Fructose 2,6-bisphosphate induces irreversible transitions in cell-free extracts of rat liver

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Abstract The effect of fructose 2,6-bisphosphate on the dynamics of the 6-phosphofructo-1-kinase/fructose-1,6-bisphosphatase cycle is investigated in a cell-free extract of rat liver under steady-state conditions. Bistability emerges on the basis of the reciprocal allosteric modulation of 6-phosphofructo-1-kinase and fructose-1,6-bisphosphatase. Under conditions of bistability fructose 2,6-bisphosphate may cause transitions between alternative steady states. However, in contrast to what is frequently observed in bistable systems, within a broad range of experimental conditions these transitions proceed irreversibly from states with high ATP to states characterized by low ATP.

Key words: Bistability; Glycolysis; Hysteresis; Irreversible transition; Nonlinear dynamics; Phosphofructokinase/fructose-1,6-bisphosphatase cycle

1. Introduction

Fructose 2,6-bisphosphate (Fru-2,6-P_2) is the most powerful activator of 6-phosphofructo-1-kinase (6PF-1-K, EC 2.7.1.11) and an inhibitor of fructose-1,6-bisphosphatase (Fru-1,6-P_2ase, EC 3.1.3.11) [1–5]. It acts as an important modulator of glycolysis and gluconeogenesis. In liver, synthesis and degradation of Fru-2,6-P_2 are catalyzed by a bifunctional enzyme, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (6PF-2-K/Fru-2,6-P_2ase, EC 2.7.1.105/3.1.3.46) [6]. The two enzyme activities are reciprocally regulated by cAMP-dependent phosphorylation and allosteric modulators [7,8]. Gene expression of the bifunctional enzyme is subject to multihormonal regulation [9].

The steady-state level of Fru-2,6-P_2 is determined by the ratio between 6PF-2-K and Fru-2,6-P_2ase activities. Appropriate concentration changes of Fru-2,6-P_2 may revert the direction of substrate flux in the 6PF-1-K/Fru-1,6-P_2ase cycle and thereby contribute to switch between glycolysis and gluconeogenesis and to ensure efficient glycolytic or gluconeogenic pathway fluxes [9–12].

Some insights into the regulatory potential of Fru-2,6-P_2 were gained from in vitro studies allowing the separate investigation of the cycles formed by 6PF-2-K/Fru-2,6-P_2ase and 6PF-1-K/Fru-1,6-P_2ase. In the co-operation between 6PF-2-K and Fru-2,6-P_2ase a non-linear dynamic trigger is formed which causes a threshold response of Fru-2,6-P_2 with respect to the concentration of fructose 6-phosphate and the phosphorylation state of the bifunctional enzyme [13]. Fru-2,6-P_2, in turn, may affect non-linear dynamic structures in the 6PF-1-K/Fru-1,6-P_2ase cycle which are the result of the reciprocal allosteric modulation of 6PF-1-K and Fru-1,6-P_2ase by AMP, fructose 6-phosphate (Fru-6-P), and fructose 1,6-bisphosphate (Fru-1,6-P_2). Fru-2,6-P_2 was also shown to modulate oscillatory states in glucose metabolism [14–16].

In the present paper the effect of Fru-2,6-P_2 on dynamic phenomena related to bistability in the 6PF-1-K/Fru-1,6-P_2ase cycle is investigated in a cell-free extract of rat liver.

Conditions are chosen under which 6PF-2-K/Fru-2,6-P_2ase is omitted and the concentration of Fru-2,6-P_2 becomes adjustable experimentally. The occurrence of bistability has been shown under these conditions [17]. In this paper it will be demonstrated that Fru-2,6-P_2 may cause transitions between alternative and functionally different states of the 6PF-1-K/Fru-1,6-P_2ase cycle. However, in fundamental contrast to what is frequently observed in bistable systems, within a broad range of experimental conditions these transitions are irreversible. This result provides an experimental verification in a biochemical reaction system of the hitherto theoretically predicted phenomenon of irreversible transitions [18].

2. Materials and methods

2.1. Preparation of cell-free extracts

Male albino Wistar rats, weighing 200–280 g and fed normally, were used. Cytosol was prepared from liver as described previously [18]. For adjustment of a defined ratio of 6PF-1-K/Fru-1,6-P_2ase the extracts were supplemented by rat liver 6PF-1-K partially purified according to Kagimoto and Uyeda [19] and Van Schaftingen et al. [2].

