Bistability in the Isocitrate Dehydrogenase Reaction: An Experimentally Based Theoretical Study

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ABSTRACT The enzyme isocitrate dehydrogenase (IDH, EC 1.1.1.42) can exhibit activation by one of its products, NADPH. This activation is competitively inhibited by the substrate $NADP^+$, whereas NADPH competes with $NADP^+$ for the catalytic site. Experimental observations briefly presented here have shown that if IDH is coupled to another enzyme, diaphorase (EC 1.8.1.4), which transforms NADPH into $NADP^+$, the system can attain either one of two stable states, corresponding to a low and a high NADPH concentration. The evolution toward either one of these stable states depends on the time of addition of diaphorase to the medium containing IDH and its substrate $NADP^+$. We present a theoretical and numerical analysis of a model for the IDH-diaphorase bienzymatic system, based on the regulatory properties of IDH. The results confirm the occurrence of bistability for parameter values derived from the experiments. Depending on the total concentration of $NADP^+$ plus NADPH and the concentration of IDH, the system can either admit a single steady state or display bistability. We obtain an expression for the critical time t^*, before which diaphorase addition leads to the lower steady state and after which addition of the enzyme leads to the upper steady state of NADPH. The analysis is extended to the case where the second substrate of IDH, isocitrate, is consumed in the course of the reaction without being regenerated. Bistability occurs only as a transient phenomenon in these conditions.

INTRODUCTION

Regulated enzymatic reactions can exhibit a variety of nonlinear phenomena, the most common of which are sustained oscillations and multistability. The latter behavior denotes the coexistence between multiple (generally two) stable steady states (bistability) or stable rhythms (birhythmicity). In addition to their possible physiological significance, such self-organization phenomena have the additional interest of providing insights into the regulatory mechanisms that underlie oscillatory or bistable behavior.

Bistability has been observed experimentally in a number of biochemical systems (Degn, 1968; Naparstek et al., 1974; Eschrich et al., 1980; Frenzel et al., 1995). Theoretical studies of biochemical models show that the phenomenon can originate in enzymatic reactions either from positive feedback by a reaction product or from substrate inhibition (Edelstein, 1970; Boiteux et al., 1980; Lisman, 1985; Goldbeter and Moran, 1987; Hervagault and Canu, 1987). Similarly, bistability has recently been shown to occur, as a result of autocatalysis, in theoretical models for the conformational transition of the PrP^{Sc} protein in prion diseases (Kacser and Rankin Small, 1996; Laurent, 1996). Enzyme activation by a reaction product can also give rise to oscillatory behavior. Thus glycolytic oscillations in yeast and muscle result from the autocatalytic regulation of phosphofructokinase (Hess and Boiteux, 1971; Goldbeter and Caplan, 1976), whereas positive feedback on adenylate cyclase is involved in the generation of cAMP oscillations in *Dictyostelium* cells (Martiel and Goldbeter, 1987; Goldbeter, 1996).

Other nonlinear processes may also give rise to bistability or oscillations. Thus the phenomena may arise in membrane processes from cooperative transport properties (Blumenthal et al., 1970), variable permeability (Hahn et al., 1973), or electrostatic interactions (Katchalsky and Spangler, 1968; Mulliert et al., 1990). The physiological interest for cellular dynamics of transitions between multiple steady states further stems from the possibility that the phenomenon might also occur in genetic regulatory networks (Babloyantz and Nicolis, 1972; Thomas and d'Ari, 1990), and could thereby play a major role in cell differentiation. Moreover, bistability is repeatedly encountered in immunological models which show that key aspects of the immune response may be comprehended in terms of transitions between multiple steady states (Kaufman and Thomas, 1987; Lefever et al., 1989; Segel and Jäger, 1994). Finally, other theoretical studies point to a possible role of bistability in tumor progression; thus the phenomenon might arise as a result of autocrine stimulation in cells responding to growth factors for which they possess membrane receptors (Schepers, 1997; Schepers and Goldbeter, manuscript in preparation).

Although bistability is by now a well-established phenomenon in chemical and biological systems, the number of experimental examples in biochemistry remains reduced. Given that enzymatic systems provide the best opportunities for studying the phenomenon in vitro in well-defined conditions, it is therefore of interest to find new examples of bistable behavior in enzymatic reactions. Indeed, the coexistence between two stable steady states raises the question

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of how the system eventually falls into one or the other state. This question, which is of general relevance to all cases of bistable behavior, can best be studied in in vitro enzymatic systems admitting a multiplicity of steady-state solutions.

In this paper we present a theoretical analysis of bistability in a cyclical bienzymatic system involving isocitrate dehydrogenase (IDH) (isocitrate dehydrogenase (NADP), threo-Ds-Isocitrate: NADP oxidoreductase (decarboxylating), EC 1.1.1.42.) and diaphorase (lipoyl dehydrogenase, EC 1.8.1.4.). Bistable behavior arises from the regulation of IDH by its substrate $NADP^+$ and product NADPH; these regulatory properties were characterized in a series of in vitro experimental studies (Carlier and Pantaloni, 1976a,b). We compare the theoretical results with unpublished experimental observations carried out on this enzyme reaction in former work by one of the authors (Carlier, 1976). The experimental observations show that when IDH is coupled to the second enzyme, diaphorase, which transforms NADPH into $NADP^+$, the system can attain either one of two stable states, corresponding to a low and a high NADPH concentration. The evolution toward either one of these stable states depends on the time of addition of diaphorase to the medium containing IDH and its substrate $NADP^+$. Such a demonstration by the addition of the same amount of one of the two enzymes of a cyclical bienzymatic system at different times represents an original approach for bringing to light the existence of different basins of attraction associated with the two stable steady states.

We first present the bienzymatic system and, in a brief section, the main experimental observations on bistability. We then propose a theoretical model for the IDH-diaphorase coupled reactions, based on the regulatory properties of IDH. We show that depending on the total concentration of $NADP^+$ plus NADPH and the concentration of IDH, the system can either admit a single steady state or display bistability. We obtain an expression for the critical time t^*, before which diaphorase addition leads to the lower steady state and after which addition of the enzyme leads to the upper steady state of NADPH. These results are compared with experimental observations.

To be maintained in the course of time, oscillations or multistability must occur in systems displaced from thermodynamic equilibrium. However, as shown for oscillatory behavior, these phenomena can also occur as long-lived transients in closed isothermal systems, before the unique state of thermodynamic equilibrium is eventually reached (Lefever et al., 1988). We conclude our study by considering the effect of the consumption of the second substrate of IDH, isocitrate, on the occurrence of bistability and bring to light the transient nature of the phenomenon in these conditions.

