REGULATION OF *Escherichia coli* PHOSPHOFRUCTOKINASE *in situ*

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SUMMARY. The activity of *E. coli* phosphofructokinase *in situ* has been studied in cells permeabilized to its substrates, products and effectors by a toluene-freezing treatment. The *in situ* enzyme exhibits moderate cooperativity in respect to F6P (n_H up to 2.0), rather low affinity for ATP (with Km up to 1 mM when saturated with F6P), activation by ADP, and inhibition, within the physiological range of concentrations, by high ATP and phosphoenolpyruvate. This behaviour of the enzyme *in situ* at concentrations of the effector metabolites as those reported in intact cells in glycolytic and gluconeogenic conditions could account for the changes of phosphofructokinase activity needed for metabolic regulation *in vivo*.

The great development in the knowledge of mechanisms of modulation of enzyme activities, particularly through allosteric effects, makes it very important to ascertain to what extent enzyme modulation as observed *in vitro* can have a significant counterpart in metabolic regulation *in vivo*. One of the factors in which assay conditions *in vitro* tend to be markedly different from those *in vivo* is the concentration of the enzyme itself. Phosphofructokinases(EC 2.7.1.11) are key regulatory enzymes in which a variety of potentially regulatory effects have been reported. Moreover, the concentration of the enzyme markedly affects the degree of aggregation (1, 2) and allosteric properties (3, 4) in preparations from eukaryotic organisms. The *E. coli* enzyme has been isolated and studied by Atkinson and Walton (5) and Blangy, Buc and Monod (6). Their results point to the positive effector ADP, the negative effector phosphoenolpyruvate (PEP), the cooperative substrate fructose-6-P (F6P) and possibly the negative effector ATP as likely to be involved in the metabolic regulation of this enzyme.

We have succeeded in revealing the activity of the phosphofructokinase in *E. coli* by a method which makes it directly accessible to external substrates while leaving it within a damaged cell membrane (or at least within

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the cell wall), conditions which we call in situ. In principle the in situ enzyme approximates the physiological condition of the enzyme more closely than does enzyme obtained from cell homogenates, particularly with respect to enzyme concentration and possible interactions with other cellular proteins. The present report deals with the regulation of the activity of in situ phosphofructokinase by the concentrations of its substrates and known allosteric effectors.

EXPERIMENTAL. E. coli (K12, strain 3300, lac$^-$, obtained from Dr. C. Asensio) is grown aerobically at 30° in minimal medium (7) containing 20 mM glucose or 40 mM glycerol. Cells are harvested at the end of exponential growth. Cell concentration, in mg dry wt. cells/ml is taken to equal 0.68 × absorbancy at 680 nm in a cuvette of 1 cm optical path (7). The sedimented cell pellet is washed once in a buffer prepared by adjusting a solution of KH_2PO_4 to pH 7.2 with NaOH, adding a calculated amount of $MgCl_2$, and diluting to a final concentration of 0.1 M phosphate and 0.005 M $MgCl_2$. The washed cells are resuspended in buffer to a concentration of 1 mg dry wt. cells/ml, chilled, briefly treated with one hundredth volume of 10% (v/v) toluene in abs. ethanol as described previously (8), and frozen in conveniently small lots in a -20° freezer.

The nucleotides, sugar phosphates, and the sodium salt of PEP were from Sigma. The auxiliary enzymes were from Boehringer; for use, the suspensions in ammonium sulfate were centrifuged, the excess supernatant fluid was removed, and the sediment was dissolved in 1 mM EDTA, pH 7.0.

For the standard enzyme assay the cuvettes contain in a volume of 0.39 ml: 40 μmoles imidazole-HCl buffer, pH 6.8; 2 μmoles $MgCl_2$; 40 μmoles KCl; 0.8 μmoles ATP; 0.05 μmole NADH; 0.5 units of each, aldolase, glycerol-3-P dehydrogenase, and triosephosphate isomerase; water; and 10 μl of the cell suspension. After thermal equilibration 10 μl water is added to a control cuvette and, in the experimental cuvettes, reaction is initiated by adding 10 μl of 60 mM F6P. The absorbancies at 340 nm are recorded at 30° in a Gilford spectrophotometer equipped with a thermostated cell compartment. The reaction rate is linear with time until the NADH is consumed. After correction for the small NADH oxidase activity exhibited by the control cuvette the reaction rate is expressed as nmoles FDP formed per min per mg dry wt cells, taking into consideration that one mole of FDP results in the oxidation of two moles of NADH. Identical reaction rates were observed when the cuvettes contained 5-25 μg dry wt cells. In some instances an ATP-regenerating system (2) was added.

