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INFLUENCE OF FRUCTOSE 2,6-BISPHOSPHATE ON THE PHOSPHOFRUCTOKINASE/FRUCTOSE 1,6-BISPHOSPHATASE CYCLE

Wolfgang Schellenberger, Klaus Eschrich and Eberhard Hofmann

Institute of Physiological Chemistry, Karl-Marx-University,
Liebigstrasse 16, 7010 Leipzig,
German Democratic Republic

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SUMMARY: In a reconstituted enzyme system multiple stationary states and oscillatory motions of the substrate cycle catalyzed by phosphofructokinase and fructose 1,6-bisphosphatase are significantly influenced by fructose 2,6-bisphosphate. Dependending on the initial conditions, fructose 2,6-bisphosphate was found either to generate or to extinguish oscillatory motions between glycolytic and gluconeogenic states. In general, stable glycolytic modes are favored because of the efficient activation of phosphofructokinase by this effector. The complex effect of fructose 2,6-bisphosphate on the rate of substrate cycling correlates with its synergistic cooperation with AMP in the activation of phosphofructokinase and inhibition of fructose 1,6-bisphosphatase.

The fructose 6-phosphate (F-6-P)/fructose 1,6-bisphosphate (F-1,6-P_2) cycle is of importance for the coordination of glycolysis and gluconeogenesis. When phosphofructokinase (PFK) and fructose 1,6-bisphosphatase (FBPase) are simultaneously active a net hydrolysis of ATP occurs which is critical for ATP-homeostasis and reduces the net flow of substrate in either the glycolytic or gluconeogenic direction. Because of this the control of the two cycle enzymes by epigenetic, covalent and allosteric mechanisms is of great significance (1-3). With respect to the allosteric regulation the reciprocal effects of AMP and fructose 2,6-bisphosphate (F-2,6-P_2) on the two enzymes are of special interest (4). While AMP is directly connected with the glycolytic pathway by the action of adenylate kinase, the formation and degradation of F-2,6-P_2 is effected through a bypass of glycolysis catalyzed by a bifunctional enzyme, the PFK 2/FBPase 2, subject to covalent regulation (5,6). Despite extensive

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knowledge about the regulation of the individual cycle enzymes, the control of the F-6-P/F-1,6-P_2 cycle is still a matter of debate.

Recently, in a reconstituted enzyme system operating far from thermodynamic equilibrium we have been able to show the occurrence of sustained oscillations in the PFK/FBPase cycle (7). In addition to these two enzymes also pyruvate kinase (PK), adenylate kinase (AK) and glucose 6-phosphate isomerase (GPI) are constituents of the reconstituted reaction system. The generation of oscillations was found to be caused by the reciprocal kinetic effects of AMP on the activities of PFK and FBPase.

This paper deals with the effects of F-2,6-P_2 on the dynamic properties of the stationary states and the oscillatory pattern of the system.

MATERIALS AND METHODS

Conditions: The experiments were carried out at 25° C in 0.1 M imidazole/HCl buffer, pH 6.6, containing 20 mM K_2HPO_4, 20 mM $MgCl_2$, 100 mM KCl and 2.5 mM mercaptoethanol. The substrates, coenzymes and buffer substances were purchased from Boehringer, Mannheim (FRG). F-2,6-P_2 was prepared according to (8). All other chemicals were of analytical grade.

Enzymes: Phosphofructokinase (EC 2.7.1.11) was prepared from baker's yeast according to (9) and fructose 1,6-bisphosphatase (EC 3.1.3.11) from pig liver according to (10). Pyruvate kinase (EC 2.7.1.40), adenylate kinase (EC 2.7.4.3) and glucose 6-phosphate isomerase (EC 5.3.1.9) were products of Boehringer, Mannheim (FRG).

Experimental approach: The cooperation of PFK, FBPase, PK, AK and GPI is investigated in an open and homogeneous system (7). The reaction chamber is fed continuously from two reservoirs containing the substrates and enzymes, respectively. According to the purpose of the individual experiment the influx concentrations of the substrates ($[F\text{-}6\text{-}P]_{IN}$, $[F\text{-}1,6\text{-}P_2]_{IN}$, $[PEP]_{IN}$, $[ATP]_{IN}$), the maximum activities of the enzymes (V_{PFK}, V_{FBPase}, V_{PK}) and the rate of flow through the reactor (τ = reactor volume/pump rate) can experimentally be varied. The metabolite concentrations in the reaction chamber were determined in the efflux solution (7). In comparison with PFK, FBPase and PK the maximum activities of AK and GPI are in excess to maintain quasi-equilibration of their reactants (1,7).

Mathematical model: The time evolution of the metabolite concentrations in the reaction chamber is governed by a set of differential equations, which take the flow processes and enzymatic conversions into account. The latter are characterized by rate laws deduced from the kinetics of the individual enzymes (7,9,10). The stationary solutions of the model are classified according to the stability and multiplicity of the stationary states. The occurrence of oscillations correlates with unique and unstable stationary solutions. Functionally, stationary states can be categorized according to their energy status (high energy states, H, low energy states, L)(11) and according to the direction of the net flow of substrates (glycolytic states, GLY, if $v_{PFK} > v_{FBPase}$, and gluconeogenic states, GN, if $v_{PFK} < v_{FBPase}$).

