Rose, I. A. (1971) *Exp. Eye Res. 11*, 264–272.
Saez, M. J., & Lagunas, R. (1976) *Mol. Cell. Biochem. 13*, 73–78.
Salas, M. L., Vinuela, E., Salas, M., & Sols, A. (1965) *Biochem. Biophys. Res. Commun. 19*, 371–376.
Schatz, G. (1965) *Biochim. Biophys. Acta 96*, 342–345.
Serrano, R., & DelaFuente, G. (1974) *Mol. Cell. Biochem. 5*, 161–171.
Sols, A. (1967) in *Aspects of Yeast Metabolism* (Mills, A. K., & Krebs, H. A., Eds.) pp 47–66, Blackwell Scientific Publications, Oxford.
Sols, A. (1976) in *Reflections on Biochemistry* (Kornberg, A., Horecker, B. L., Cornudella, L., & Oro, J., Eds.) pp 199–206, Pergamon Press, Oxford.
Sols, A. (1981) *Curr. Top. Cell. Regul. 19*, 77–101.
Sols, A., Gancedo, C., & DelaFuente, G. (1971) in *The Yeasts* (Rose, A. H., & Harrison, J. S., Eds.) Vol. 2, pp 271–307, Academic Press, London.
Stickland, L. H. (1956a) *Biochem. J. 64*, 498–503.
Stickland, L. H. (1956b) *Biochem. J. 64*, 503–515.
Tejwani, G. A. (1978) *Trends Biochem. Sci. (Pers. Ed.) 3*, 30–33.
Tortora, P., Birtel, M., Lenz, A. G., & Holzer, H. (1981) *Biochem. Biophys. Res. Commun. 100*, 688–695.
Vary, M. J., Edwards, C. L., & Stewart, P. R. (1969) *Arch. Biochem. Biophys. 130*, 235–243.
Vinuela, E., Salas, M. L., & Sols, A. (1963) *Biochem. Biophys. Res. Commun. 12*, 140–145.
Wales, D. S., Cartledge, T. G., & Lloyd, D. (1980) *J. Gen. Microbiol. 116*, 93–98.
Weibel, K. E., Mor. J.-R., & Fiechter, A. (1974) *Anal. Biochem. 58*, 208–216.

Studies on the Regulation of Yeast Phosphofructo-1-kinase: Its Role in Aerobic and Anaerobic Glycolysis†

D. Reibstein,‡ J. A. den Hollander,‡,§ S. J. Pilkis,‖ and R. G. Shulman*,‡
Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, Connecticut 06511, and Vanderbilt University School of Medicine, Nashville, Tennessee 37235
Received October 12, 1984; Revised Manuscript Received July 26, 1985

ABSTRACT: The kinetics of yeast phosphofructo-1-kinase has been studied in vitro. Effector concentrations (Fru-6-P, ATP, ADP, AMP, P_i, Fru-1,6-P_2, and Fru-2,6-P_2) and pH were adjusted so as to mimic intracellular concentrations in yeast. Under these conditions we were able to reproduce the measured in vivo rate of PFK. In addition, by reconstituting the intracellular conditions existing during aerobic and anaerobic glycolysis, we were able to reproduce in vitro the changes in the rate of PFK observed under these conditions. Without the addition of the newly discovered effector Fru-2,6-P_2, in vitro rates of PFK are much lower than its in vivo rate. Changes in Fru-2,6-P_2, Fru-1,6-P_2, ATP, AMP, P_i, and pH in going from aerobic to anaerobic conditions all contributed somewhat to the change in the rate of PFK observed during the Pasteur effect, with no contribution coming from ADP. These studies show that the control of PFK under the condition of the Pasteur effect cannot be ascribed to changes in any one particular effector but rather to contributions from a variety of effectors. Also, the *net* change in the rate of PFK in the switch from anaerobic to aerobic glycolysis is small compared with the change in its dependence upon its substrate Fru-6-P, indicating a compensation mechanism.

Phosphofructo-1-kinase (PFK)[1] has been implicated as an important control point in the glycolytic pathway (Passonneau & Lowry, 1962, 1963; Vinuela et al., 1963; Tejwani, 1978; Banuelos et al., 1977; Sols, 1976; Ramaiah, 1974; Chance, 1959), in particular in explanations of the Pasteur effect (Krebs, 1972; Racker, 1974; Rose, 1971; Sols et al., 1971). The activity of PFK from yeast is known to be altered in vitro by many physiologically relevant effectors. Among these are ATP, ADP, AMP, NH_4^+, fructose 1,6-bisphosphate (Fru-1,6-P_2), fructose 2,6-bisphosphate (Fru-2,6-P_2), pH, P_i, and possibly citrate (Banuelos et al., 1977; Atzpodien & Bode, 1970; Hess, 1973; Ghosh & Chance, 1964; Moore et al., 1965; Betz & Chance, 1965a,b; Hofmann & Kopperschlaeger, 1982; Salas et al., 1965). In addition, PFK shows sigmoidal kinetics with respect to its substrate Fru-6-P (Sols, 1981). It is clearly possible that any of these effectors might be important in changing the activity of PFK in response to changing environmental and metabolic conditions. In fact, the literature abounds in claims that one or another of these effectors is *the* controller of PFK activity, and there is a history of conflict between proponents of different effectors.

Two questions emerge from these facts: First, why are there so many effectors of PFK? Second, how important is each of these effectors in vivo? In order to answer these questions, at least two requirements must be satisfied: (1) the in vivo

† The work done at Yale University was supported by National Institutes of Health Grant AM 27121.
‡ Yale University.
§ Present address: Philips Medical Systems, 5600 MD Eindhoven, The Netherlands.
‖ Vanderbilt University School of Medicine.

[1] Abbreviations: Fru-6-P, fructose 6-phosphate; ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate; P_i, inorganic phosphate; Fru-1,6-P_2, fructose 1,6-bisphosphate; Fru-2,6-P_2, fructose 2,6-bisphosphate; PFK, phosphofructokinase; NADH, reduced nicotinamide adenine dinucleotide; PMSF, phenylmethanesulfonyl fluoride; PIPES, 1,4-piperazinediethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; G6P, glucose 6-phosphate.

0006-2960/86/0425-0219$01.50/0

concentrations of all relevant effectors and substrates must be accurately known for a given set of metabolic states, and (2) the in vitro rate of enzyme catalysis measured in the presence of these known concentrations must equal the in vivo rate.

It is well established that the response of PFK to many of its effectors depends critically on the concentrations of the other effectors [for review, see Sols (1981)]. Therefore, in order to establish the physiological importance of each effector, it is necessary to duplicate in vivo conditions during in vitro assays. Although several measurements of intracellular metabolites and changes in concentrations under the conditions of the Pasteur effect have been reported (Holzer et al., 1958; Holzer & Freytag-Hilf, 1959; Holzer, 1961; Lynen et al., 1959), there was a need for a comprehensive set of measurements. We recently made ^{31}P and ^{13}C NMR measurements of the concentrations of many metabolites in the glycolytic pathway (den Hollander et al., (1985a,b) and of the intracellular pH (den Hollander et al., 1981) in anaerobically and aerobically glycolyzing yeast grown under a variety of conditions. We have thus satisfied the first of the two requirements set out above.

The second requirement, that in vitro rates be compared with those in vivo, is the subject of this paper. Our NMR work (den Hollander et al., 1985a,b) measured yeast glycolytic rates in the presence or absence of oxygen (the Pasteur effect), demonstrating that they depend critically on previous cell growth conditions and on the conditions of measurement. In those studies we established sets of well-defined conditions for measuring glycolytic rates and made estimates of the rate through PFK. In this paper we focus on one set of conditions, namely, glucose-grown yeast depleted of glucose and refed glucose for a relatively short period in a minimal medium.