RESULTS. The decryptification procedure revealed, in glucose-grown cells, activity amounting to 160 nmoles FDP formed/min/mg dry wt cells when determined by the standard enzyme assay. For glycerol-grown cells the corresponding value was 85 nmoles/min/mg dry wt cells. Only a fraction of these activities were revealed by the toluene treatment alone.

The remainder became evident after the freezing and thawing step. Inverting the order of toluene and freezing treatments revealed little of the activity. The use of greater amounts of toluene gave enzyme which, on some occasions, was not sedimented completely with the cells. No more enzyme activity was obtained in sonic homogenates prepared from intact cells.

The activity of the enzyme in glucose-grown cells was explored as a function of F6P concentration, both in the absence and in the presence of allosteric effectors. For this work F6P was provided by the action of an excess of glucosephosphate isomerase in the cuvettes acting on added glucose-6-P (G6P). Some of the results of these experiments are shown in Fig. 1. Curve 1 of this figure shows the essentially hyperbolic curve obtained in the presence of the positive effector GDP, an analogue of ADP as allosteric effector and not a substrate for adenylate kinase. In other experiment (not shown) the effect of ADP was observed to be similar to that of GDP. Curve 2 shows the sigmoid relationship which is obtained in the absence of allosteric effectors. The Hill coefficient for this curve is $n_H = 2$. Blangy et al. (6) had found a Hill coefficient of 3.8 for the purified enzyme in the absence of effectors. Curves 3 and 4 depict the increase in $S_{0.5}$ for F6P produced by increasing concentrations of the negative effector, PEP.

Fig. 2 shows the activity of the in situ enzyme as a function of the concentration of ATP. In this experiment the concentration of F6P was low, 0.2 mM. Curves of this general shape were also obtained with GTP as phosphoryl donor. The reciprocal plot (insert), gives an apparent Km for ATP of 0.4 mM. At greater F6P concentrations the apparent Km for ATP increased to 1 mM. The Vmax was greater when ATP was the donor, but the Km for GTP was approximately the same under the conditions tested.

We next explored the effects of substrates and effectors on enzyme activity in concentrations which simulated in vivo growth conditions. For this work we selected, from the data presented by Lowry et al. (9), metabolite concentrations approximately corresponding to four different growth conditions: growth on glucose-NH_3, on glucose-glycine, on succinate-NH_3 and on glycerol-NH_3. In the first two cases the cells are