The analysis of bistability in the IDH-diaphorase bienzymatic system is of special interest for metabolic regulation, particularly in cases where different enzymes catalyze opposite reactions. Thus the switch between glycolysis and gluconeogenesis has been discussed in terms of multiple steady states arising from the operation of phosphofructokinase and fructose-1,6-bisphosphatase in opposite directions (Boiteux et al., 1980; Eschrich et al., 1980; Schellenberger and Hervagault, 1991; Frenzel et al., 1995). More generally, the present results bear on the possible occurrence of bistable behavior in a large variety of cellular regulatory systems, given that many important physiological processes—including those involving some oncogene products, signal transduction, and cell proliferation—are controlled by kinases and phosphatases that are organized in a cyclical manner, like the enzymatic system considered here.

BIENZYMATIC SYSTEM CONSIDERED FOR BISTABILITY

Central to the occurrence of bistability in biochemical systems are the nonlinearities associated with the regulatory properties of enzymes. It is therefore of particular significance that enzyme activation and inhibition by different chemical species can occur in the IDH reaction. In an earlier study, Carlier and Pantaloni (1976a) already suggested that the nonlinear kinetics of IDH is capable of producing oscillations in an open system. The enzyme studied by these authors was the dimeric, cytoplasmic form of $NADP^+$-dependent isocitrate dehydrogenase purified from beef liver (Carlier and Pantaloni, 1973). The substrates of the IDH reaction are $NADP^+$ and two possible forms of isocitrate: either complexed with a divalent metal or in a tribasic form (Carlier and Pantaloni, 1976b); these substrates are transformed by the enzyme into NADPH and α-ketoglutarate plus CO_2.

When the enzyme operates in a reaction medium deprived of divalent metal cations, it exhibits autocatalytic activation by the product NADPH (Carlier et al., 1976). In the absence of NADPH, the enzyme possesses a minimal catalytic activity; this minimum turnover (k_0) leads to the accumulation of product, which in turn brings about catalytic activation and the establishment of the maximum turnover (k_{max}) of the fully active enzyme. This activation seems to be due to the binding of NADPH to an activation site different from the catalytic site of the enzyme (Carlier and Pantaloni, 1976a). The experiments to be described below were carried out with IDH purified from beef liver. Activation by NADPH has also been reported for the mitochondrial isoform of IDH (Sanner and Ingebretsen, 1976), which has structural properties different from those of the cytoplasmic isoenzyme (Illingworth and Tipton, 1970; Colman et al., 1970; Henderson, 1973).

Besides the autocatalytic regulation, the product NADPH and the substrate $NADP^+$ both inhibit the enzyme from beef liver, through different mechanisms. Because of the similarity between the two species, each one can interfere with binding of the other, both at the activation and the catalytic sites. Thus NADPH gives rise to product inhibition through competing at the catalytic site with the substrate $NADP^+$ when it is present in concentrations much higher than those

that produce the activation phenomenon, whereas $NADP^+$ can interfere at the activation site with the binding of NADPH, retarding in this way the autocatalytic activation of the enzyme (Carlier and Pantaloni, 1976a).

The second substrate, isocitrate, does not influence the kinetics of the enzyme through inhibition or activation. Indeed, the enzyme displays a saturation kinetics of the Michaelis-Menten type with respect to isocitrate (Carlier and Pantaloni, 1976b). For a relatively long time in the course of the experiments, the relative variation of isocitrate concentration is much less than that of the $NADP^+$ and NADPH concentrations. Therefore, in the theoretical study carried out below, we shall assume, to a first approximation, that the isocitrate concentration remains constant. This assumption will be relaxed in the last section of this study, where we show that when the isocitrate consumption is taken into account, the final state is reached slowly enough to allow for the observation of (transient) multistability.

The overall reaction scheme for IDH is represented in Fig. 1, which also indicates the regulatory interactions. The values of the dissociation constant characterizing the activation and inhibition by NADPH and $NADP^+$, as well as the Michaelis constant for $NADP^+$, are listed in Table 1. Moreover, the Michaelis constant of isocitrate is 35 μM; the values of the minimum and maximum turnover numbers k_0 and k_{max} are, respectively, 2 s^{-1} and 14.3 s^{-1}. All of these parameter values were determined experimentally (Carlier, 1976; Carlier and Pantaloni, 1976a).

To ensure regeneration of the substrate of IDH, a second enzyme is used in the experiments (see Fig. 2). This enzyme, diaphorase, functions as a NADPH oxidase and thus transforms NADPH into $NADP^+$, reversing the effect of IDH. Oxygen bubbled into the reaction medium was used as an electron acceptor. The K_m of diaphorase for NADPH seems to be less than 1 μM, and the catalytic constant equals 0.8 s^{-1} (Carlier, 1976).

When the two enzymes work in the same reaction medium, NADPH and $NADP^+$ are interconverted at various velocities; at the same time, isocitrate is transformed into α-ketoglutarate without being resynthesized. In Fig. 2 the reaction is illustrated in the presence of the two enzymes, without considering the consumption of the second substrate of IDH, isocitrate, which is supposed to remain constant (see above). The concentration of O_2 is likewise considered to remain constant in the reaction medium. In such conditions, the cyclical system of Fig. 2 will reach a steady state when the rates of the reactions catalyzed by IDH and by diaphorase become equal.

TABLE 1 Experimental values of the dissociation constants for $NADP^+$ and NADPH at the catalytic and activation sites

Ligand	Catalytic site	Activator site
$NADP^+$	$K_m^I = 17\ \mu M$	$K_I' = 7\ \mu M$
NADPH	$K_I = 35\ \mu M$	$K_A = 0.25\ \mu M$

References: Carlier (1976) and Carlier and Pantaloni (1976a).

EXPERIMENTAL OBSERVATIONS ON BISTABILITY

Before developing a theoretical model for the IDH-diaphorase reactions, we shall present experimental observations (Carlier, 1976) that point to the occurrence of bistability in this bienzymatic system. For multiple steady states to occur, IDH and diaphorase must be coupled in the reaction medium. The evolution of the IDH reaction is determined in the presence of a given initial concentration of the substrate $NADP^+$ alone. During the reaction, $NADP^+$ is transformed by IDH into NADPH, but the total concentration $[NADP^+] + [NADPH]$ remains constant. When diaphorase is added, the system reaches a steady state characterized by constant levels of $NADP^+$ and NADPH. In the experiments, the final steady state is determined as a function of the time at which diaphorase is added. For every experiment, the kinetics of IDH is also determined in the absence of diaphorase.