The question of matching the in vitro rate of PFK to the in vivo glycolytic rate has previously been addressed by Banuelos et al. (1977). They noted that in previous publications by other authors the in vitro PFK rate was always at least 2 orders of magnitude lower than the in vivo glycolytic rate under conditions thought to simulate those found in vivo. They reported that P_i is a stimulator of PFK below pH 7.5, in contrast to its inhibitory behavior at higher pH (Atzpodien & Bode, 1970). In the presence of the concentrations of P_i, ATP, ADP, AMP, and Fru-6-P that they had measured chemically in cell extracts and accepting an intracellular pH of 6.4 [which had been measured in deenergized yeast by Salhany et al. (1975)], Banuelos et al. obtained an in vitro PFK rate that was equal to the in vivo glycolytic rate.

However, our later ^{31}P NMR measurements on intact energized yeast cells under similar growth conditions (Navon et al., 1979; den Hollander et al., 1981) were in disagreement with those of Banuelos et al. in at least three respects. We found that (a) in the presence of an adequate supply of glucose the pH in cells is considerably higher than 6.4, ranging from about 7.0 to about 7.5, depending on growth conditions and whether oxygen is present, (b) intracellular ATP concentrations are of the order of 3 mM rather than 1.5 mM, and (c) during aerobic glycolysis the concentration of AMP (derived on the assumption of adenylate kinase equilibrium) is only one-fourth that measured by Banuelos et al. When these factors are taken into account, the in vitro PFK rate should be almost 2 orders of magnitude lower than that found by Banuelos et al. because ATP inhibits and AMP activates PFK and because the higher pH values we found are well above the pH optimum of the enzyme. Hence, there remained a large discrepancy between in vitro and in vivo rates.

Recently, Fru-2,6-P_2 has been discovered to be a potent stimulator of PFK (Hers & Van Schaftingen, 1982; Clifton & Fraenkel, 1983). The presence of this compound in liver, muscle, and yeast (Lederer et al., 1981; Furuya et al., 1982) has been firmly established, as has its effect on PFK from each of these sources. Avigad (1981) and Bartrons et al. (1982) have shown that yeast PFK is greatly stimulated by micromolar concentrations of Fru-2,6-P_2, and Kessler et al. (1982) and Nissler et al. (1983) have studied its effect on yeast PFK in the presence of AMP. The relative amounts of Fru-2,6-P_2 in anaerobic and aerobic glucose-growing yeast in the logarithmic phase were measured by Furuya et al. (1982). They found that the concentration of this effector is about 4 times higher under anaerobic conditions than it is aerobically. These results suggest that Fru-2,6-P_2 might be the missing effector that would resolve the discrepancy between in vivo and in vitro rates and that it might be involved in changing this rate in response to the presence or absence of oxygen. In the present work, we have measured Fru-2,6-P_2 concentrations in cells grown and incubated under the same conditions as in our NMR studies.

In light of the above-cited new information on the in vivo concentrations of PFK effectors, we have reinvestigated the in vitro activity of PFK. In this paper we quantitatively assess the contribution of each effector to the change in PFK properties brought about by the presence or absence of oxygen.

Materials and Methods

Growth of Yeast. *Saccharomyces cerevisiae* strain NCYC 239 was grown on 2% glucose to saturation (24 h at 30 °C) and harvested as previously described (den Hollander et al., 1981). These cells are glucose-derepressed (Mahler et al., 1981).

Preparation of Crude Enzyme Extract. We initially extracted the enzyme by the method of Banuelos et al. (1977). Later extractions, as pointed out under Results, were performed as follows (all operations carried out at 4 °C). After harvesting, the yeast cells were washed twice with cold deionized water. They were transferred to 17 × 150 mm test tubes (1 g of wet yeast per tube), to each of which was added 5 g of glass beads (0.45–0.50 mm, B. Braun Melsungen AG) and 2 mL of 40 mL 2-(*N*-morpholino)ethanesulfonic acid (MES)/KOH buffer, pH 6.4, containing 0.1 M KCl, 1 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 2 mM Fru-6-P, 50 mM potassium phosphate, and 1 mM phenylmethanesulfonyl fluoride (PMSF). The cells were disrupted by vortexing at top speed in a desk-top vortexer for four 30-s periods, the tubes being cooled on ice between these periods. The supernatant was poured off, and the beads were washed with the same buffer (3 mL per tube). Cell debris and glass beads were removed by centrifugation at 27000*g* for 20 min. The yield of protein was approximately 60 mg/g of wet yeast.

Partial Purification of PFK. The following procedure is based upon the methods of Banuelos et al. (1977) and of Stellwagen & Wilgus (1975). To the crude extract was added a protamine sulfate solution (Sigma grade X from salmon), pH 6.4, containing 1 mM PMSF, to a final concentration of 0.13 mg of protamine sulfate/mg of protein. The resulting precipitate was removed by centrifugation at 27300*g* for 20 min, and the supernatant was subjected to ammonium sulfate fractionation. Protein precipitating between 35 and 55% saturation was redissolved in a minimal volume of 40 mM MES/KOH buffer, pH 6.8, containing 0.1 M KCl, 1 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 25 mM potassium phosphate, and 0.2 mM ATP. This was applied to a 2.5 × 25 cm column of Sephadex G-200 (Pharmacia) equilibrated with the

same buffer. Upon elution with this same buffer, a sharp peak of PFK activity appeared just after the void volume, as predicted for the reported molecular weight of ca. 8×10^5 (Kopperschlaeger et al., 1977).

There was a substantial tail of PFK activity after the main peak, containing slightly less than half the total activity recovered. In different preparations we pooled either all fractions showing significant PFK activity or only those in the main peak of activity. There was no apparent difference between the properties of these two types of pool. The pooled fractions were assayed immediately by method I (see below) at pH 7.2 in the presence of 1.4 mM Fru-6-P, 3.6 mM ATP, and 5 μM Fru-2,6-P_2. The pool consisting of only the fractions in the main peak had a specific activity of about 7.2 μmol min^{-1} (mg of protein)$^{-1}$ [protein assayed by the method of Lowry et al. (1951)], representing a 13-fold purification with a 27% yield relative to the crude extract. Pooled fractions were divided into small portions and stored at −15 °C. There was no detectable ATPase activity in either type of pool.

Assay of PFK. Two assay methods were used in the course of this work. Method I is that of Racker (1947), in which the production of Fru-1,6-P_2 is followed. The reaction mixture, in a quartz cuvette of 1-cm path length, contained 0.2 mM NADH, 2.7 units/mL aldolase, 48 units/mL triosephosphate isomerase, 4.7 units/mL α-glycerophosphate dehydrogenase, 50 mM PIPES, 0.1 mM KCl, 8 mM $MgCl_2$, 10 mM NH_4Cl, 2.5 mM 2-mercaptoethanol, and varying concentrations of Fru-6-P, ATP, and other additions as described in the text and figure legends. The pH of the assay was controlled in the following way. Substrates and effectors were dissolved in a PIPES buffer at pH 6.9 having the same composition as given above (except for Fru-2,6-P_2, which was dissolved in 5 mM glycine at pH 9). Aliquots of each of these were added to a PIPES buffer/salts mixture having a pH that we had empirically determined would yield the desired final pH. The actual pH of the assay mixture was measured with a Corning Model 135 pH meter. There was no change in pH during the course of the assay.

The reaction was initiated by the addition of sufficient PFK to produce a decrease of absorbance at 340 nm of 0.1–0.4/min, the range over which the rate was found to be proportional to enzyme concentration. The absorbance change was followed in a Bausch and Lomb Spectronic 2000 recording spectrophotometer at ambient temperature. An extinction coefficient of 6.22 mM^{-1} cm^{-1} for NADH was used. Most of the data presented are the averages of at least two determinations. Duplicate assays differing by more than 10% were repeated.