2.2. Experimental study of the 6PF-1-K/Fru-1,6-P_2ase cycle

The experiments were carried out in a buffer consisting of 50 mM HEPES-KOH (pH 7.2), 100 mM KCl, 5 mM KH_2PO_4, 2.5 mM $MgCl_2$, 1 mM NH_4Cl, 0.2 mM EGTA, 0.25 mM PMSF, 2.5 mM DTT, and 1 μM E-64 at 37°C. The experimental set-up was described in detail elsewhere [17]. The reaction chamber is fed continuously with cell-free extract and substrates from two reservoirs through two separate channels. Glycolytic flow was restricted to the upper part of the pathway by omission of NAD. The maximum activities of the enzymes in the reaction chamber, the concentration of the substrates in the influx solution, and the residence time characterizing the flow rate through the reactor (τ=reactor volume/pump rate) can experimentally be varied. The state of the system is defined by the actual metabolite concentrations which are determined in samples taken from the efflux solution leaving the reaction chamber. Hexose monophosphates, Fru-1,6-P_2, ATP, and pyruvate (Prv) were determined by standard enzymatic methods [20]. Fru-2,6-P_2 was determined by the pyrophosphate-dependent 6PF-1-K activation assay [21].

2.3. Mathematical modelling

The dynamics of the reaction web formed in the cell-free extract is described by a set of ordinary differential equations for the metabolite concentrations

$$dx/dt = f(x,p)$$

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which take into consideration the substrate conversion by the enzymes involved as well as flow processes which are related to the experimental design. x denotes the vector of the metabolite concentrations and p the vector of control parameters of the system [17]. Qualitative dynamic properties of the reaction system as the multiplicity and the stability of the stationary states were analyzed [22].

3. Results

In Fig. 1 the results of an experiment are shown in which bistability is demonstrated. The experiment was started with high initial concentrations of PEP and ATP without added Fru-2,6-P_2. A stable stationary state is approached in phase I which is characterized by high concentrations of hexose monophosphates and ATP. The latter is connected with low

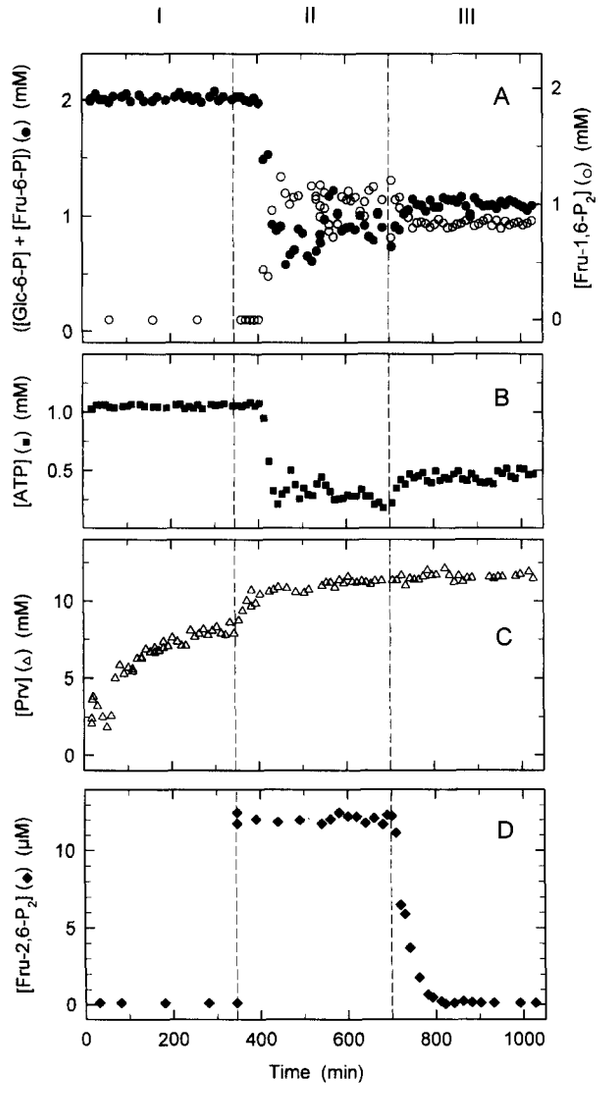

Fig. 1. Experimental evidence for an irreversible transition induced by fructose 2,6-bisphosphate: Time evolution of metabolite concentrations in rat liver cytosol. (A) ●, ([Glc-6-P]+[Fru-6-P]); ○, [Fru-1,6-P_2]. (B) ■, [ATP]. (C) △, [Prv]. (D) ◆, [Fru-2,6-P_2]. Control parameters – enzyme activities: $V_{6PF-1-K} = 0.38$ U/ml; $V_{Fru-1,6-P2ase} = 1.1$ U/ml; $V_{pyruvatekinase} = 39$ U/ml; substrate supply: $([Glc\text{-}6\text{-}P] + [Fru\text{-}6\text{-}P])_{IN} = 2$ mM; $[ATP]_{IN} = 1$ mM; $[PEP]_{IN} = 11$ mM; $\tau = 41$ min. Phases I and III: $[Fru\text{-}2,6\text{-}P_2]_{IN} = 0$; phase II: $[Fru\text{-}2,6\text{-}P_2]_{IN} = 10$ μM.