In the first experiment (Fig. 3), referred to below as experiment A, a given amount of diaphorase is added at different times indicated by arrows, corresponding to dif-

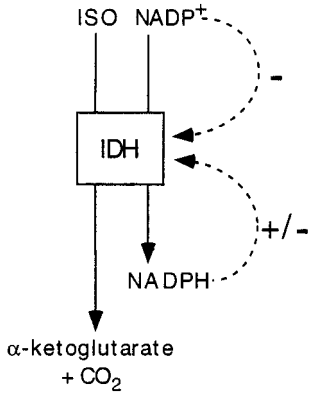

FIGURE 1 The isocitrate dehydrogenase (IDH) reaction. The enzyme transforms tribasic isocitrate (ISO) and $NADP^+$ into α-ketoglutarate plus CO_2 and NADPH, respectively. Also indicated are the inhibition by $NADP^+$ and the inhibition and activation by NADPH.

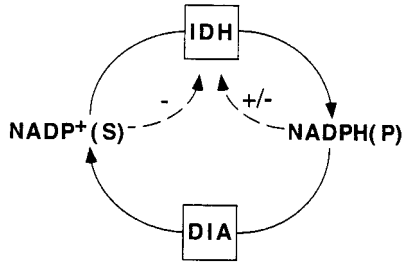

FIGURE 2 Bienzymatic system considered for bistability. The cyclical system for the reversible interconversion of $NADP^+$ into NADPH contains the two enzymes isocitrate dehydrogenase (IDH) and diaphorase (DIA). The transformation of the second substrate of IDH, isocitrate, into α-ketoglutarate is not considered (see text). The dashed arrows refer to the positive and negative regulation of IDH by $NADP^+$ and NADPH in the absence of divalent metal ions.

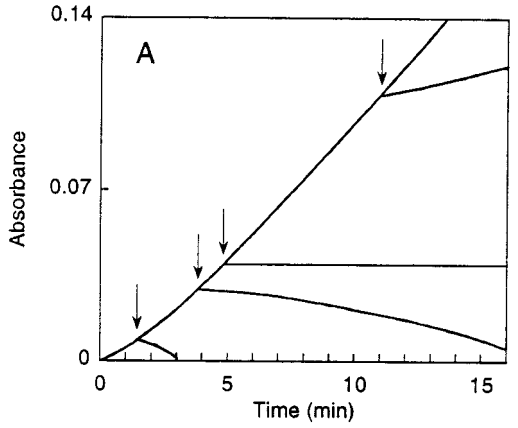

FIGURE 3 Experimental demonstration of bistability (*experiment A*). Diaphorase (0.031 μM) was added in a reaction medium containing IDH (0.002 μM) at the times indicated by arrows. As in the following experiment (Fig. 4), the kinetics of NADPH production by IDH, measured by the absorbance at 340 nm (0.006 unit = 1 μM NADPH), is also determined as a control in the absence of diaphorase. The net production of NADPH vanishes when diaphorase is added at t^*, precisely when NADPH has reached the value of the intermediate steady state. If diaphorase is added at times smaller than or greater than t^*, the NADPH concentration decreases or increases to the low- or high-concentration steady state, respectively. The cuvette contained 4.46 mM DL-isocitrate and 220 μM $NADP^+$ (Carlier, 1976).

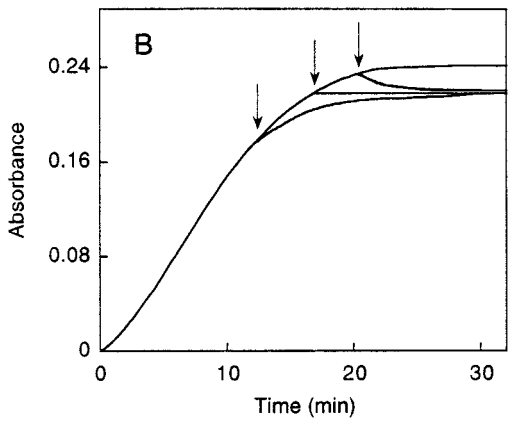

FIGURE 4 Evolution to a high NADPH steady state (*experiment B*). The effect of diaphorase addition on NADPH production is shown for a different set of experimental conditions. Diaphorase was added at the times indicated by arrows, at the final concentration of 0.021 μM. The cuvette contained 8.3 mM DL-isocitrate, 53 μM $NADP^+$, and 0.014 μM isocitrate dehydrogenase (Carlier, 1976).

ferent NADPH concentrations. When diaphorase is added before a time t^* of ~5 min from the beginning of the reaction, the net production of NADPH decreases and the system reaches a steady state corresponding to a product concentration slightly greater than zero. If diaphorase is added after this critical time, the production of NADPH grows until it reaches a steady state (not shown in this figure), at a much higher value of the product concentration. These results indicate that an unstable steady state probably separates these two stable steady states of low and high NADPH concentration; this unstable steady state would be reached if diaphorase were added exactly at the time t^*. The experiment shows that in this case the resulting NADPH concentration is stabilized for at least 10 min at the value reached at that time. Obtaining an analytical expression for the critical time t^* will be one of the goals of our theoretical analysis.

In the second series of experiments (Fig. 4), referred to below as experiment B, the establishment of the stable steady state corresponding to the higher NADPH level is shown. In this case, the system evolves toward the same stable steady state independently of the time of diaphorase addition. In both Figs. 3 and 4, the sigmoidal shape of the curve representing the NADPH concentration in the absence of diaphorase reflects the autoactivation of IDH by its product NADPH.

In this cyclical biochemical system, a steady state is reached when the two enzymes function at the same rate; the existence of three steady states thus corresponds to three distinct situations in which the rate of NADPH production by IDH equals the rate of NADPH consumption by diaphorase. Because of the autocatalytic regulation of IDH, one can assume that the low NADPH steady state is established when the NADPH concentration has not reached a level sufficient to activate IDH, which thus functions at a low rate with the minimum turnover number k_0. In contrast, the steady state characterized by a high NADPH concentration is reached when IDH functions with a turnover number approaching k_{max}. The activation phenomenon, which affects the maximum rate of the enzyme, is then limited by the fact that the NADPH concentration is sufficiently high to inhibit IDH because of competition with its substrate $NADP^+$. At an intermediate NADPH level, inhibition by NADPH is not significant, whereas activation by NADPH is not maximal. Further increase in NADPH is then prevented by the presence of diaphorase, and by the competition between $NADP^+$ and NADPH for the (activating) regulatory site of IDH.

The intermediate steady state turns out to be unstable because any displacement from it in the direction of greater NADPH concentration enhances the autoactivation phenomenon, so that the rate of IDH will exceed the rate of diaphorase, whereas any displacement in the direction of smaller NADPH concentration further lowers the activation phenomenon, because the rate of diaphorase then exceeds that of IDH.

In the following section we account for these experimental observations by analyzing a kinetic model for bistability in the coupled IDH-diaphorase reactions, based on the regulatory properties of IDH.