In method II (Jauch et al., 1970), the production of ADP was followed by substituting 8.25 units/mL pyruvate kinase and 21.0 units/mL lactic dehydrogenase for the auxiliary enzymes of method I. The reaction buffer and other conditions were the same as in method I. The two assay methods gave identical results when both were carried out under the same conditions, i.e., in the absence of both Fru-1,6-P_2 and ADP.

The specific activity of PFK is expressed either as μmol of Fru-6-P min^{-1} (mg of protein)$^{-1}$ or as μmol of Fru-6-P min^{-1} (g of wet yeast)$^{-1}$. For the latter calculation, we took into account the yield of protein in the extract per gram of wet yeast and the yield of PFK in the Sephadex eluate relative to the crude extract. Those results which are expressed per gram of yeast have been corrected to 90 mg of protein/g of wet yeast pellet. This figure was derived from an estimate of 150 mg of protein/g of yeast cells and by accepting the measurement by Gancedo & Gancedo (1973) that 1.67 g of wet yeast pellet contains 1 g of cell water.

Measurement of Fru-2,6-P_2 Content of Cells. The following extraction procedure mimics that used by den Hollander et al. (1981) for NMR determinations of metabolite levels, except for the requirement that Fru-2,6-P_2 be extracted into an alkaline medium. It is also similar to the method used by Lederer et al. (1981). Yeast cells that had been grown to saturation in 2% glucose and harvested as described above were resuspended to 20% wet weight in minimal medium buffered with 50 mM MES, pH 6.0 (den Hollander et al., 1981). Five milliliters of this suspension was equilibrated at 20 °C for 8 min with continuous bubbling at 500 mL/min of either N_2 or O_2 saturated with water vapor. Glucose was then added to a final concentration of 100 mM, and incubation with bubbling was continued. At 13 and 30 min, aliquots of this incubation mixture were added to 1.5 volumes of 100 mM glycine, pH 10, at 90 °C, and held for 1 min at 80 °C. The mixture was transferred to a mortar containing liquid nitrogen, ground to a powder, and subjected to two more freeze/thaw cycles. Cell debris were removed by centrifugation at 26400*g*. Fru-2,6-P_2 was measured in these extracts by its ability to act as an acid-labile stimulator of rat liver PFK (Pilkis et al., 1982a).

Materials. Fructose 2,6-bisphosphate and purified rat liver 6-phosphofructo-1-kinase were prepared as described previously (Pilkis et al., 1982a,b). Biochemicals were of the highest grade from Sigma. The auxiliary enzymes aldolase, triosephosphate isomerase, α-glycerophosphate dehydrogenase, pyruvate kinase, and lactic dehydrogenase (all from rabbit muscle) were obtained as suspensions in ammonium sulfate and were dialyzed before use against 900 volumes of 50 mM PIPES/KOH, pH 6.9, containing the same salts as in the assay mixture. Other chemicals were of at least reagent grade. All water was treated in a Milli-Q system (Millipore Co.).

RESULTS

When we attempted to duplicate the extraction procedure for preparing PFK of Banuelos et al. (1977), we found that our cell-free extract rapidly lost PFK activity, even at 4 °C. About 64% was lost overnight, and ca. 75% of the remaining activity was gone after another day. The activity that remained was not stimulated by 10 mM P_i. The loss of activity overnight, and about half of the loss after 2 days, could be prevented by the addition of 50 mM P_i and 1.0 mM Fru-6-P immediately after the extraction was completed. However, addition of these same concentrations of P_i and Fru-6-P after 1 day's storage restored only 6% of the loss activity. It must be noted that these concentrations added only 0.5 mM P_i and 0.01 mM Fru-6-Pto the assay mixtures. Hence, any effect found cannot be due to stimulation of the enzyme by these substances.

It has been pointed out by Stellwagen & Wilgus (1975) that the maintenance of yeast PFK activity during purification requires the presence of substrates and/or effectors of the enzyme (presumably to protect against proteolysis). We therefore repeated the extraction in the presence of 50 mM P_i, 2 mM Fru-6-P, and 1 mM PMSF. We then obtained an extract with high and stable activity, which could be purified further by the methods already described. These additions of substrates and effectors during purification added only negligible increments to the assay mixture, since the enzyme is diluted at least 50-fold in this mixture.

Figure 1, panel A, shows the activity of this partially purified preparations under the assay conditions employed by Banuelos et al. (curves A and B). We found several significant differences between the behavior of our preparation and that of Banuelos et al. The specific activity of PFK in the presence

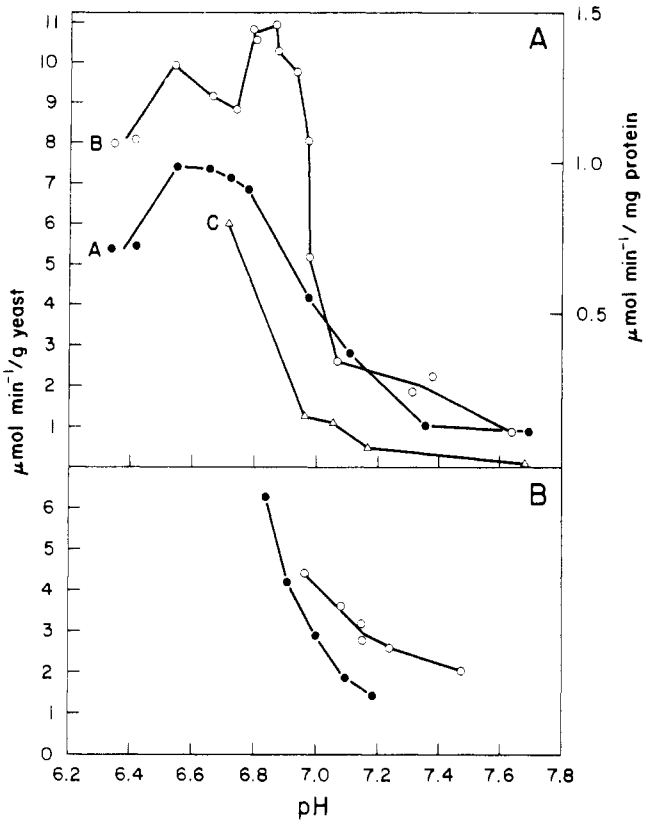

FIGURE 1: Panel A shows the activity of partially purified phosphofructo-1-kinase, measured under the conditions of Banuelos et al. (1977). Curve A is the activity in the presence of 0.4 mM P_i and curve B in the presence of 10 mM P_i. Increasing the concentration of ATP from 1.5 to 3.1 mM lowers the PFK activity (curve C). Under these conditions the in vitro rate is 2 orders of magnitude smaller than the in vivo rate. Panel B shows the results under the conditions presented in Table I, with the omission of Fru-2,6-P_2 and of Fru-1,6-P_2. Under these conditions the PFK rates are 10–20 times lower than the in vivo rate. The closed circles correspond to "anaerobic" conditions and the open circles to "aerobic" conditions. Note that with the omission of Fru-2,6-P_2 the anaerobic rates are lower than the aerobic rates.

of 0.4 mM P_i (curve A) was 8–9-fold higher than that of their partially purified preparation under identical assay conditions. Moreover, this activity was stimulated only very little by the addition of 10 mM P_i (curve B), even at pH 6.4, where these authors had found a 100-fold stimulation by 10 mM P_i. In agreement with Banuelos et al., we found that the activity was highest at pH 6.5–6.9, with a very steep drop of activity between pH 6.9 and pH 7.1. At pH values higher than about 7.1, there was no stimulation by 10 mM P_i, in agreement with the findings of Banuelos et al. and also with those of Atzpodien & Bode (1970) in this pH range.

