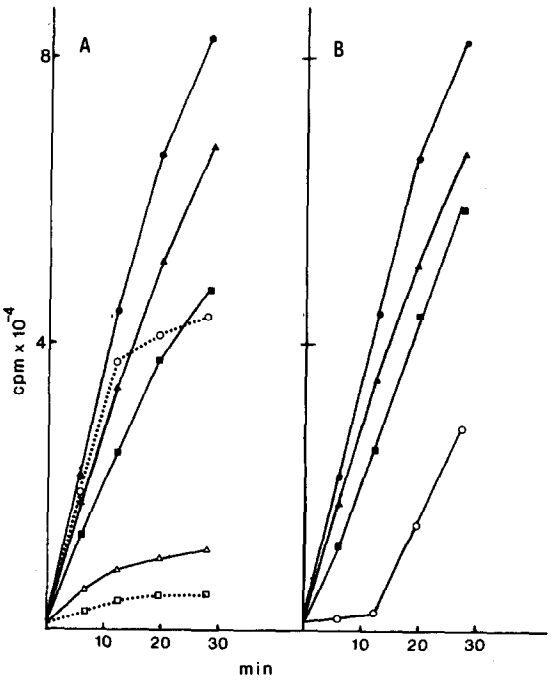

Fig.1. Time courses of protein synthesis (5 μl samples) in lysates incubated with [^{35}S]methionine (100 μCi/ml final conc.) at 30°C with different ATP-generating systems. The two panels relate to the same experiment. The ATP-generating systems used were: none (□. . . .□); 2 mM fructose 1,6-bisphosphate in the absence of NAD (△——△); 0.5 mM NAD and 0.5 mM sodium pyruvate (○. . . .○); and 10 mM creatine phosphate with 50 μg/ml creatine phosphokinase ((●——●), the same curve plotted in each panel). All other assays contained 0.5 mM NAD and 0.5 mM sodium pyruvate, and either fructose 1,6-bisphosphate (A) at 1.5 mM (▲——▲) and 4 mM (■——■), or glucose 6-phosphate (B) at 1 mM (▲——▲), 2 mM (■——■), and 4 mM (○——○).

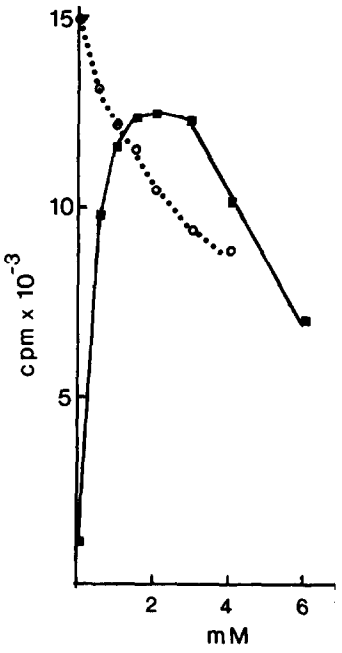

Fig.2. The efficiency of protein synthesis in gel-filtered lysates incubated with varying concentrations of fructose 1,6-bisphosphate and either 0.5 mM NAD and 0.5 mM sodium pyruvate (■——■), or 10 mM creatine phosphate with 50 μg/ml creatine phosphokinase (○. . . .○). [^{3}H]Leucine (200 Ci/mol) was used at 80 μM final conc. and samples (5 μl) were taken after 40 min incubation at 30°C. Samples taken after 12 min incubation gave curves almost identical in shape to those shown.

phosphate together with NAD allows more sustained protein synthesis but at the expense of a decrease in rate which is more severe the higher the concentration of fructose 1,6-bisphosphate (fig.1). The inhibitory effect of the sugar phosphate implied by this observation is also seen in situations where its presence is redundant because creatine phosphate and creatine phosphokinase serve as the ATP-generating system (fig.2). Attempts to overcome this inhibition by varying Mg^{2+} concentration were unsuccessful.

We therefore recommend the addition of 0.5 mM NAD, 0.5 mM sodium pyruvate, and the minimum level (usually 1.5–2.0 mM) of fructose 1,6-bisphosphate to sustain protein synthesis for the duration of the experiment. A lower concentration of NAD (0.1 mM) was found to be sufficient in most

lysates, but since higher levels are not inhibitory we routinely use 0.5 mM. These conditions were also found to be optimal when gel-filtered lysates were used. The only difference caused by gel-filtration is to reduce or eliminate the short burst of protein synthesis effected by NAD in the absence of hexose phosphates (fig.2). We have found no stimulation of protein synthesis on adding aldolase, pyruvate kinase or other glycolytic enzymes, so the endogenous enzyme levels must be more than sufficient.

Figure 1 shows that low concentrations of glucose 6-phosphate served as a suitable ATP-generating system (in the presence of NAD). High concentrations were again less effective, but this differs from the inhibitory effect of fructose 1,6-bisphosphate and is due to a lag period before protein synthesis starts at its maximum rate (fig.1). When the nucleotide and phosphate pools were analysed by the methods described below, 4 mM glucose 6-phosphate was found to cause severe depletion of the ATP and phosphate pools, most probably as a result of the formation of fructose 1,6-bisphosphate. As would be expected, the lag can be reduced or eliminated by the addition of phosphate, but since this prejudices the study of the labelling of phosphoproteins, and since phosphate itself is slightly inhibitory to protein synthesis, we do not recommend the use of glucose 6-phosphate except in short-term experiments when low concentrations are sufficient.

