

# Thermodynamic Analysis of the Emergence of New Regulatory Properties in a Phosphoribulokinase-Glyceraldehyde 3-Phosphate Dehydrogenase Complex\*

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Emmanuelle Graciet, Sandrine Lebreton, Jean-Michel Camadro, and Brigitte Gontero‡

From the Institut Jacques Monod, UMR 7592 CNRS-Universités Paris VI–VII, 2 Place Jussieu, 75 251 Paris Cedex 05, France

Glyceraldehyde 3-phosphate dehydrogenase and phosphoribulokinase exist as stable enzymes and as part of a complex in *Chlamydomonas reinhardtii*. We show here that phosphoribulokinase exerts an imprinting on glyceraldehyde 3-phosphate dehydrogenase, which affects its catalysis by decreasing the energy barrier of the reactions with NADH or NADPH by  $3.8 \pm 0.5$  and  $1.3 \pm 0.3$  kJ·mol<sup>-1</sup>. Phosphoribulokinase and glyceraldehyde 3-phosphate dehydrogenase within the complex are regulated by NADP(H) but not by NAD(H). The activities of the metastable phosphoribulokinase and glyceraldehyde 3-phosphate dehydrogenase released from the complex preincubated with NADP(H) are different from those of the metastable enzymes released from the untreated complex. NADP(H) increases phosphoribulokinase and NADPH-glyceraldehyde 3-phosphate dehydrogenase activities with a  $K_{0.5}$ (NADP) of  $0.68 \pm 0.16$  mM and a  $K_{0.5}$ (NADPH) of  $2.93 \pm 0.87$  mM and decreases NADH-dependent activity. 1 mM NADP increases the energy barrier of the NADH-glyceraldehyde 3-phosphate dehydrogenase-dependent reaction by  $1.8 \pm 0.2$  kJ·mol<sup>-1</sup> and decreases that of the reactions catalyzed by phosphoribulokinase and NADPH-glyceraldehyde 3-phosphate dehydrogenase by  $3 \pm 0.2$  and  $1.2 \pm 0.3$  kJ·mol<sup>-1</sup>, respectively. These cofactors have no effect on the independent stable enzymes. Therefore, protein-protein interactions may give rise to new regulatory properties.

Enzymes do not exist as separate, independent entities in the cell but interact with many components, including membranes and other proteins, to form more complex structures. These supramolecular edifices are involved in many metabolic pathways (1) such as the Benson-Calvin cycle, which is responsible for CO<sub>2</sub> assimilation in plants (2–5). Protein-protein interactions can affect several of the enzymes belonging to this pathway. We have purified (6) a bi-enzyme complex (460 kDa) from a green alga, *Chlamydomonas reinhardtii*. This complex is made up of two tetramers of glyceraldehyde 3-phosphate dehydrogenase (EC.1.2.1.13) and two dimers of phosphoribulokinase (EC.2.7.1.19). These two enzymes from the Benson-Calvin cycle do not catalyze consecutive reactions. The kinase catalyzes the ATP-dependent phosphorylation of ribulose 5-phosphate to form ribulose 1,5-bisphosphate, the CO<sub>2</sub> acceptor

in photosynthetic organisms; the dehydrogenase catalyzes the reversible reduction and dephosphorylation of 1,3-bisphosphoglyceric acid (BPGA)<sup>1</sup> to give glyceraldehyde 3-phosphate and phosphate using NADPH or NADH (7). We have also purified independent, stable, phosphoribulokinase (PRK) (8) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which are not included in the complex.

The main functional advantage of supramolecular structures is “channeling” (9), the direct transfer of intermediates from one active site to the next active site and so avoiding diffusion into the cell medium. Any functional advantage provided by the physical association of enzymes that do not catalyze consecutive reactions is likely to result from a change in their kinetic properties. For example, in the green alga, the association of two enzymes in a complex enables oxidized PRK to be active, although this enzyme is inactive in its stable, independent state (10). In spinach, the specific catalytic activity of ribulose-bisphosphate carboxylase-oxygenase (EC.4.1.1.39) is about 10 times greater when it is within a five-enzyme complex than when it is alone (11). Similarly, reduced thioredoxin can act on independent PRK and on the same enzyme within the five-enzyme complex, but the latter is activated 12 times faster than the enzyme in its independent state (12). These differences are linked to a transfer of information (13) between the enzymes in supramolecular edifices.