Our earlier NMR measurements of the internal pH of glucose-grown derepressed yeast cells (den Hollander et al., 1981) had shown that the pH varies between about 7.2 (anaerobic) and 7.5 (aerobic) while glycolyzing. In this pH range the activity of PFK, when assayed under the conditions of curves A or B of Figure 1, panel A, is about an order of magnitude lower than the in vivo rate of glycolysis, which is about 20 μmol min^{-1} (g of yeast)$^{-1}$ (den Hollander et al., 1979). Furthermore, we had found by ^{31}P NMR (den Hollander et al., 1981) that the concentration of ATP in these cells is about 3 mM. When we increased the concentration of ATP in the assay mixture to 3.1 mM (Figure 1, panel A, curve C), the activity of PFK fell even lower, the relative inhibition by ATP being most marked above pH 7. Under this set of conditions, in vitro PFK activity is about 0.2 μmol min^{-1} (g of yeast)$^{-1}$, which is 2 orders of magnitude lower than the in vivo glycolytic rate.

Table I: Intracellular Metabolite Levels and pH in Glucose-Grown Derepressed Yeast While Glycolyzing

parameter	anaerobic value	aerobic value
[Fru-6-P] (mM)[a]	0.33	1.37
[ATP] (mM)[b]	2.8	3.6
[ADP] (MM)[b]	0.68	0.30
[AMP] (mM)[a]	0.16	0.025
[P_i] (mM)[d]	10.0	4.0
[Fru-1,6-P_2] (mM)[e]	3.1	4.2
[Fru-2,6-P_2][f]	3.2	0.6
pH[d]	7.23	7.52

[a] Derived from ^{31}P NMR measurement of Glu-6-P concentration in vivo (J. A. den Hollander et al., unpublished results) by assuming the glucosephosphate isomerase catalyzed reaction is at equilibrium, with [Fru-6-P] = (1/3)[Glu-6-P]. [b] From nucleoside di- and triphosphate peaks in extracts (J. A. den Hollander et al. unpublished results), assuming that the larger peak in each case is from the adenine nucleotide. [c] From ATP and ADP concentrations, assuming adenylate kinase is at equilibrium (Chiu et al., 1967) and $K = 1$ (Su et al., 1968). [d] den Hollander et al. (1981), from ^{31}P NMR measurements in vivo. [e] den Hollander et al. (1985), from ^{31}P NMR measurements of extracts. [f] Measured as described under Materials and Methods.

As noted above, we had found in our NMR experiments that the concentrations of several other important metabolites are different from those measured chemically by Banuelos et al. Table I summarizes these data, as well as the results of our determinations of Fru-2,6-P_2 concentrations, for glucose-grown derepressed yeast glycolyzing anaerobically or aerobically. At this point we wish to emphasize that assay method I does not allow for the addition of Fru-1,6-P_2 and method II does not allow the addition of ADP. We chose to begin our experiments with method I, omitting Fru-1,6-P_2, because the literature suggested that ADP was more likely to be an important effector of PFK than was Fru-1,6-P_2. The results of PFK assays carried out under the conditions shown in Table I (but omitting Fru-2,6-P_2 and Fru-1,6-P_2) are presented in Figure 1, panel B. These results show that the effectors mentioned thus far account for neither the absolute glycolytic rate nor the difference in rates between anaerobic and aerobic conditions. The rates found are about 10–20 times lower than the measured in vivo glycolytic rates. Moreover, when PFK activity under anaerobic conditions at the anaerobic pH of 7.2 is compared with the rate under aerobic conditions at the aerobic pH of 7.5, one does not see the predicted 2-fold higher anaerobic rate; indeed, the "anaerobic" rate is slightly lower than the "aerobic" rate.

We therefore concluded that there must be one or more other effectors that strongly stimulate PFK and that might also account in large measure, for the difference in PFK activity between anaerobic and aerobic glycolysis. The recently discovered fructose 2,6-bisphosphate seemed the likely candidate. Using the extraction and assay methods described above, we found that the concentrations of Fru-2,6-P_2 in yeast cells reach steady-state values of 3.2 μM anaerobically and 0.60 μM aerobically by 13 min after addition of glucose and that these concentrations are the same at 30 min after glucose addition. A 13-min time point was chosen because our ^{31}P NMR studies had shown that the concentrations of other metabolites reach steady-state values in this time period. Lederer et al. (1981) have reported that, under similar conditions, Fru-2,6-P_2 reaches ca. 93% of its maximal value by 10 min after glucose addition and 100% after 20 min. Our findings are therefore consistent with their results.

Furuya et al. (1982) have reported that the concentration of Fru-2,6-P_2 is about 4 times higher in anaerobically growing

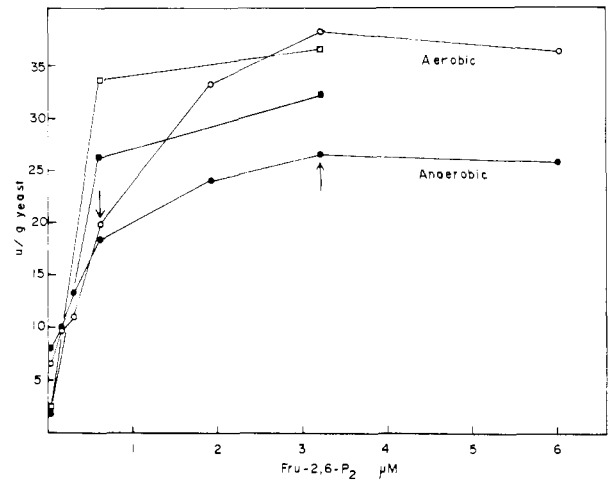

FIGURE 2: Effect of Fru-2,6-P_2 and Fru-1,6-P_2 on the activity of partially purified PFK, under the conditions of Table I except as noted: (○) aerobic; (□) aerobic without Fru-1,6-P_2; (●), anaerobic; (■) anaerobic without Fru-1,6-P_2. Experiments without Fru-1,6-P_2 were performed by either assay method I (with or without ADP) or assay method II (without ADP), yielding identical results. Assays with Fru-1,6-P_2 were done by method II. Arrows show the anaerobic (3.2 mM) and aerobic (0.6 mM) concentrations of Fru-2,6-P_2.

yeast than in those growing aerobically. They reported their data in terms of picomoles of Fru-2,6-P_2 per milligram of protein. Assuming 90 mg of protein/g of wet yeast (see above) and assuming that 1.67 g of wet yeast corresponds to 1 mL of internal cell volume (Gancedo & Gancedo, 1973), one can calculate intracellular concentrations of 5.2 μM anaerobically and 1.35 μM aerobically from their data. Our values are reasonably close to theirs, bearing in mind the different conditions (log phase cells vs. glucose-depleted cells refed glucose).

Figure 2 shows the effect of Fru-2,6-P_2 on the anaerobic and aerobic reaction mixtures in the absence and presence of Fru-1,6-P_2. Fru-2,6-P_2 is indeed a powerful stimulator of PFK under in vivo conditions. In the absence of Fru-1,6-P_2 (experiments initially performed by assay method I), the degree of stimulation is on the order of 20-fold at V_{max}, yielding absolute rates that are in good agreement with the in vivo glycolytic rates [ca. 20 μmol min^{-1}) (g of yeast)$^{-1}$]. The concentrations required for half-maximal stimulation of the enzyme (less than 1 μM) are lower than the 2 μM reported by Bartrons et al. (1982) at pH 6.4 with 2.5 mM ATP and 0.5 mM Fru-6-P. They are, however, close to the range reported by Avigad (1981), 0.2–0.5 μM, using a commercially purified yeast PFK at pH 7.4 with 0.2 mM ATP and 0.2 mM Fru-6-P. When we assayed our PFK preparation under exactly the same conditions as those of Avigad (data not shown), we obtained the same values as he did for the half-saturating concentration of Fru-2,6-P_2, the extent of stimulation at V_{max}, and the absolute rate at V_{max}.