We have had no success using glucose instead of hexose phosphates. Although the ability of glucose to promote the reduction of oxidised glutathione [5] argues that it can be converted to glucose 6-phosphate in the lysate, either the rate of phosphorylation of glucose is too low to sustain reasonable rates of protein synthesis, or there may again be problems over depletion of the ATP and phosphate pools.

The advantage of the glycolytic system is that in the presence of [^{32}P]phosphate labelled nucleotides are generated, since the γ-phosphate of ATP is assimilated from the phosphate pool at the glyceraldehyde 3-phosphate dehydrogenase/3-phosphoglycerate kinase steps. Analysis of the nucleotide pools at various stages during the incubation shows that even the zero time sample exposed to [^{32}P]phosphate for a few seconds at 0°C shows partial equilibration of label, and that equilibration is complete within 1 min incubation (fig.3). The radioactivity in the

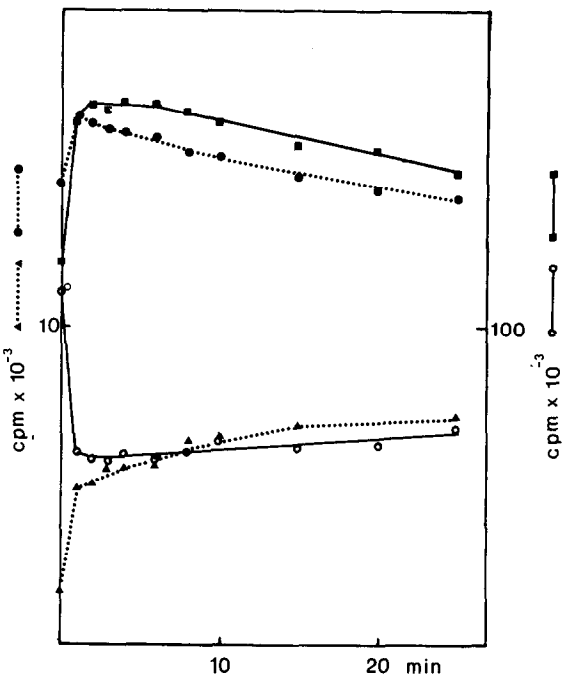

Fig.3. Labelling of the pools of phosphate (○——○), ATP (■——■), ADP (▲. . . .▲) and GTP (●. . . .●) in lysates incubated with 2 mM fructose 1,6-bisphosphate, 0.5 mM NAD, 0.5 mM sodium pyruvate and 0.2 mCi/ml carrier-free [^{32}P]phosphate at 30°C under protein synthesis conditions. Note that the results for ADP and GTP are plotted on a different scale from those relating to ATP and phosphate.

nucleotides declines gradually during further incubation, probably as the phosphates of the added fructose 1,6-bisphosphate are assimilated into the ATP pool at the pyruvate kinase step. Experiments using [^{3}H]ATP and [^{3}H]GTP showed that the pool sizes of ATP, ADP and GTP did not change appreciably during the incubation (data not shown), so that the ^{32}P-labelling can be taken as a measure of the specific activities of the various nucleotides. The fact that the small ADP pool becomes labelled (fig.3) implies that ^{32}P is assimilated into the β-phosphate of ATP: since the AMP pool in lysates incubated under these conditions is extremely small (data not shown), the AMP produced in the aminoacyl-tRNA synthetase reactions must be rapidly reconverted into ATP, and this would result in the incorporation of ^{32}P into the β-phosphate of ATP. The label in ATP is therefore likely to be equally divided between the β- and γ-phosphates.

Baglioni and Weber [1] found that fructose 1,6-bisphosphate (in the absence of added NAD) serves as an efficient ATP-generating system in extracts from several types of tissue culture cells, but not in reticulocyte lysates. They speculated that this difference reflected the paramount importance of the glycolytic pathway in the overall metabolism of tumour cells in contrast to reticulocytes. Our results show that the distinction seems to lie entirely in the size of the NAD pool or the overall availability of NAD, and that reticulocyte lysates have the full potential to generate ATP through glycolysis. Although the rate of protein synthesis in the presence of 2 mM fructose 1,6-bisphosphate, 0.5 mM NAD and 0.5 mM sodium pyruvate is only 70–80% that obtained using creatine phosphate and creatine phosphokinase, this lower rate is not due to an inadequacy of glycolysis as an ATP-generating system but to an inhibitory effect of the hexose phosphate. The regulation of initiation in response to haem deficiency, double-stranded RNA and other effectors [3] is essentially the same whichever ATP-generating system is used (data not shown). Since [^{32}P]ATP of more or less constant specific activity can be generated using the glycolytic system, it has the advantage of allowing the phosphorylation of lysate proteins to be studied under conditions in which protein synthesis takes place.

Acknowledgements

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References

[1] Baglioni, C. and Weber, L. A. (1978) FEBS Lett. 88, 37–40.
[2] Balkow, K., Hunt, T. and Jackson, R. J. (1975) Biochem. Biophys. Res. Commun. 67, 366–375.
[3] Farrell, P. J., Balkow, K., Hunt, T., Jackson, R. J. and Trachsel, H. (1977) Cell 11, 187–200.
[4] Collier, R. J. (1967) J. Mol. Biol. 25, 83–98.
[5] Kosower, N. S., Vanderhoff, G. A., Benerofe, B., Hunt, T. and Kosower, E. M. (1971) Biochem. Biophys. Res. Commun. 45, 816–821.