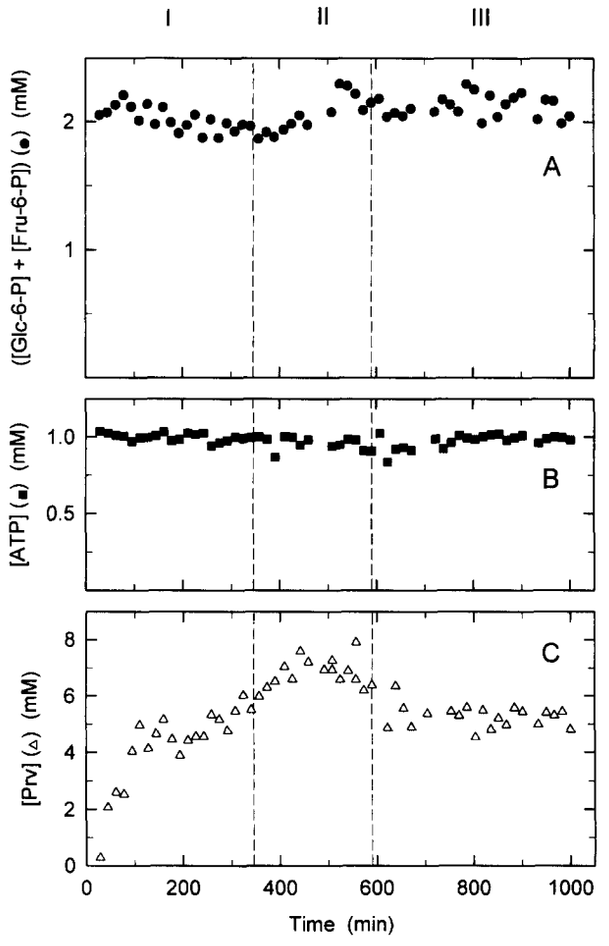

Fig. 2. Low concentrations of Fru-2,6-P_2 are not capable of inducing transitions to low-energy states: Time evolution of metabolite concentrations in rat liver cytosol. (A) ●, ([Glc-6-P] + [Fru-6-P]); ○, [Fru-1,6-P_2]. (B) ■, [ATP]. (C) △, [Prv]. Experimental conditions refer to those in Fig. 1 with the exception of $[PEP]_{IN} = 7$ mM. Phases I and III: $[Fru\text{-}2,6\text{-}P_2]_{IN} = 0$; phase II: $[Fru\text{-}2,6\text{-}P_2]_{IN} = 0.2$ μM.

concentrations of ADP and AMP and, correspondingly, a high adenylate energy charge. This state is therefore denoted as high-energy stationary state (S_H). In phase II the system was perturbed by the addition of Fru-2,6-P_2 to both the influx and the reaction chamber. This causes primarily efficient activation of 6PF-1-K and inhibition of Fru-1,6-P_2ase. In the beginning of phase II, transitorily homeostasis of ATP is observed, which is at the expense of PEP. When the rephosphorylation capacity of pyruvate kinase is exhausted, the concentrations of ATP and hexose monophosphates decrease. A steady state characterized by low concentrations of ATP and hexose monophosphates is approached. It differs qualitatively from that approached in phase I and is denoted as the low-energy stationary state (S_L). In phase III, the experimental conditions applied in phase I were restored. Fru-2,6-P_2 is removed from the reaction chamber according to first-order kinetics. Nevertheless, also in the absence of Fru-2,6-P_2 a low-energy stationary state is approached which differs qualitatively from that observed in phase I.