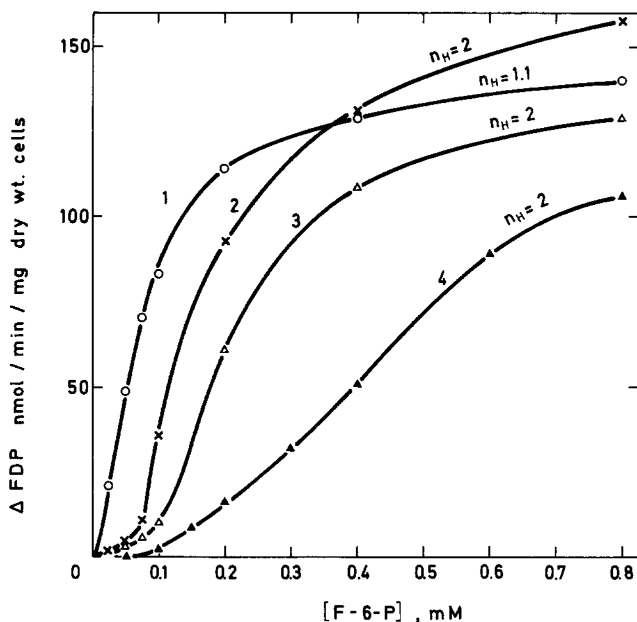

Fig. 1. The influence of F6P and allosteric effectors on the activity of E. coli phosphofructokinase in situ. All cuvettes contained the imidazole buffer, KCl, $MgCl_2$, ATP, NADH, and the auxiliary enzymes as specified in the standard assay (see Experimental section), plus 1 unit glucosephosphate isomerase and G6P at 4 times the concentration indicated for F6P on the abscissa in a volume of 0.39 ml. After equilibration for 10 min at 30° reaction was initiated by the addition of glucose-grown cells which had received the standard decryptification treatment (10 μg dry wt cells in 0.01 ml of the phosphate-$MgCl_2$ buffer). The control cuvette lacked added G6P. Conditions of monitoring the rate of reaction were as specified for the standard assay. Curve 1; cuvettes contained 1 mM GDP. Curve 2; cuvettes contained 1 mM creatine-P and 0.5 unit creatine kinase. Curves 3, 4; cuvettes contained 0.2 and 0.8 mM PEP, respectively. The calculated Hill coefficient (n_H) for each curve is indicated in the Figure.

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metabolizing, principally, via the glycolytic pathway, while in the last two cases the cells must depend upon gluconeogenesis to produce the hexose residues essential to growth. For each set of metabolic concentrations enzyme activity was determined at 4 different concentrations of added Mg^{2+}, ranging from 0.5 to 5.0 mM. The results of the above experiments are presented in Fig. 3.

DISCUSSION. The phosphofructokinase activity studied in situ exhibits kinetic properties distinct from those found by prior investigators (5, 6)

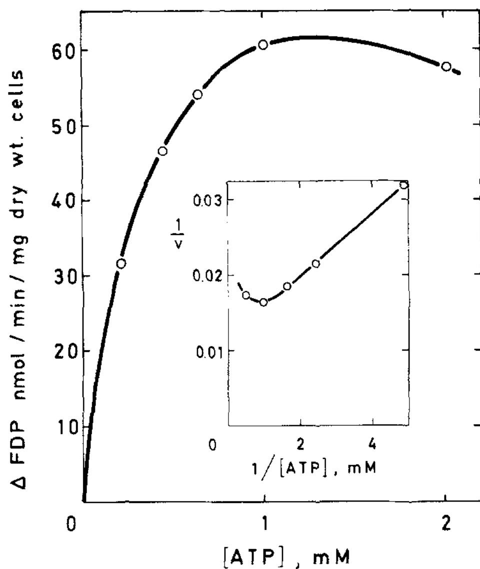

Fig. 2.

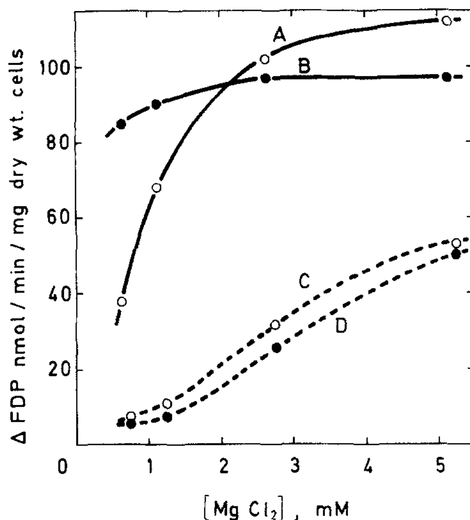

Fig. 3.

Fig. 2. The influence of ATP concentration on the activity of E. coli phosphofructokinase in situ. All cuvettes contained the imidazole buffer, KCl, $MgCl_2$, NADH, and the auxiliary enzymes as specified for the standard assay (see Experimental section). In addition each contained 0.8 mM G6P, 1.0 mM creatine-P, 1 unit glucosephosphate isomerase, 1 unit creatine kinase, and ATP as indicated in a volume of 0.39 ml. After equilibration for 10 min at 30° reaction was initiated by the addition of glucose-grown cells which had received the standard decryptification treatment. The insert shows a reciprocal plot of the data.