The dissociated or metastable enzymes produced by dissociating a multi-enzyme complex by dilution may retain for a while some features of the conformation they had in the associated form. This is the imprinting effect. It has been shown that PRK produced by dissociation of the complex from *C. reinhardtii*, transiently adopts a metastable conformation highly competent for catalysis as a consequence of the conformational constraints exerted on it by GAPDH (10, 14). A theory developed earlier describes in terms of statistical thermodynamics how the arrangements and mutual constraints of identical or different polypeptide chains may facilitate catalysis by PRK (15).

Conversely, PRK may be expected to exert an imprinting on GAPDH, and one of the aims of this work was to answer this question. The imprinting effects could also be modified by metabolites. NAD(P)(H) has been shown to regulate an oligomeric 600-kDa form of GAPDH in spinach (16, 17), and this form is absent from *C. reinhardtii*. Thus, one may wonder (i) if the GAPDH inserted in the complex we have purified could be regulated by these metabolites and (ii) if they alter the imprinting effects between these two enzymes. This report examines these questions using kinetic and thermodynamic analyses.

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‡ To whom correspondence should be addressed. Tel.: 33-1-44-27-63-56; Fax: 33-1-44-27-57-16; E-mail: meunier@ijm.jussieu.fr.

<sup>1</sup>The abbreviations used are: BPGA, 1,3 bisphosphoglyceric acid; PRK, phosphoribulokinase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

## EXPERIMENTAL PROCEDURES

**Enzyme Purification**—The complex was purified to apparent homogeneity from *C. reinhardtii* (WM3<sup>-</sup>) cells as previously described (6). Stable PRK was purified according to Ref. 8. The purified complex was reduced for 1 h with 20 mM dithiothreitol and dissociated. The resulting enzymes were applied to a DEAE-Trisacryl column (2.5 × 13 cm) equilibrated in 30 mM Tris, 4 mM EDTA, 0.1 mM NAD, 5 mM dithiothreitol, pH 7.9 (column flow rate of 40 ml/h). The washout fractions contained pure stable GAPDH. The purified enzymes were stored at -80 °C in 10% aqueous glycerol.

**Preincubation Experiments**—The purified complex and the stable enzymes were incubated at 30 °C with different concentrations of NAD(P)(H) in the absence of any reducing agent. Aliquots were withdrawn after specific times and placed in the assay cuvettes at the same temperature.

**Enzyme Assays and Protein Measurements**—The assay cuvettes used to test NADH- or NADPH-dependent activities of GAPDH contained 2 units of pyruvate kinase, 1 mM phosphoenolpyruvate, 1 mM ATP, 0.17 mM NAD(P)H, 2 mM phosphoglyceric acid, 4.5 units of phosphoglycerate kinase, and 5 mM dithiothreitol (18). PRK activity was coupled to NADH oxidation via pyruvate kinase and lactate dehydrogenase. The assay cuvette contained 3 units of lactate dehydrogenase, 2 units of pyruvate kinase, 2 units of ribose isomerase, 1 mM phosphoenolpyruvate, 1 mM ATP, 1 mM ribose 5-phosphate, 0.17 mM NADH, and 5 mM dithiothreitol (19). All activities were recorded using a Pye Unicam UV2 spectrophotometer. The experimental data were fitted to theoretical curves using Sigma Plot 5.0. Protein concentration was assayed with the Bio-Rad protein dye reagent, using bovine serum albumin as a standard (20).

**Aggregation States of the Enzymes**—The dissociation or integrity of the complex in the preincubation mixture was checked by native PAGE performed on 4–15% minigels in a Amersham Biosciences Phastsystem apparatus. The proteins were transferred to nitrocellulose filters (0.45 μm; Schleicher & Schüll) by passive diffusion for 16 h. The filters were then immunoblotted with a rabbit antiserum directed against spinach PRK. Antibody binding was revealed using alkaline phosphatase (21).