THEORETICAL ANALYSIS

Kinetic model

Two kinetic schemes were proposed by Carlier and Pantaloni (1976a) to describe the properties of IDH. In the first

one, activation is explained by the existence of a very rapid association-dissociation equilibrium of NADPH in the intermediate enzyme-oxalosuccinate-NADPH complex, whereas in the second model activation is assumed to be due to the asymmetry between the subunits of this dimeric enzyme with regard to the rate of binding of NADPH in the presence of isocitrate.

From a mathematical point of view, the two reaction schemes are strictly identical; the transformation of $NADP^+$ into NADPH by IDH is governed by the rate function $f(S, P)$:

$$f(S, P) = k_0 \frac{E_I S}{K_m^I(1 + (P/K_I)) + S} \times \times \left[1 + \frac{k_1}{k_0} \frac{P}{K_A(1 + (S/K_I')) + P}\right] \quad (1)$$

where P, S, and E_I indicate the concentrations of NADPH, $NADP^+$ and IDH, respectively, and the other constants are defined in Table 1.

The first term in the product accounts for the kinetic properties of the enzyme working with the minimum turnover number k_0, and includes the competitive inhibition of IDH by its product NADPH (P) with an inhibition constant K_I. The term between square brackets describes the change in turnover number due to the activation by the reaction product NADPH with an activation constant K_A (this activation leads to the maximum turnover $k_{max} = k_0 + k_1$), and includes the inhibition by the substrate $NADP^+$ (S) characterized by the inhibition constant K_I'.

Diaphorase can be regarded as a Michaelian enzyme, showing a high affinity for its substrate NADPH. The kinetic expression describing the transformation of NADPH into $NADP^+$ by diaphorase is thus given by the function $g(P)$:

$$g(P) = \frac{k_{cat} E_{II} P}{K_m^{II} + P} \quad (2)$$

where $k_{cat} = 0.8\ s^{-1}$ and E_{II} indicates the diaphorase concentration, whereas K_m^{II} denotes the Michaelis-Menten constant of the enzyme. In the following we have chosen $K_m^{II} = 0.01\ \mu M$ (experimentally, the value of K_m^{II} was not determined precisely, but, as indicated above, was found to be less than 1 μM).

The evolution of the system consisting of the two coupled enzyme reactions can be represented by the following system of two nonlinear kinetic equations:

$$\frac{dP}{dt} = f(S, P) - g(P)$$
$$\frac{dS}{dt} = -f(S, P) + g(P) \quad (3)$$

where $f(S, P)$, given by Eq. 1, represents the transformation of $NADP^+$ into NADPH catalyzed by IDH, and $g(P)$, given by Eq. 2, pertains to the reverse transformation catalyzed by diaphorase. As the enzymes operate in a closed system with respect to $NADP^+$ and NADPH, the following conservation relation holds:

$$S + P = Z \quad (4)$$

where Z is the total, constant concentration of $NADP^+$ plus NADPH.

Equation 4 allows us to reduce the two differential equations (Eq. 3) to a single kinetic equation for P, whereas the second variable S is expressed as a function of P and of the total concentration Z:

$$S = Z - P$$
$$\frac{dP}{dt} = f(Z - P, P) - g(P). \quad (5)$$

By definition, the system is at steady state when $dP/dt = 0$, i.e., when both enzymes operate at the same velocity, so that the net production of NADPH and $NADP^+$ goes to zero:

$$f(Z - P, P) - g(P) = 0. \quad (6)$$

The NADPH concentration at steady state is given by the solutions to Eq. 6. In solving that algebraic equation, two physical constraints must be satisfied: solutions have to be real and positive to represent chemical concentrations, and must be lower than Z to satisfy the conservation relation in Eq. 4. The latter relation then yields the steady-state concentration of $NADP^+$.

Once Z is fixed, Eq. 6 reduces to a third-order polynomial equation in P (Eq. A1 in Appendix 1), which admits one or three physically acceptable steady states, depending on parameter values.

Bistability

To test whether the system of Eq. 5 can account for experimental observations on the dynamics of the IDH-diaphorase system, we have performed numerical simulations corresponding to the experiments described in Figs. 3 and 4.

Clearly, the results of experiment A (Fig. 3) correspond to the existence of three steady-state solutions, whereas experiment B (Fig. 4) can be explained by a situation of bistability or by the existence of only one physically acceptable solution. In Figs. 5 and 6 we have simulated these experiments by numerical integration of Eq. 5, using the experimental values of the constants reported in Table 1.

Let us first address the case where diaphorase (E_{II}) is added only when a high level of NADPH is reached because of the activity of IDH alone; good agreement between experiment B and numerical simulations is then obtained (Fig. 6). In contrast, for the simulation (Fig. 5) of experiment A, in which diaphorase is added sooner, we had to choose a value for E_I that was twice as high as the one used in the experiment (0.004 μM versus 0.002 μM); in these conditions, good agreement with the experiment is also

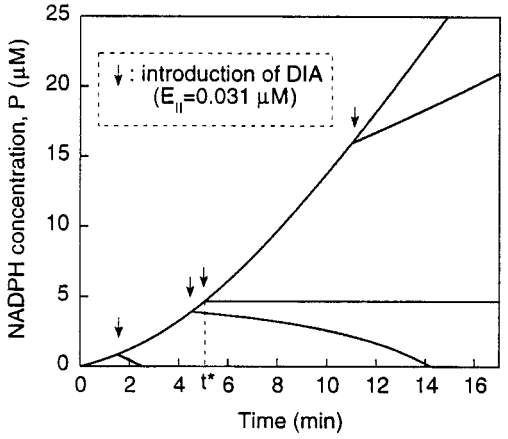

FIGURE 5 Bistability: simulation of experiment A (Fig. 3). The curves are generated by numerical integration of Eqs. 3 or 5. Diaphorase (E_{II} = 0.031 μM) is added at different times, as in Fig. 3. The initial $NADP^+$ concentration (Z) is equal to 220 μM; the IDH concentration (E_I) is 0.004 μM; $k_0 = 2\ s^{-1}$; $k_1 = 12.3\ s^{-1}$; $K_m^I = 17\ \mu M$. The turnover number (k_{cat}) and the Michaelis constant (K_m^{II}) of diaphorase are 0.8 s^{-1} and 0.01 μM, respectively; other parameter values are given in Table 1.

obtained. The reason for the change in the E_I value will be discussed below. The results of numerical simulations in Fig. 5 confirm that the observations of Fig. 3 correspond to the coexistence of two stable steady states (bistability). This conclusion will be further corroborated by the construction of bifurcation diagrams, which also indicate that the results of Fig. 4 (experiment B) and Fig. 6 correspond to the evolution toward a unique stable steady state.