Returning to Figure 2, one sees that in the absence of Fru-1,6-P_2 the difference in concentration of Fru-2,6-P_2 between anaerobic and aerobic states has virtually no effect on the rate of PFK. The enzyme is saturated, or nearly so, at both concentrations and in both metabolic states. Therefore, while Fru-2,6-P_2 is clearly necessary to establish the observed in vivo rate, it is also clear that the difference of Fru-2,6-P_2 concentrations does not, by itself, account for the different glycolytic rates.

We found later (see below) that ADP has no effect on these curves. When ADP was omitted entirely and these same assays in the absence of Fru-1,6-P_2 were carried out by assay method II, results identical with those shown in Figure 2 were obtained. The other curves in Figure 2 will be discussed later.

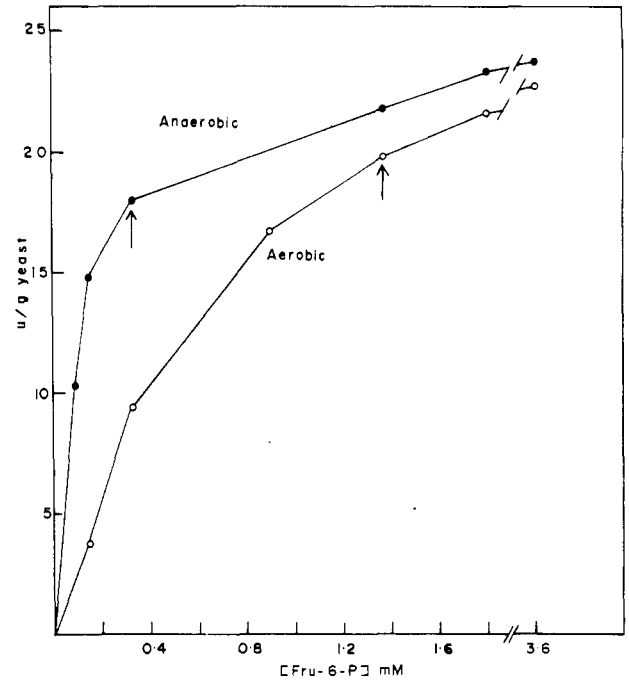

FIGURE 3: Activity of PFK as a function of Fru-6-P concentration, with the effector concentrations given in Table I but omitting Fru-1,6-P_2. Assay method I was used. Arrows show anaerobic and aerobic concentrations of Fru-6-P.

Having obtained rates in vitro that closely match those observed in vivo, we next asked how the kinetic properties of PFK in the anaerobic and aerobic states are controlled by the individual effectors, including the substrate Fru-6-P. Figure 3 shows PFK activity as a function of Fru-6-P concentration when the other effectors are held constant at their observed anaerobic and aerobic values (Fru-1,6-P_2 again omitted). The arrows in this figure show, respectively, the rates at the experimentally determined in vivo anaerobic and aerobic concentrations of Fru-6-P. It is clear that the difference in Fru-6-P concentration between anaerobic and aerobic conditions contributes significantly to the control of PFK in vivo. If, for example, [Fru-6-P] remained at 0.33 mM (its anaerobic value), the aerobic rate would be only about half the anaerobic rate. With the change in substrate concentration, however, the two rates are about equal under the set of conditions shown in Figure 3.

In addition to the role of the substrate, we could now measure the relative importance of each of the effectors of PFK. Of several possible methods for accomplishing this, we chose the following. As can be seen from Figure 3, there is very little difference between the anaerobic and aerobic rates at the aerobic Fru-6-P concentration of 1.37 mM. However, at the anaerobic Fru-6-P concentration of 0.33 mM there is an approximately 2-fold difference between the two curves. Our approach was to determine the contribution of each effector to this 2-fold difference. First, holding the concentration of all effectors constant at their anaerobic values (Table I), we varied the concentration of one effector at a time from its anaerobic to its aerobic value and measured the change in rate produced by this variation. Then, the procedure was reversed: holding all variables constant at their aerobic values (except Fru-6-P, which was kept at 0.33 mM), we varied at a time from its aerobic to its anaerobic value. The results of these experiments are shown in Table II. We notice first that the sum of the effects due to all effectors shown in this table adds up to less than 100% for the anaerobic conditions and more than 100% for aerobic conditions. These results can be ac-

Table II: Percentage of the Observed Difference in Glycolytic Rate between Anaerobic and Aerobic in Vivo Conditions, Fru-1,6-P_2 Omitted, Contributed by Particular Allosteric Effectors[a]

effector	anaerobic condition	aerobic condition
Fru-2,6-P_2	0	81
ATP	23	11
ADP	0	0
AMP	35	34
P_i	−12	−29
pH	13	47
total	59	144

[a] The concentration of Fru-6-P was fixed at 0.33 mM. Observed difference in rate between anaerobic and aerobic in vivo conditions, Fru-1,6-P_2 omitted.

counted for in the following way. Under anaerobic conditions, at [Fru-6-P] = 0.33 mM, PFK is virtually saturated with Fru-6-P (Figure 3). Therefore, it is not surprising that changes in effector concentrations have only minimal effect. For the aerobic conditions, on the other hand, the point we are considering is very far down on the curve of rate vs. [Fru-6-P] (Figure 3). Therefore, small changes in effector concentrations can have disproportionately large effects on the rate.

Several important conclusions emerge from the data presented in Table II. First, no single effector accounts for all of the difference in PFK activity, as has been claimed by many authors. Second, in contrast to what others have found in many cases, ADP has no effect on PFK when assayed in the presence of in vivo concentrations of the other effectors. This fact is important not only in itself but also because it allowed us to proceed to the following set of experiments. Since ADP could be omitted entirely from the reaction mixture without affecting the activity of PFK, we could then investigate the role of Fru-1,6-P_2 by using assay method II.

Returning to Figure 2, we see the effects of Fru-1,6-P_2 at its in vivo concentrations on the response of PFK to Fru-2,6-P_2. Fru-1,6-P_2 has two effects: it decreases the maximal activation caused by Fru-2,6-P_2 in the anaerobic case and increases the concentration of Fru-2,6-P_2 necessary for half-maximal stimulation in both cases. The changes in the latter property are from 0.07 to 0.53 μM in the anaerobic case and from 0.04 to 0.14 μM in the aerobic. These curves were not altered at all if the anaerobic concentration of Fru-1,6-P_2 was added to the aerobic mixture, or vice versa. In other words, the effect of Fru-1,6-P_2 upon the activation of PFK by Fru-2,6-P_2 is concentration-independent over the in vivo range of Fru-1,6-P_2 levels. The result of these interactions is that the rate in the presence of the complete set of in vivo effectors and substrates (except ADP) is 1.4 times higher in the anaerobic case than in the aerobic, approximating the estimated 1.8-fold difference in the in vivo rate of PFK.

Figure 4 shows the PFK rate as a function of [Fru-6-P] when all effectors, including Fru-1,6-P_2 but omitting ADP, are present at their in vivo values. There are large differences in both $[S]_{0.5}$ for Fru-6-P and V_{max}. The $[S]_{0.5}$ values are 0.12 mM anaerobically and 0.96 mM aerobically, while the V_{max} values are 35 and 23 μmol min^{-1} (g of yeast)$^{-1}$, respectively. PFK is about 77% saturated with Fru-6-P in the anaerobic in vivo situation and almost completely saturated aerobically. Again, we see the large role played by the substrate concentration. Here, the aerobic rate would be less than 10% of the anaerobic rate if the concentration of Fru-6-P did not rise.