Fig. 3. The activity of E. coli phosphofructokinase in situ under conditions simulating glycolytic or gluconeogenic metabolism. Each cuvette contained in a final volume of 0.40 ml: 40 μmoles imidazole-HCl buffer, pH 6.8; 40 μmoles KCl; 0.5 μmole NADH; 0.5 units of each, aldolase, glycerol-3-P dehydrogenase, triosephosphate isomerase, and glucose-phosphate isomerase; glucose-grown cells which had received the standard decryptification treatment; and $MgCl_2$ as indicated on the abscissa. In addition cuvettes of Curve A contained 2.4 mM ATP, 0.8 mM ADP, 0.1 mM PEP and 0.35 mM F6P (calculated as one-fourth the concentration of added G6P). Curve B: 1.2 mM ATP, 0.25 mM ADP, 0.1 mM PEP, and 0.45 mM F6P. Curve C: 2.8 mM ATP, 1.2 mM ADP, 1.0 mM PEP, and 0.25 mM F6P. Curve D: 2.2 mM ATP, 1.0 mM ADP, 0.3 mM PEP, and 0.1 mM F6P. In all instances reaction was initiated by adding the cells after a 10 min period of equilibration at 30°. The controls lacked F6P (G6P). The conditions for curves A-D were chosen to simulate, approximately, the substrate and effector concentrations in: (A) cells grown on glucose-NH_3, (B) on glucose-glycine, (C) on succinate-NH_3, and (D) on glycerol-NH_3. These metabolite concentrations are based on the data of Lowry et al. (9).

with enzyme obtained by sonic disruption of the cells. In the absence of allosteric effectors the Hill coefficient reflecting substrate cooperativity is markedly smaller for the *in situ* enzyme, suggesting important differences in protein interactions for *E. coli* phosphofructokinase *in situ* and *in vitro*. The Km for ATP is approximately one order of magnitude greater for the *in situ* enzyme and it is no longer independent of F6P concentrations. The effect of the concentration of F6P on the Km for the nucleosidetriphosphate substrate suggests for *E. coli* phosphofructokinase *in situ* the possibility of a ping-pong mechanism, as seems to be the case for the enzyme from several eukaryotic organisms (1).

It is noted that with concentrations of substrates and the allosteric effectors PEP and ADP corresponding to glycolytic metabolism, the *in situ* enzyme displays sufficient activity to account for the flow of glucose carbon along the glycolytic pathway. Furthermore, at concentrations of the above metabolites corresponding to growth on gluconeogenic carbon sources the *in situ* enzyme activity is greatly reduced, particularly so at low Mg^{2+} concentrations, which probably reflect more accurately those available in the living cell (10). After taking into account the lower maximal activity of the enzyme in gluconeogenic cells (glycerol-grown cells) it is evident that actual phosphofructokinase activity might be, in these cells, as little as about 5% of that obtained in cells grown on glucose. Thus, it seems that the *in situ* enzyme can be well regulated by physiological concentrations of its substrates and its two best characterized allosteric effectors.

The marked effect of changes in activity when varying added Mg^{2+} around the millimolar range in three out of the four sets of metabolite concentrations tested may be related to the fact that in these cases there was a significant excess of ATP. This observation is consistent with the report by Atkinson and Walton (5) that *E. coli* phosphofructokinase can be feedback inhibited by ATP.

Blangy *et al*. (6) have reported kinetic parameters for their purified enzyme which permit the calculation of the fraction of total enzyme activity expressed at any particular concentration of F6P, ADP, and PEP, provided ATP is saturating (Km 0.06 mM for the purified enzyme) and sufficient Mg^{2+} is present to fully complex the ATP. This last condition

would be approximately fulfilled in those experiments of Fig. 3 having 5 mM added $MgCl_2$. Calculations based on these parameters for the concentrations of effector metabolites in glycolytic and gluconeogenic conditions here tested lead to the anomalous conclusion that phosphofructokinase would operate essentially at its Vmax in the two conditions.*

Mansour had recently ended a review on phosphofructokinase indicating that "future advances in our knowledge of the regulation of phosphofructokinase will have to await studies on the nature of the enzyme in the resting cell" (11). The present work fulfills a substantial part of this objective. The difference between calculated values for purified enzyme regulation and our findings concerning in situ enzyme regulation serves to emphasize that whenever there is a possibility to study a regulatory enzyme in situ the opportunity should be seized as a means to improve understanding of the function of the enzyme in metabolic regulation in the living cell.

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*A different conclusion was reached in a recent publication (9). We have received a personal communication from one of the authors of that publication (L.G.) which notes that the reported calculations are in error.

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