The conditions for the occurrence of bistability can be clarified by means of bifurcation diagrams established as a function of the main control parameters such as Z and E_I. We first investigate the effect of the initial $NADP^+$ concentration (Z) at zero initial NADPH on the steady-state concentration of the product NADPH (P) for parameter

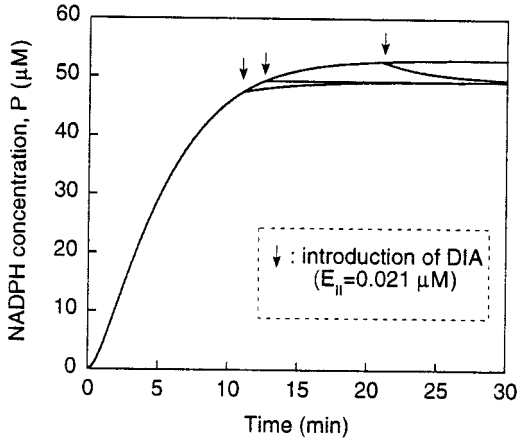

FIGURE 6 Evolution to a single, high NADPH steady state: simulation of experiment B (Fig. 4). The curves are obtained as in Fig. 5, with an initial $NADP^+$ concentration (Z) of 53 μM. Diaphorase (E_{II} = 0.021 μM) is added at different times, as in Fig. 4. The IDH concentration (E_I) is 0.014 μM. Other parameter values are as in Fig. 5.

values corresponding to the simulation (Fig. 5) of experiment A. Fig. 7 clearly shows that, if the initial concentration Z is less than ~24.7 μM, the system admits a single steady state corresponding to a low concentration of P. For Z larger than this threshold value, bistability occurs; there are indeed three steady states, two of which are stable—they correspond to a low and a high concentration of P, respectively—whereas the median state is unstable. At Z = 220 μM, a value that was used both in experiment A in Fig. 3 and in the corresponding simulation of Fig. 5, we obtain three steady states.

Shown in Fig. 8 is another bifurcation diagram yielding the steady-state concentration of NADPH as a function of the IDH concentration (E_I), for the two sets of parameter values corresponding to experiment A (Z = 220 μM) and B (Z = 53 μM). The data indicate that for Z = 220 μM, bistability occurs only for values of E_I ranging from 0.0024 μM to 0.0113 μM. For the E_I value of 0.002 μM used in experiment A, the results predict that there is only one low concentration steady state for NADPH. This is why we chose for the corresponding simulation (Fig. 5) the value E_I = 0.004 μM, so as to account for the experimental observation of bistability and to best reproduce the results of experiment A, particularly with respect to the value of t^* (see below).

Fig. 8 also indicates that for Z = 53 μM, there is no bistability for an IDH concentration equal to 0.014 μM, which is the value corresponding to experiment B. This is

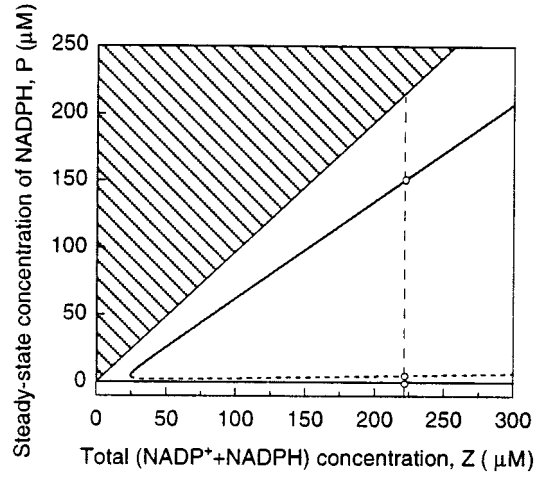

FIGURE 7 Bistability as a function of the total concentration (Z) of $NADP^+$ + NADPH. The NADPH steady-state concentration is shown as a function of Z. For low values of Z, the system admits only one stable steady state, whereas for medium and high Z values it admits three steady states: the low and high concentration steady states are stable, whereas the medium state is unstable (*dashed line*). For the value Z = 220 μM (*vertical dashed line*) considered in experiment A, the system admits three steady states. The curve is obtained by solving numerically the third-degree Eq. A1 obtained for P at steady state. Parameter values are as in Fig. 5. The hatched domain is not physically accessible, because NADPH > Z in that region. For the sake of clarity, the vertical axis extends slightly below zero, so that the lower branch of NADPH steady states, which lies slightly above zero, is not confused with the horizontal axis.

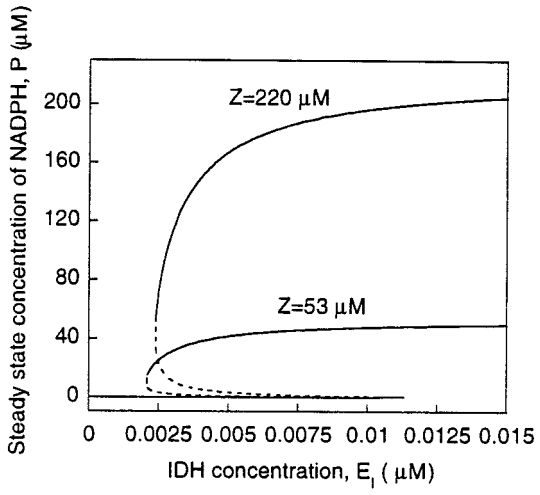

FIGURE 8 NADPH steady state as a function of IDH concentration, for the parameter values of experiments A ($Z = 220\ \mu M$, $E_{II} = 0.031\ \mu M$) and B ($Z = 53\ \mu M$, $E_{II} = 0.021\ \mu M$). For low and high values of the IDH concentration, the system admits a single steady state. In both cases, bistability occurs in a range of IDH concentration bounded by two critical values.

confirmed by Fig. 9, which is the equivalent of Fig. 7 for parameter values corresponding to experiment B. There the three steady states are obtained in a region that ranges from $Z = 2.94\ \mu M$ to $Z = 8.5\ \mu M$. Therefore, for $Z = 53\ \mu M$, the system can only reach a high-concentration steady state, as observed both in the experiment (Fig. 4) and in the model (Fig. 6).

Selection between the two stable steady states: critical time (t^*) for diaphorase addition

We have seen that the evolution toward either one of the two stable steady states in experiment A and in the corresponding simulation depends on the time of addition of diaphorase. When the enzyme is added before a critical time t^*, the system evolves toward the low-NADPH steady state, whereas adding diaphorase after t^* results in the evolution toward the high-concentration steady state. When diaphorase is added precisely at t^*, the unstable steady state corresponding to an intermediate NADPH level is maintained for a significant time interval (longer than 10 min in the experiment; see Fig. 3).