Discussion

The present study has shown how it is possible to use our knowledge of metabolite levels, determined mostly by NMR, to set up an in vitro system that has the same PFK rate as that

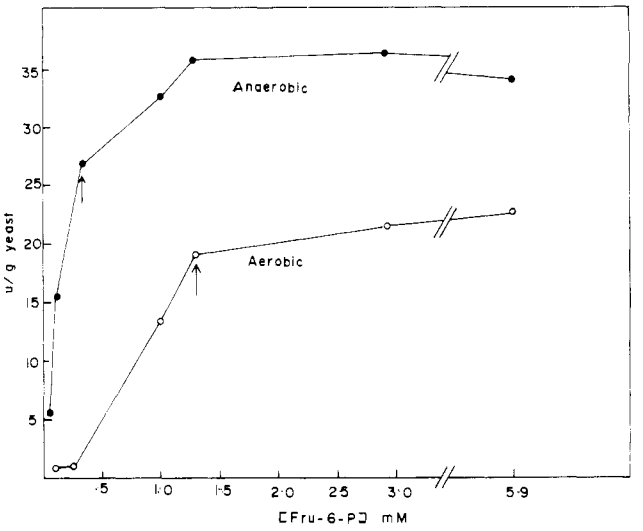

FIGURE 4: Activity of PFK as a function of [Fru-6-P] concentration, with effector concentrations given in Table I, including Fru-6-P but omitting ADP. Assay method II was used. Arrows are as in Figure 3.

observed in vivo. In a previous attempt, Banuelos et al. (1977) had taken the best estimation of in vivo conditions available to them and reproduced the in vivo rate in an in vitro experiment. As we pointed out earlier, however, their values of intracellular pH and ATP levels were incorrect, as shown by our recent ^{31}P NMR experiments, and their AMP levels were too high compared to the values we have determined in perchloric acid extracts. Their low pH gave an order of magnitude increase in the rate, which compensated for their omission of Fru-2,6-P_2, a compound that had not been discovered at the time of their experiments. Hence, their agreement with in vivo rates was obtained by a cancellation of sizable errors. We have also reproduced in vitro to a reasonable approximation the small difference in PFK rate between anaerobic and aerobic conditions, using measured in vivo concentrations of substrates and effectors.

The effect on PFK of changing intracellular conditions can be viewed from two perspectives. One is the effect of these changes on the responsiveness of the enzyme to Fru-6-P. This property can be analyzed in terms of what may be called apparent rate constants. These are defined as k_{app} = rate/[Fru-6-P] at any given point on either curve of Figure 4 (k_{app} would be the slope of a straight line drawn from the origin to the given point). The apparent rate constants at the anaerobic and aerobic points (the points shown by the arrows in Figure 4) are 81.2 and 14.2 μmol min^{-1} (g of yeast)$^{-1}$ mM^{-1}, respectively. Thus, one can say that in the anaerobic state the enzyme is about 5.7 times more responsive to Fru-6-P than it is in the aerobic state. However, since the Fru-6-P concentration is 4.2-fold lower anaerobically than aerobically, the net change in PFK reaction rate is only 1.4-fold. These curves clearly show the effect on PFK of the buildup of Fru-6-P when the cell is changed from anaerobic to aerobic conditions. If the concentration of Fru-6-P were to remain at its anaerobic value (0.33 mM), PFK activity would actually decrease 12-fold. However, the accumulation of Fru-6-P to a concentration of 1.37 mM (a factor of 4.2) increases PFK activity by a factor of 8.8, yielding a net reduction in the rate of reaction by only 1.4-fold. Thus, it is seen that the regulation of PFK keeps its catalytic rate equal to those of the other enzymes of the glycolytic pathway by substrate-level control. Because the sensitivity of the PFK rate to substrate levels is sigmoidal, when the enzyme is slowed down under aerobic conditions by effector

control, the increase of Fru-6-P levels by 4.2 times increases the enzymatic rate by a factor of 8.8, thereby almost restoring the anaerobic value.

The other way to view the change in PFK activity is in terms of the overall in vivo change in rate of the order of a factor of almost 2 in going from aerobic to anaerobic conditions. Our present understanding of this change is that it is not brought about by any single effector. We see this clearly in the data shown in Table II. Likewise, in Figure 2 we see that the presence of Fru-1,6-P_2 modulates the effect of Fru-2,6-P_2 (presumably by competition for the same binding site). We do not believe that either of these (or other) effectors can be called *the* controller of PFK activity. It is rather the combination of changes in effector concentrations that produces a large change in the responsiveness of the enzyme to Fru-6-P. This altered responsiveness is than nearly cancelled by the change in Fru-6-P concentration. What we do see is appreciable homeostasis, a near cancellation of what would otherwise be large changes in rates.

We do find great significance in the almost complete *lack* of any effect due to changes in ADP, P_i, and ATP, since it has been proposed by many writers that changes in one or more of these effectors are major factors in the Pasteur effect. The near absence of any effect due to ATP has two causes. First, the change in ATP concentration is quite small (2.8–3.5 mM). Second, at the intracellular concentrations of the other effectors that exist under these conditions, the enzyme is already maximally inhibited by 2.8 mM ATP. In fact, we found (data not shown) that under either anaerobic or aerobic conditions the activity of PFK is virtually independent of ATP concentration from 2 to 5 mM.

It is not surprising that ATP levels are relatively high in these cells, since the medium contained 100 mM glucose when our in vivo measurements of glycolytic rates were made. One can postulate conditions for which ATP levels might be substantially lower, for example, near starvation. Furthermore, in this kind of state Fru-2,6-P_2 is known to be low or absent (Lederer et al., 1981). The combination of low Fru-2,6-P_2 and low ATP (i.e., in an inhibitory range) might well be a situation in which ATP *is* a dominant controller of PFK.

In this paper we have studied one set of conditions for which some effectors of PFK previously thought to be important (ADP, P_i, ATP) are, in fact, insignificant in regulating the enzyme. This points out the importance of duplicating in vivo conditions for establishing which effectors are physiologically relevant. We believe that, for an enzyme such as PFK (which is responsive to so many effectors in in vitro assays), the relative importance of each effector may well be different under different metabolic conditions. This is not to say that ADP, P_i, ATP, and other effectors play no role in setting the level of PFK activity, even under the present conditions. The complete absence of these effectors, or their presence at very different concentrations, might very well change the control profile of the enzyme or the absolute value of the rates. This is almost certainly true of ATP as discussed above. We have also seen (Figure 2) that, although the difference in Fru-1,6-P_2 concentration between anaerobic and aerobic states has no effect on the activity of PFK, the presence of this effector at 3–4 mM alters the effect of Fru-2,6-P_2 so as to account for the observed in vivo difference in glycolytic rate. What we are saying is that *changes* in the concentrations of certain compounds have little or no effect on PFK in the particular conditions studied here.

In contrast to Banuelos et al. (1977), we found only a small stimulation of PFK by P_i, even at low pH and at their concentrations of Fru-6-P, AMP, ADP, and ATP (Figure 1, panel A), in contrast to the 100-fold stimulation they reported at pH 6.4. We can speculate that this discrepancy arises from the difference in the stability of our extract as compared to theirs and that perhaps the drastic loss of enzyme activity in a medium lacking stabilizers is accompanied by changes in the enzyme's structure that render it more responsive to P_i at low pH. In any case, we agree with these authors in finding an absence of any effect of P_i in the physiologically relevant pH range (7.0–7.6).

ADP has been found to be either a positive or a negative effector, depending on the concentration of ATP and other effectors. The effect of ADP on PFK has been reported to be a shift to the right in the curve of activity vs. [ATP]. Thus, at low ATP concentrations, ADP stimulates the enzyme, while the reverse is true at high ATP concentrations. We find no effect whatsoever of ADP under in vivo conditions (but with Fru-1,6-P_2 omitted).

The homeostasis we have observed, in which there are large changes in the rate caused by substrate-level control, has several significant consequences for the overall picture of the control of glycolysis, of which the control of PFK is just a part. These larger issues are as follows.