RESULTS AND DISCUSSION

In Fig. 1A a parameter plane of the enzyme system is shown formed from the maximum activities of PFK and FBPase and from the influx concentrations of F-6-P and F-1,6-P_2. For the calculation the total concentration of the hexosephosphates (([F-6-P]+[G-6-P]+[F-1,6-P_2]) = ([F-6-P]$_{IN}$+[F-1,6-P_2])$_{IN}$ = 6 mM) and the sum of the maximum activities of PFK and FBPase (V_{PFK}+V_{FBPase} = 1.5 U/ml) were kept constant. In this plane F-2,6-P_2 is absent. In the horizontal hatched area alternate steady states occur, while the states outside of this region are unique. By the solid curve (v_{PFK} = v_{FBPase}) glycolytic and gluconeogenic states are separated. The unique unstable stationary states occurring at intermediate maximum activities of PFK and FBPase correlate to the emergence of sustained oscillations. At low V_{PFK} the character of the states is not influenced by variation of the substrate supply, whereas at high V_{PFK} glycolytic and gluconeogenic states can arise, when the supply of substrate is changed. Both the stable gluconeogenic and the stable glycolytic stationary states are of the high energy type, i.e. they exhibit ATP-homeostasis (11).

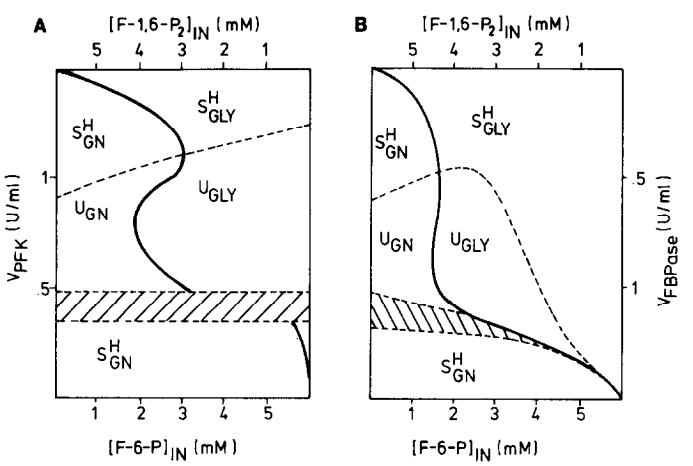

Fig. 1. Dynamic and functional characterization of the stationary states. Control parameters: $[ATP]_{IN}$ = 3 mM, $[PEP]_{IN}$ = 9 mM, τ = 40 min, V_{PK} = 7.5 U/ml, V_{PFK} + V_{FBPase} = 1.5 U/ml, ($[F\text{-}6\text{-}P]_{IN}$ + $[F\text{-}1,6\text{-}P_2]_{IN}$) = 6 mM. S and U mean stable and unstable steady states, respectively. For further explanation see the text.
A: Absence of F-2,6-P_2; B: $[F\text{-}2,6\text{-}P_2]_{IN} = 3.3\times10^{-4}\times[F\text{-}6\text{-}P]_{IN}$

The kinetic organization of the PFK and FBPase reactions is caused mainly by the reciprocal effects of AMP on the activities of the two enzymes. Hence, ATP-homeostasis is related to a low activity of PFK. This diminishes the rate of substrate cycling in the gluconeogenic states. ATP-homeostasis in the glycolytic states is attained at the expense of their efficiency, this means, that the rate of substrate cycling is not negligible. In the unstable steady states a high rate of substrate cycling is proceeding as calculated from the steady state activities of the enzymes (not shown). However, during the sustained oscillations correlated to these unstable states glycolytic and gluconeogenic phases alternate. By the oscillations the mean rate of substrate cycling is diminished when compared with that of the corresponding unstable steady state.

Yeast PFK was found to be efficiently activated by F-2,6-P_2 (12). Furthermore, it could be shown, that AMP and F-2,6-P_2 act synergistically in the activation of the enzyme. F-2,6-P_2 is capable of increasing both the AMP sensitivity and its maximum extent of activation. Accordingly, the half activation constant for F-2,6-P_2 is decreased by AMP (13). Interactions between AMP and F-2,6-P_2 have also been reported in their inhibitory action on liver FBPase (14,15). Owing to the reciprocal actions of the two effectors on PFK and FBPase a rather complex pattern of the F-6-P/F-1,6-P_2 cycle can be expected when they are present simultaneously.

In Fig. 2 the effect of F-2,6-P_2 on an oscillatory state of the cycle is shown. The experimental conditions in phase I correspond to the center of Fig. 1A. According to the instability of the stationary state, sustained oscillations emerge. As theoretically predicted these proceed in a two-dimensional subspace of the phase space. The concentration of phosphoenolpyruvate (PEP) remains permanently below the limit of detection. Then, in phase II F-2,6-P_2 is added and a constant level of 2 uM maintained in the course of the experiment. The oscillations are rapidly extinguished and a stable stationary low energy glycolytic state is approached by damped oscillations.