Clearly, the critical time t^* will depend on the amount of diaphorase added, as well as on the concentration of IDH and on the total amount of substrate (Z) present in the reaction medium. To obtain an analytical expression for t^*, we first determine by means of Eq. A1 the intermediate steady-state concentration of NADPH, denoted below by P_0, corresponding to the unstable steady state. Then we determine the time t^* needed for IDH to produce this amount P_0 of NADPH, starting from a zero level of NADPH (i.e., $NADP^+ = Z$ at time 0). Thus t^* is given by Eq. 7:

$$t^* = \int_0^{P_0} \frac{dP}{f(Z - P, P)} \tag{7}$$

The solution of this equation is given as Eq. A2 in Appendix 2.

Plotted in Fig. 10 is the value of the critical time t^* computed according to Eqs. 7 and A2 as a function of the IDH concentration, for three distinct concentrations of diaphorase. That each curve spans only a finite domain of IDH concentration results from the fact that bistability occurs in a range bounded by two critical value of the enzyme concentration (see Fig. 8). The theoretical curves indicate that the value of t^* diminishes as the IDH concentration rises, at a given diaphorase concentration. Moreover, t^* increases with diaphorase concentration at a given IDH level. The variation of t^* with IDH can be very significant. Thus, when the diaphorase concentration is equal to 0.031 μM, as in experiment A, the model predicts that t^* will drop from ~30 min to ~6 s when the IDH concentration is progressively varied over the range yielding bistability, i.e., 0.0024–0.0113 μM (see also Fig. 8 for $Z = 220\ \mu M$).

How t^* depends on Z is shown in Fig. 11 for three distinct values of the IDH concentration. The curves indicate that t^* passes through a minimum as Z increases, but the rise in t^*

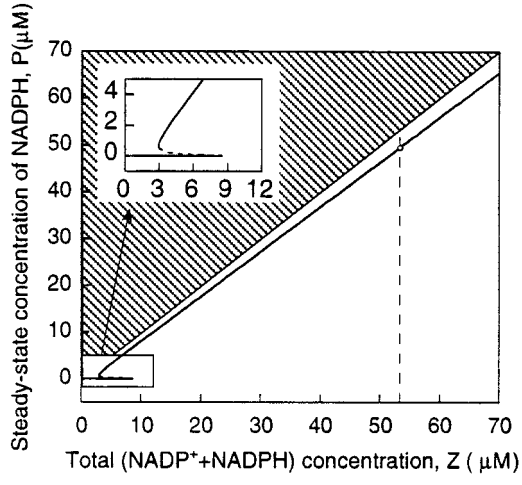

FIGURE 9 NADPH steady-state concentration as a function of the initial concentration of $NADP^+$ (Z), for the same parameter values as in experiment B. The situation is similar to the one described in Fig. 7, but in this case for high initial concentrations of substrate, because of the high IDH concentration considered in experiment B, the IDH velocity can equal the diaphorase rate only at a high NADPH level. When the value of Z is 53 μM (*vertical dashed line*), as considered in experiment B, the system admits a single steady state. For the same reasons as in Fig. 7, the horizontal axis has been displaced downwards; the hatched area has the same meaning as in Fig. 7.

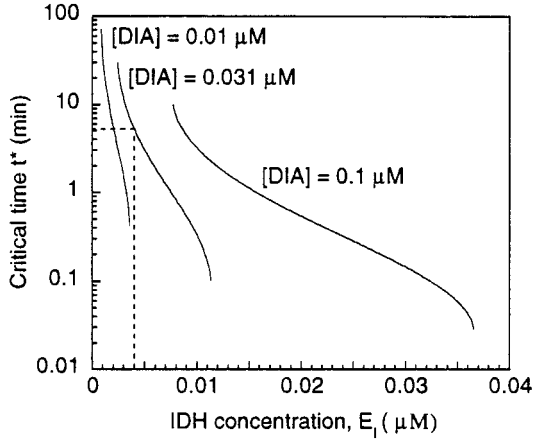

FIGURE 10 Dependence of the critical time t^* for diaphorase addition on the concentration of IDH. The theoretical curves are generated according to Eq. A2 for three distinct concentrations of diaphorase. Other parameter values are as in Fig. 5. The dashed line indicates the value of t^* corresponding to the value [IDH] = 0.004 μM used in the simulation (Fig. 5) of experiment A (Fig. 3).

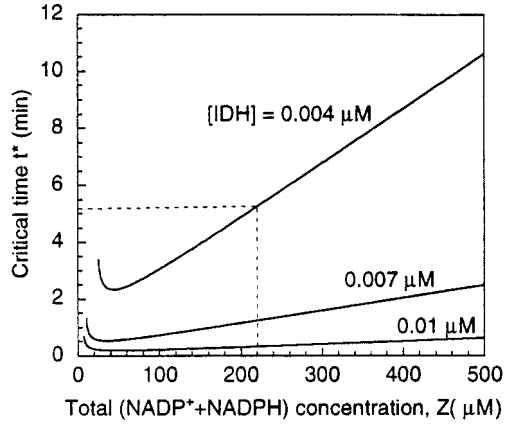

FIGURE 11 Dependence of the critical time t^* on the total concentration of substrate, Z. The theoretical curves are obtained by means of Eq. A2 for three distinct values of the IDH concentration. Other parameter values are as in Fig. 5. The critical time corresponding to Fig. 5 is indicated by the dashed line.

beyond this minimum is more significant at lower IDH levels.

Long-term behavior: effect of isocitrate consumption on bistability

So far we have assumed that the level of the second substrate of IDH, isocitrate, remains constant in the course of the experiments. To determine the long-time behavior, it is necessary to examine how the consumption of the second substrate, isocitrate, modifies the above analysis.

The kinetic equations become

$$\begin{aligned}\frac{dP_1}{dt} &= f_1(S_1, S_2, P_1) - g(P_1)\\ \frac{dP_2}{dt} &= f_1(S_1, S_2, P_1)\\ \frac{dS_1}{dt} &= -f_1(S_1, S_2, P_1) + g(P_1)\\ \frac{dS_2}{dt} &= -f_1(S_1, S_2, P_1)\end{aligned} \tag{8}$$

where S_1, S_2, P_1, and P_2 represent the concentrations of $NADP^+$, isocitrate, NADPH, and α-ketoglutarate, respectively. These equations have to be supplemented with the two conservation relations for [$NADP^+$] + [NADPH] and for [isocitrate] + [α-ketoglutarate]:

$$S_1 + P_1 = Z_1, \quad S_2 + P_2 = Z_2 \tag{9}$$

In the rate function f_1 for IDH, we now include the contribution of isocitrate, which was so far included as a constant close to unity in the numerator of function $f(S, P)$ in Eq. 1:

$$f_1(S_1, S_2, P_1) = k_0 \frac{E_I \cdot S_1 \cdot S_2}{[K_m^I(1 + (P_1/K_I)) + S_1] \cdot [K_m^{III} + S_2]} \left(1 + \frac{k_1}{k_0} \frac{P_1}{K_A(1 + (S_1/K_I')) + P_1}\right) \tag{10}$$

The experimental value of the Michaelis constant K_m^{III} for isocitrate is 35 μM. The value of Z_2 considered in experiments A and B is 4.46 mM and 8.3 mM, respectively.