The qualitative dynamic properties of the stationary states as calculated from a mathematical model of the reaction network are shown in Fig. 3A. In the absence of Fru-2,6-P_2 two

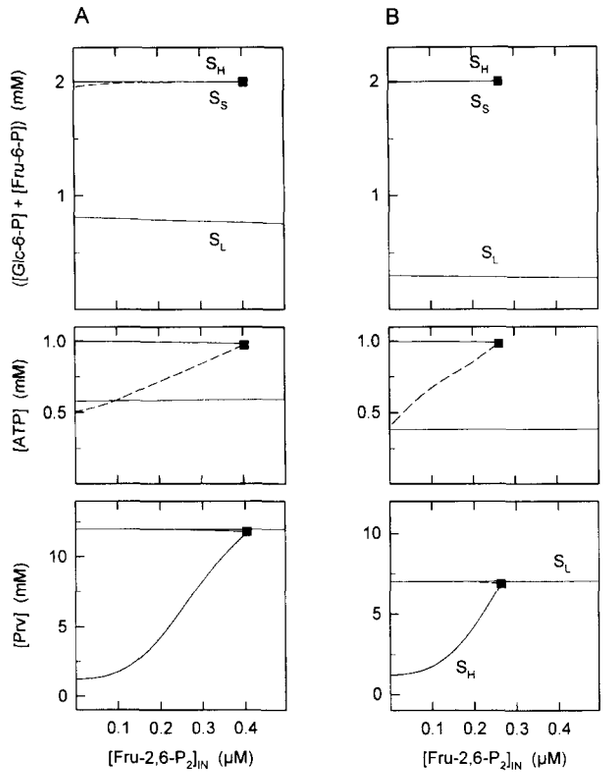

Fig. 3. Stationary metabolite concentrations as a function of [Fru-2,6-P_2]$_{IN}$. The control parameters refer to Figs. 1A and 2B. S_H, high-energy steady states; S_L, low-energy states; S_S, saddle points. ■, limit points; solid curves, stable stationary states; dashed curves, unstable steady states.

Alternative stationary states (S_H and S_L) coexist with a saddle point (S_S). Which of the stable states is actually approached depends on the initial conditions or on the history of the system. The initial metabolite concentrations used in Fig. 1 ensure the attainment of an high-energy stationary state in phase I. By the addition of Fru-2,6-P_2 in phase II of the experiment a limit point is crossed, at which the high-energy branch of stationary states disappears. Hence, the metabolic state switches to the remaining low-energy branch of stationary states. At decreasing levels of Fru-2,6-P_2 the metabolic states rest on the low-energy branch of stationary states which persists even in the absence of Fru-2,6-P_2.

In Fig. 2 the results of a control experiment are shown which differs from that in Fig. 1 essentially by the lower influx concentration of PEP used throughout the experiment and the much lower concentration of Fru-2,6-P_2 applied in phase II. In accordance with model calculations shown in Fig. 3B this concentration of Fru-2,6-P_2 is unable to induce a transition towards the low-energy stationary state. Hence, in phase III the same high-energy steady state as that approached in phase I is attained.

4. Discussion

The investigation of glucose metabolism in cell-free extracts enables the analysis of well-defined reaction networks by adding and/or removing enzymes and cofactors and by determining the composition of substrate supply. Removal of 6PF-2-K/Fru-2,6-P_2ase from the cell-free extract simplifies the reaction web by excluding the interaction of the Fru-6-P/Fru-1,6-P_2 and the Fru-6-P/Fru-2,6-P_2 cycle and makes Fru-2,6-P_2 experimentally adjustable. This reduces the biochemical and dynamic complexity and provides the basis to study experimental paradigms of stepwise increasing complexity.

Already in this simplified metabolic web complex dynamic pattern as multistability emerge [17]. Fru-2,6-P_2 is able to trigger transitions between alternative stationary states. In contrast to what is observed in classical bistable systems, only one limit point can be passed by changes of the concentration of Fru-2,6-P_2. Hence, variations of Fru-2,6-P_2 may cause transitions from the high-energy branch of stationary states (S_H) towards the low-energy branch of stationary states (S_L), while the reverse transition cannot be attained by any change of Fru-2,6-P_2. The transitions $S_H \Rightarrow S_L$ induced by Fru-2,6-P_2 are irreversible. Consequently, from any state on the low-energy branch of steady states transitions to high-energy steady states can only be induced by mechanisms which do not act via Fru-2,6-P_2 on the 6PF-1-K/Fru-1,6-P_2ase cycle. Possible candidates for such mechanisms acting in vivo are changes in the concentrations of adenine nucleotides or changes of the substrate supply.

The occurrence of irreversible transitions in bistable metabolic systems creates a qualitatively new dynamic pattern. Irreversible transitions induce a preference between coexisting functionally different stationary states in that the system is finally arrested in one stationary mode. To overcome such restrictions in cellular metabolism, mechanisms of multiple control of substrate cycles had to be developed to rescue dynamic flexibility.

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