First, in a perfused heart it has been shown that "when the heart is made to work, the flux through PFK is increased about four-fold, whereas the content of ATP is decreased by only 16%" [p 108 of Newsholme & Start (1973)], and similar results are reported for blowfly muscle. Newsholme and Start, continuing to discuss muscle, point out quite correctly that this small change in ATP level cannot possibly, on the basis of its allosteric control, be responsible for the large change in PFK flux. Hence, they conclude that amplification of the ATP control is needed and present evidence showing that the [AMP] increases several fold while the [ATP] decreases by only 10–20%. This can occur because hydrolysis of a small fraction of ATP could increase the [AMP] severalfold, since the latter has a much lower steady-state concentration. We do not wish to suggest that the present results on yeast contradict these studies of muscle—in fact, there are some similarities between both sets of data. We do say, though, that amplification of ATP control is not happening in yeast. Furthermore, the large changes induced mainly by the changing Fru-2,6-P_2 concentration are not *amplified* by related changes but are in fact *reduced* by Fru-6-P changes and competition by Fru-1,6-P_2. Hence, instead of amplification, we see a compensation.

Second, we are now able to understand how there is control of the glycolytic flux at more than one point and to suggest a possible function for this seemingly excessive amount of control. Our NMR results, in general agreement with previous reports, show that there is control of glucose uptake, occurring either at the glucose transport step (Serrano & DelaFuente, 1974; Azam & Kotyk, 1969; Kotyk & Kleinzeller, 1967; Sols, 1967) or at hexokinase (HK). In addition, there is control at PFK, as discussed here, and we have two kinds of experiments (den Hollander et al., 1985a,b) that show that there is control below PFK in the glycolytic pathway. Presumably, the lower control occurs at pyruvate kinase, but that is beyond the scope of this paper.

We bear in mind that the flow through these control steps is the same. Control at several points so as to maintain a continuity of flux could occur in three ways, which we illustrate, for simplicity, for the particular control points of glucose utilization and PFK.

(I) There could be independent control of these two points by their effectors. Presumably this would reflect the energy

state of the cell, and although independently exercised, it would be arranged so that the two controlled steps track over a variety of cellular conditions.

(II) There could be control at a step in the pathway such as PFK that, when slowed down, would increase the concentrations of intermediates such as Fru-6-P and G6P above it. These intermediates would decrease glucose utilization, by allosteric control or by mass action.

(III) There could be allosteric control of PFK that, when slowed down, would cause an increase in the concentration of the substrate Fru-6-P (and possibly also of G6P). The increased Fru-6-P level would increase the flux through PFK by substrate-level control.

Both cases II and III will exhibit an increase in the concentration of the substrate, Fru-6-P, when the flux through PFK decreases. This satisfies the criterion for considering PFK to be a regulatory enzyme as formulated by Newsholme & Start (1973, p 16). Hence, PFK activity must be under the control of allosteric effectors, and Table II and Figure 2 show that this is indeed true. Our cases II and III differ in the effect that the increased substrate concentration has upon the flux; in case II the enzyme is not under substrate-level control but is operating in the V_{max} region, while in case III the enzyme is operating under substrate-level control and the increased substrate level will increase the flow through the enzyme. Case III clearly fits the measurements presented in this paper.

Finally, we see from the nature of the control exercised at PFK that there is indeed a real function for having control at more than one point along a pathway. If, for example, there were only control at the first stage of the pathway, e.g., glucose utilization, then all rates below the first stage must be fast and all metabolite levels must be very low. However, by introducing control at PFK the cell has managed to keep levels of intermediates above PFK at a high level, while it has adjusted the flux to be continuous in the manner described in this paper.

Registry No. PFK, 37278-03-8; Fru-6-P, 643-13-0; Fru-1,6-P_2, 488-69-7; Fru-2,6-P_2, 79082-92-1; 5′-ATP, 56-65-5; 5′-ADP, 58-64-0; 5′-AMP, 61-19-8; P_i, 14265-44-2.

References

Atzpodien, W., & Bode, H. (1970) *Eur. J. Biochem. 12*, 126–132.

Avigad, G. (1981) *Biochem. Biophys. Res. Commun. 102*, 985–991.

Azam, F., & Kotyk, A. (1969) *FEBS Lett. 2*, 333–335.

Banuelos, M., Gancedo, C., & Gancedo, J. M. (1977) *J. Biol. Chem. 252*, 6394–6398.

Bartrons, R., Van Schaftingen, E., Vissers, S., & Hers, H.-G. (1982) *FEBS Lett. 143*, 137–140.

Barwell, C. J., & Hess, B. (1971) *FEBS Lett. 19*, 1–4.

Betz, A., & Chance, B. (1965a) *Arch. Biochem. Biophys. 109*, 579–584.

Betz, A., & Chance, B. (1965b) *Arch. Biochem. Biophys. 109*, 585–594.

Chance, B. (1959) in *Ciba Foundation Symposium on the Regulation of Cell Metabolism* (Wolstenholme, G. E. W., & O'Connor, C. M., Eds.) pp 90–121, Churchill, London.

Chiu, C.-S., Su, S., & Russell, P. J. (1967) *Biochim. Biophys. Acta 132*, 361–369.

Clifton, D., & Fraenkel, D. G. (1983) *J. Biol. Chem. 258*, 9245–9249.

den Hollander, J. A., Brown, T. R., Ugurbil, K., & Shulman, R. G. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 6096–6100.

den Hollander, J. A., Ugurbil, K., Brown, T. R., & Shulman, R. G. (1981) *Biochemistry 20*, 5871–5880.

den Hollander, J. A., Ugurbil, K., Brown, T. R., Bednar, M., Redfield, C., & Shulman, R. G. (1985a) *Biochemistry* (first paper of three in this issue).

den Hollander, J. A., Ugurbil, K., & Shulman, R. G. (1985b) *Biochemistry* (second paper of three in this issue).

Furuya, E., Kotaniguchi, H., & Hagihara, B. (1982) *Biochem. Biophys. Res. Commun. 105*, 1519–1523.

Gancedo, J. M., & Gancedo, C. (1973) *Biochimie 55*, 205–211.

Ghosh, A., & Chance, B. (1964) *Biochem. Biophys. Res. Commun. 16*, 174–181.

Hers, H.-G. & Van Schaftingen, E. (1982) *Biochem. J. 206*, 1–12.

Hess, B. (1973) in *Symposia of the Society for Experiment of Biology*, pp 105–131, Cambridge University Press, Cambridge.

Hofmann, E., & Kopperschlaeger, G. (1982) *Methods Enzymol. 90*, 49–60.

Holzer, H. (1961) *Cold Spring Harbor Symp. Quant. Biol. 26*, 227–288.

Holzer, H., & Freytag-Hilf, R. (1959) *Hoppe-Seyler's Z. Physiol. Chem. 316*, 7–30.

Holzer, H., Witt, J., & Freytag-hilf, R. (1958) *Biochem. Z. 329*, 467–475.

Jauch, R., Riepertinger, C., & Lynen, F. (1970) *Hoppe-Seyler's Z. Physiol. Chem. 351*, 74.

Kessler, R., Nissler, K., Schellenberger, W., & Hofmann, E. (1982) *Biochem. Biophys. Res. Commun. 107*, 506–510.

Kopperschlaeger, G., Baer, J., Nissler, E., & Hofmann, E. (1977) *Eur. J. Biochem. 81*, 315–325.

Kotyk, A., & Kleinzeller, A. (1967) *Biochim. Biophys. Acta 135*, 106–111.

Krebs, H. A. (1972) *Essays Biochem. 8*, 1–34.

Lederer, B., Vissers, S., Van Schaftingen, E., & Hers, H.-G. (1981) *Biochem. Biophys. Res. Commun. 103*, 1281–1287.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Lynen, F., Hartmann, G., Netter, K. F., & Schuegraf, A. (1959) in *Ciba Foundation Symposium on the Regulation of Cell Metabolism* (Wolstenholme, G. E. W., & O'Connor, C. M., Eds.) pp 236–237, Churchill, London.