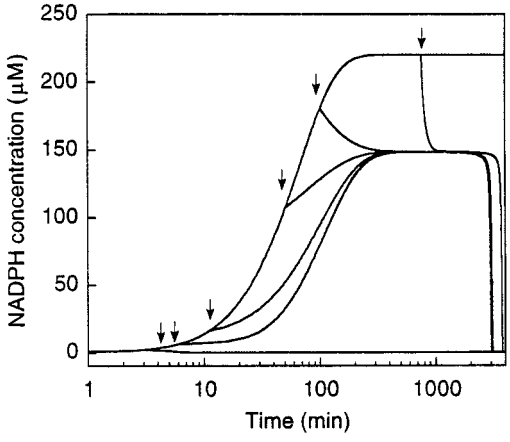

FIGURE 12 Simulation of experiment A, taking into account isocitrate consumption according to Eqs. 8 and 9. The initial $NADP^+$ concentration (Z) is equal to 220 μM; the initial isocitrate concentration equals 4.46 mM. Parameter values are E_I = 0.004 μM, E_{II} = 0.031 μM, k_{cat} = 0.8 s^{-1}, K_m^{II} = 0.01 μM. Other parameter values are given in Table 1. Depending on the time of introduction of diaphorase (*arrows*), the NADPH concentration reaches at first one of the two stable pseudo-steady-state concentrations. Such bistable behavior is only transient, since after a sufficiently long time, because of isocitrate consumption, the NADPH concentration becomes progressively smaller as NADPH is transformed into $NADP^+$.

Because isocitrate is progressively consumed and not regenerated in the course of the reaction, after some (relatively long) time the reaction will reach a unique, final steady state. Bistability can nevertheless occur as a transient phenomenon in these conditions (Fig. 12). Upon the addition of diaphorase, if the initial NADPH concentration is not high enough to activate IDH, after a short time on the order of some minutes, the NADPH level drops and the system reaches a low steady-state value. In contrast, if the enzyme is sufficiently activated upon addition of diaphorase, NADPH will reach a high steady-state concentration; the system will remain in that state as long as enough isocitrate is available. Because of the isocitrate consumption by IDH, the rate of the enzyme will eventually begin to decrease. This, however, will not occur before a long time, on the order of many hours, under the conditions of Fig. 12. The NADPH concentration will then begin to drop and will finally reach a low NADPH level.

DISCUSSION

Bistability is the phenomenon in which two distinct stable steady states can coexist in a given set of experimental conditions. By means of a theoretical model, we have analyzed the conditions in which bistability occurs in an enzymatic system containing isocitrate dehydrogenase (IDH), which transforms $NADP^+$ into NADPH, and diaphorase, which catalyzes the reverse transformation. We showed that the phenomenon of bistability occurs in this bienzymatic system as a result of the positive and negative regulation of IDH by NADPH and $NADP^+$. The theoretical analysis accounts for the experimental observation of bistable behavior reported in this paper.

Bistability has previously been shown to occur in a number of chemical or biochemical systems, both experimentally (Degn, 1968; Naparstek et al., 1974; Geiseler and Föllner, 1977; Eschrich et al., 1980; Ganapathisubramanian and Showalter, 1983; Frenzel et al., 1995) and in theoretical models (Edelstein, 1970; Babloyantz and Nicolis, 1972; Boiteux et al., 1980; Lisman, 1985; Goldbeter and Moran, 1987; Hervagault and Canu, 1987; Gray and Scott, 1994). The phenomenon is due to the nonlinearity of kinetic equations. In biochemistry such a nonlinearity primarily arises from the regulatory properties of the enzymes. Thus, substrate inhibition and activation of an enzyme by a reaction product are two prominent modes of control capable of producing a multiplicity of stationary states. Such positive and negative feedback processes also underlie the occurrence of bistability in the IDH reaction.

The present theoretical analysis accounts qualitatively and, in a large measure, quantitatively for the experimental observations on bistability in the coupled IDH-diaphorase reactions reported in this paper, with respect to the levels of NADPH reached and to the value of the critical time t^* for the selection between the two stable steady states upon the addition of diaphorase. Parameter values used in the numerical simulations match those determined from the available experiments (Carlier, 1976; Carlier and Pantaloni, 1973, 1976a,b; Carlier et al., 1976; see also text, Table 1, and the legends to Figs. 3 and 4), except for the value of the IDH concentration (E_I) in experiment A (Fig. 3), which was taken to be twice as large in the corresponding simulation (Fig. 5). This discrepancy could be due to an understimation of the value of the turnover number k_1; indeed, the range of E_I producing bistability is shifted to lower values when k_1 rises.

Generally, when bistability occurs, the domain of multiple steady states is bounded by two critical values of a given control parameter. Such a situation arises in Figs. 8 and 9, as a function of IDH concentration or of the total constant amount Z of [$NADP^+$ + NADPH]. In these conditions, a hysteresis phenomenon can be observed by varying the control parameter. Consider, for example, the curve obtained for $Z = 220\ \mu$M in Fig. 8. When starting from a low IDH concentration, the system moves to the right along the lower branch of the S-shaped curve giving the NADPH steady-state level. Then, when IDH reaches the critical value of 0.0113 μM, the system abruptly "jumps" to the upper branch of the steady-state curve corresponding to higher NADPH levels. When the IDH concentration is decreased, the system moves to the left on that branch; in a certain range, e.g., for IDH = 0.005 μM, two stable steady states of NADPH thus coexist, separated by an unstable one. When the IDH concentration is further decreased below the critical value of 0.0024 μM, the system "jumps" back to the lower branch.

Such a hysteresis is not always observed. Thus, in Fig. 7, where the steady-state level of NADPH is plotted as a function of Z, the upper critical value of Z has gone to infinity, in contrast to the situation in Fig. 9, which is established for another set of parameter values. In such conditions, the hysteresis cycle cannot be performed because, as Z is varied back and forth, the system can jump from the upper to the lower branch, but cannot undergo the reverse transition. Only if the system is subjected to an appropriate perturbation (e.g., by adding a suprathreshold amount of NADPH, which will also change the value of Z but not the steady-state curve) can it switch from the lower to the upper steady state. Such "irreversible transitions" have been described in other biochemical (Hahn et al., 1973; Hervagault and Canu, 1987; Fassy et al., 1992; Schellenberger and Hervagault, 1991) and chemical (Gray et al., 1991; Guidi and Goldbeter, 1997) models, and experimental evidence for the phenomenon has been presented (Frenzel et al., 1995; Coevoet and Hervagault, 1997).