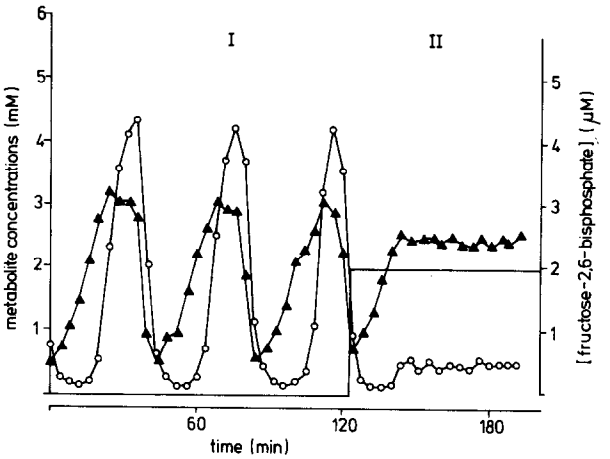

Fig. 2. The effect of fructose 2,6-bisphosphate on sustained oscillations of the fructose 6-phosphate/fructose 1,6-bisphosphate cycle.
The control parameters correspond to the center of Fig. 1.
$[F\text{-}6\text{-}P]_{IN} = [F\text{-}1,6\text{-}P_2]_{IN} = 3$ mM, $V_{PFK} = V_{FBPase} = 0.75$ U/ml.
I: $[F\text{-}2,6\text{-}P_2] = 0$, II: $[F\text{-}2,6\text{-}P_2] = 2$ μM.
▲ :[ATP], ○ :([F-6-P] + [G-6-P]).

In the experiment shown in Fig. 3 the initial conditions fit to the region of stable gluconeogenic stationary states as explained in Fig. 1A. Accordingly, a gluconeogenic state is approached in phase I. Then, $F\text{-}2,6\text{-}P_2$

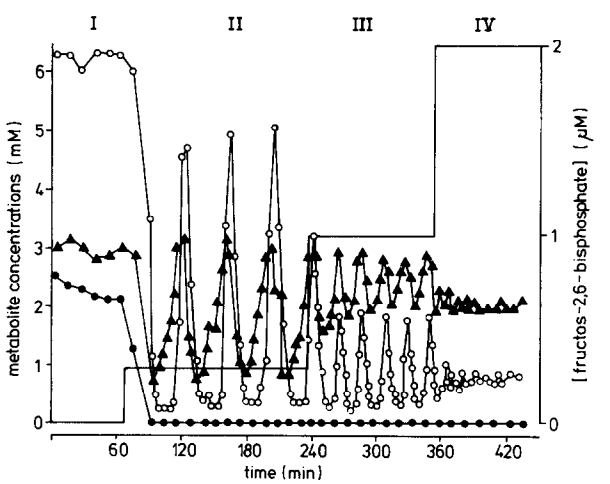

Fig. 3. The effect of fructose 2,6-bisphosphate on a stable gluconeogenic state.
$[F\text{-}6\text{-}P]_{IN} = 5$ mM; $[F\text{-}1,6\text{-}P_2]_{IN} = 1$ mM, $V_{PFK} = 0.3$ U/ml, $V_{FBPase} = 1.2$ U/ml.
The other parameters are as in Fig. 1. In I, II, III, and IV the $[F\text{-}2,6\text{-}P_2]$ are 0, 0.3, 1.0, and 2.0 μM, respectively.
▲ :[ATP], ○ :([F 6-P] + [G 6-P]), ● :[PEP].

is added and its concentration stepwise increased. At low concentration (0.3 μM) the effector gives rise to sustained oscillations. When its concentration is increased to 1 μM the oscillation frequency is enhanced and the amplitudes of the ATP and hexosemonophosphate periodicities are decreased. After further increase (2 μM) F-2,6-P_2 abolishes the oscillatory state and leads to a stable stationary state. This state is glycolytic and of low energy character.

The experiments demonstrate, that depending on the actual conditions F-2,6-P_2 is capable of either inducing or abolishing sustained oscillations. Obviously, glycolytic states are always favored by this effector. This is due to its activating action on PFK and its inhibiting action on FBPase.

Fig. 1B shows the parameter plane in the presence of F-2,6-P_2. The influx rate of the effector was linked with that of F-6-P, resembling the coupling between the glucose supply and the level of F-2,6-P_2 observed in liver (3). From a comparison with Fig. 1A it is obvious that F-2,6-P_2 increases the domain of the glycolytic states and decreases the domain of alternate stationary states as well as that of the oscillations and of the gluconeogenic states.

In contrast to AMP, which owing to the presence of adenylate kinase is a dynamic constituent of the reaction system, in this contribution the level of F-2,6-P_2 is directly fixed by the experimental conditions. For a deeper insight into the relationships between AMP and F-2,6-P_2 into the regulation of the F-6-P/F-1,6-P_2 cycle, F-2,6-P_2 must also be made a dynamic variable of the system. This is achieved by integration of the PFK 2/FBPase 2 into the reconstituted enzyme system. The actions of this enzyme in the in vitro system are currently under investigation.

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