Mahler, H. R., Jaynes, P. K., McDonough, J. F., & Hanson, D. K. (1981) *Curr. Top. Cell. Regul. 18*, 455–474.

Moore, C. L., Betz, A., & Chance, B. (1965) in *Control of Energy Metabolism Colloquium*, Philadelphia (Chance, B., Estabrook, R. W., & Yonetani, T., Eds.) pp 97–100, Academic Press, New York.

Navon, G., Shulman, R. G., Yamane, T., Eccleshall, T. R., Lam, K.-B., Baronofsky, J., & Marmur, J. (1979) *Biochemistry 18*, 4487–4499.

Newsholme, E. A., & Start, C. (1973) *Regulation in Metabolism*, Wiley, London.

Nissler, K., Otto, A., Schellenberger, W., & Hofmann, E. (1983) *Biochem. Biophys. Res. Commun. 111*, 294–300.

Passonneau, J. V., & Lowry, O. H. (1962) *Biochem. Biophys. Res. Commun. 7*, 10–15.

Passonneau, J. V., & Lowry, O. H. (1963) *Biochem. Biophys. Res. Commun. 13*, 372–379.

Pilkis, S. J., El-Maghrabi, M. R., Cumming, D. A., Pilkis, J., & Claus, T. H. (1982a) *Methods Enzymol. 89*, 101–107.

Pilkis, S. J., El-Maghrabi, M. R., McGrane, M., Pilkis, J., Fox, E., & Claus, T. H. (1982b) *Mol. Cell. Endocrinol. 25*, 245–266.

Racker, E. (1947) *J. Biol. Chem. 167*, 843.

Racker, E. (1974) *Mol. Cell. Biochem. 5*, 17–23.

Ramaiah, A. (1974) *Curr. Top. Cell. Regul. 8*, 297–345.
Rose, I. A. (1971) *Exp. Eye Res. 11*, 264–272.
Salas, M. L., Vinuela, Salas, M., & Sols, A. (1965) *Biochem. Biophys. Res. Commun. 19*, 371–376.
Salhany, J. M., Yamane, T., Shulman, R. G., & Ogawa, S. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 4966–4970.
Serrano, R., & DelaFuente, G. (1974) *Mol. Cell. Biochem. 5*, 161–171.
Sols, A. (1967) in *Aspects of Yeast Metabolism* (Mills, A. K., & Krebs, H. A., Eds.) pp 47–66, Blackwell Scientific Publications, Oxford.
Sols, A. (1976) in *Reflections on Biochemistry* (Kornberg, A., Horecker, B. L., Cornudella, L., & Oro, J., Eds.) pp 199–206, Pergamon Press, Oxford.
Sols, A. (1981) *Curr. Top. Cell. Regul. 19*, 77–101.
Sols, A., Grancedo, C., & DelaFuente, G. (1971) in *The Yeasts* (Rose, A. H., & Harrison, J. S., Eds.) Vol. 2, pp 271–307, Academic Press, London.
Stellwagen, E., & Wilgus, H. (1975) *Methods Enzymol. 42*, 78–85.
Su, S., & Russell, P. J. (1968) *J. Biol. Chem. 243*, 3826–3833.
Tejwani, G. A. (1978) *Trends Biochem. Sci. 3*, 30–33.
Vinuela, E., Salas, M. L., & Sols, A. (1963) *Biochem. Biophys. Res. Commun. 12*, 140–145.

Protonation Mechanism and Location of Rate-Determining Steps for the *Ascaris suum* Nicotinamide Adenine Dinucleotide–Malic Enzyme Reaction from Isotope Effects and pH Studies†

Dennis M. Kiick, Ben G. Harris, and Paul F. Cook*
Department of Biochemistry, North Texas State University/Texas College of Osteopathic Medicine, Denton, Texas 76203
Received June 24, 1985

ABSTRACT: The pH dependence of the kinetic parameters and the primary deuterium isotope effects with nicotinamide adenine dinucleotide (NAD) and also thionicotinamide adenine dinucleotide (thio-NAD) as the nucleotide substrates were determined in order to obtain information about the chemical mechanism and location of rate-determining steps for the *Ascaris suum* NAD–malic enzyme reaction. The maximum velocity with thio-NAD as the nucleotide is pH-independent from pH 4.2 to 9.6, while with NAD, V decreases below a pK of 4.8. V/K for both nucleotides decreases below a pK of 5.6 and above a pK of 8.9. Both the tartronate pK_i and V/K_{malate} decrease below a pK of 4.8 and above a pK of 8.9. Oxalate is competitive vs. malate above pH 7 and noncompetitive below pH 7 with NAD as the nucleotide. The oxalate K_{is} increases from a constant value above a pK of 4.9 to another constant value above a pK of 6.7. The oxalate K_{ii} also increases above a pK of 4.9, and this inhibition is enhanced by NADH. In the presence of thio-NAD the inhibition by oxalate is competitive vs. malate below pH 7. For thio-NAD, both ^DV and $^D(V/K)$ are pH-independent and equal to 1.7. With NAD as the nucleotide, ^DV decreases to 1.0 below a pK of 4.9, while $^D(V/K_{NAD})$ and $^D(V/K_{malate})$ are pH-independent. Above pH 7 the isotope effects on V and the V/K values for NAD and malate are equal to 1.45, the pH-independent value of ^DV above pH 7. From the above data, the following conclusions can be made concerning the mechanism for this enzyme. Substrates bind to only the correctly protonated form of the enzyme. Two enzyme groups are necessary for binding of substrates and catalysis. Both NAD and malate are released from the Michaelis complex at equal rates which are equal to the rate of NADH release from E–NADH above pH 7. Below pH 7 NADH release becomes more rate-determining as the pH decreases until at pH 4.0 it completely limits the overall rate of the reaction.

The NAD[1]–malic enzyme from *Ascaris suum*, isocitrate dehydrogenase, and NADP–malic enzyme from pigeon liver all catalyze the same type of reaction, a divalent metal dependent oxidative decarboxylation utilizing NAD(P) as the electron acceptor. However, the enzymes listed above are quite different with respect to their kinetic properties. The pigeon liver enzyme has an ordered kinetic mechanism (Hsu & Lardy, 1967; Schimerlik & Cleland, 1977a), while both ICDH[1] (Uhr et al., 1974) and the ascarid enzyme (Park et al., 1984) have random mechanisms. Further, release of reduced nucleotide completely limits the maximum rate for both ICDH (Uhr et al., 1974) and the pigeon liver enzyme (Schimerlik & Cleland,

† This research was supported by National Institutes of Health Grants AI-12331 (to B.G.H.) and GM-31686 (to P.F.C.), Grant BRSG S07 RR07195-03 (to P.F.C.) awarded by the Biomedical Research Grant Program, Division of Research Resources, National Institutes of Health, and Grants B-997 (to B.G.H.) and B-1031 (to P.F.C.) from the Robert A. Welch Foundation. P.F.C. is the recipient of NIH Research Career Development Award AM-01155.

[1] Abbreviations: BTP, [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)propane; Caps, 3-(cyclohexylamino)propanesulfonic acid; Ches, 2-(cyclohexylamino)ethanesulfonic acid; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EPR, electron paramagnetic resonance; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; ICDH, NADP–isocitrate dehydrogenase; Mes, 2-(*N*-morpholino)ethanesulfonic acid; NAD, nicotinamide adenine dinucleotide; NADP, nicotinamide adenine dinucleotide phosphate; Ox, oxalate; PAGE, polyacrylamide gel electrophoresis; Pipes, 1,4-piperazinediethanesulfonic acid; SDS, sodium dodecyl sulfate; Taps, 3-[[tris(hydroxymethyl)methyl]amino]propanesulfonic acid.

0006-2960/86/0425-0227$01.50/0