Bistability is often demonstrated experimentally by showing the existence of a hysteresis phenomenon, as described above. Here the experimental procedure used (see Fig. 3) relied on a different approach based on the bienzymatic nature of the cyclical system considered. The method consists of adding the second enzyme, diaphorase, at different times, to a solution containing a given amount of

IDH. Two different stable steady states are reached, depending on whether the time of addition of diaphorase is less than or greater than a critical time t^* (Fig. 3). We used the present model to obtain an analytical expression for t^*, which allows us to predict how this critical time for diaphorase addition varies with the concentration of diaphorase and IDH, and with the total amount of $NADP^+ + NADPH$ present in the medium (Figs. 10 and 11).

The phenomenon of bistability described in the present experimental and theoretical study occurs in a partially closed system. We have taken into account the slow consumption of the second substrate of IDH, isocitrate, and showed by numerical simulations (Fig. 12) that under such conditions, when the behavior of the system is followed over a much more extended time period on the order of hours, bistability occurs as a long-lived transient phenomenon before the system reaches a unique final state. We have not attempted to determine the thermodynamic equilibrium state in the model, because this would require changing the kinetic equations to include all reversible steps in the bienzymatic reaction system. The present study suggests that it should be possible to observe bistability over significant periods of time in the IDH-diaphorase bienzymatic system, even if one of the substrates is consumed. Such a situation is analogous to the observation of chemical oscillations in the Belousov-Zhabotinsky reaction in a closed system. These slowly damped oscillations continue for up to 1 h before the system reaches equilibrium (see, for example, Field and Burger, 1985). The long duration of the oscillatory transient is due to the initial displacement of the system far from thermodynamic equilibrium (see also Lefever et al., 1988, for a thermodynamic analysis of oscillations in a closed system).

The present experimental and theoretical results show that the isocitrate dehydrogenase/diaphorase reaction system provides an additional biochemical example of bistability. This system is a good candidate for further experimental studies of reversible and irreversible transitions associated with bistable behavior. Furthermore, when the system is made open to a flux of isocitrate, the analysis indicates (Guidi and Goldbeter, 1998) that sustained oscillations may also develop in this system. This strengthens the interest of the IDH reaction as a potentially useful experimental model for studying nonlinear dynamic phenomena in biochemical systems.

That the present results may bear on the possible occurrence of bistability in a wide array of biochemical reactions stems from the fact that many cellular regulatory processes involve cycles of covalent modification of protein substrates, as illustrated by the most prevalent case of kinases and phosphatases. Indeed, phosphorylation-dephosphorylation regulatory systems, much as the IDH-DIA bienzymatic system, possess a cyclical organization. The study of covalent modification cycles has already revealed the potential for increased sensitivity, in the form of steep changes in the amount of phosphorylated protein as a function of the ratio of kinase versus phosphatase activity; the phenomenon, referred to as *zero-order ultrasensitivity*, occurs when the two enzymes operate under zero-order kinetic conditions (Goldbeter and Koshland, 1981, 1982). Such steep transitions were recently demonstrated in the mitogen-activated protein kinase cascade (Huang and Ferrell, 1996). As shown by the present work and other studies (Lisman, 1985; Hervagault and Canu, 1987), incorporating the regulation of any of the enzymes in a cyclical bienzymatic system by its substrate or product can readily lead from monostability to bistability. The present results might thus bear on the possible occurrence of bistability in a variety of key cellular processes related to signal transduction, the action of some oncogene products, gene expression, and cell proliferation, given that these processes are often regulated through multiple cycles of phosphorylation-dephosphorylation or of other types of protein covalent modification.

APPENDIX 1: STEADY-STATE CONCENTRATION OF NADPH

At steady state, Eq. 6 yields the following third-degree equation for the concentration of NADPH:

$$aP^3 + bP^2 + cP + d = 0 \tag{A1}$$

with

$$a = E_{II}k_{cat}K_m^I K_A + E_I K_I k_0 K_A - E_I K_I k_0 K_I' - E_{II}k_{cat}K_m^I K_I' - E_I K_I k_1 K_I' - E_{II}k_{cat}K_I K_A + E_{II}k_{cat}K_I K_I'$$

$$b = E_I K_I k_0 K_I' Z + E_I K_I k_0 K_A K_m^{II} + E_I K_I k_1 K_I' Z - E_I K_I k_0 K_A K_I' - E_I K_I k_1 K_I' K_m^{II} + E_{II}k_{cat}K_I K_A K_I' - 2E_I K_I k_0 K_A Z + 2E_{II}k_{cat}K_I K_A Z + + E_{II}k_{cat}K_m^I K_I K_A - E_{II}k_{cat}K_m^I K_A K_I' - E_{II}k_{cat}K_m^I K_I K_I' - E_{II}k_{cat}K_m^I K_A Z - E_{II}k_{cat}K_I K_I' Z - E_I K_I k_0 K_I' K_m^{II}$$

$$c = E_I K_I k_0 K_A K_I' Z + E_I K_I k_0 K_A Z^2 - 2E_I K_I k_0 K_A K_m^{II} Z + - E_{II}k_{cat}K_m^I K_I K_A K_I' + E_I K_I k_0 K_I' K_m^{II} Z - E_{II}k_{cat}K_m^I K_I K_A Z + E_I K_I k_1 K_I' K_m^{II} Z - E_{II}k_{cat}K_I K_A K_I' Z - E_I K_I k_0 K_A K_I' K_m^{II} + - E_{II}k_{cat}K_I K_A Z^2$$

$$d = E_I K_I k_0 K_A K_I' K_m^{II} Z + E_I K_I k_0 K_A K_m^{II} Z^2$$

As indicated in the text, this equation admits one or three physically acceptable solutions, depending on parameter values.

APPENDIX 2: CRITICAL TIME t^* FOR DIAPHORASE ADDITION

For the parameter values considered (see text and figure legends), integration of Eq. 7 yields

$$t^* = \frac{1}{840E_1}\left(\frac{81}{166}P_0 - 17\frac{(4Z^2 + 141Z + 35)}{143Z + 5}\ln\left(\frac{Z - P_0}{Z}\right)\right) + \frac{1}{840E_1}\left(\frac{1025}{55112}\frac{(233Z^2 + 5586Z + 27685)}{143Z + 5}\ln\left(\frac{35 + 5Z + 996P_0}{35 + 5Z}\right)\right) \quad \text{(A2)}$$

where P_0 is the intermediate (unstable) steady-state value of the product NADPH, obtained from Eq. A1. Note that P_0 is itself a function of [IDH], diaphorase concentration, and Z.

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