**Quantification of the Imprinting Effect**—The free energies of activation of a given chemical process catalyzed by free enzymes (stable forms) and by the same enzymes within a complex may be compared to determine the fraction of the energy transferred from one protein to another and thus used to alter the rate of the chemical process. The enzymes released when this complex dissociates (“metastable” forms) may retain some features of their conformation in the associated state. This is the “imprinting effect,” and it can be described thermodynamically as follows.

$$\Delta G_{\mu}^{\ddagger} = \Delta G_{\sigma}^{\ddagger} + (\Psi_{\gamma} - \Psi_{\tau}) \quad (\text{Eq. 1})$$

$$k_{\mu} = k_{\sigma} \exp(-(\Psi_{\gamma} - \Psi_{\tau})/RT) \quad (\text{Eq. 2})$$

where  $\Delta G_{\mu}^{\ddagger}$  and  $\Delta G_{\sigma}^{\ddagger}$  are the free energies of activation of a given process performed by the metastable ( $\mu$ ) and stable ( $\sigma$ ) forms of the same enzyme. The difference  $(\Psi_{\gamma} - \Psi_{\tau})$  is the fraction of energy that is stored after the dissociation of the supramolecular edifice, where  $\Psi_{\gamma}$  and  $\Psi_{\tau}$  represent the stabilization/destabilization energies of the metastable form relative to the stable form in both its ground state ( $\gamma$ ) and its transition state ( $\tau$ ). This difference may be positive or negative, and the rate of the chemical process may decrease or increase.

Let us assume that a ligand X is mixed with the complex and that the activity of the metastable form released from this complex is modified. The free energy of activation  $\Delta G_{\mu'}^{\ddagger}$  of a given chemical process performed by this metastable form ( $\mu'$ ) and the free energy of activation  $\Delta G_{\mu}^{\ddagger}$  of the metastable form ( $\mu$ ) obtained in the absence of the ligand X may be compared.

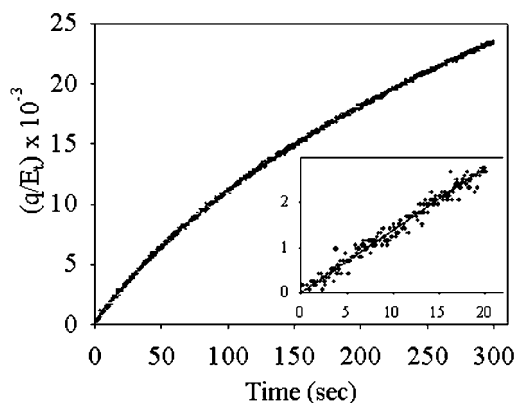
$$\Delta G_{\mu'}^{\ddagger} = \Delta G_{\mu}^{\ddagger} + \Delta X \quad (\text{Eq. 3})$$

$$k_{\mu'} = k_{\mu} \exp(-\Delta X/RT) \quad (\text{Eq. 4})$$

where  $\Delta X$  is the fraction of energy attributed to the presence of the ligand X. This difference may be negative or positive, and the metastable form released from a complex incubated with this ligand X may have more or less activity ( $k_{\mu'}$ ).

**Free Energies of Activation of the Metastable Forms and the Stable Form**—Free energies of activation are given in the following equation.

$$\Delta G_i^{\ddagger} = RT \ln \frac{k_B T}{h k_i} \quad (\text{Eq. 5})$$



**FIG. 1. Progress curve of NADH-dependent activity of GAPDH.** The complex (2.4 nM) was placed in the reaction mixture of NADH-GAPDH activity, and the appearance of products was monitored. The experimental points were fitted to a pseudo-first order equation ( $q/E_t = a \times (1 - e^{-bt})$ ), where  $a = 3.2 \times 10^4 \pm 60$ ,  $b = 4.2 \times 10^{-3} \pm 4 \times 10^{-4} \text{ s}^{-1}$  and to a Neet equation (Equation 6 in the main text), where  $p(1) = 46.5 \pm 0.3 \text{ s}^{-1}$ ,  $p(2) = 151.2 \pm 0.3 \text{ s}^{-1}$ , and  $p(3) = 0.01 \pm 10^{-4} \text{ s}^{-1}$ . Because the curvature was finally linked to the approach to equilibrium, the first 20 s were fitted to a straight line (rate constant of  $153.2 \pm 1.1 \text{ s}^{-1}$ ) (shown in the inset).

where  $k_B$  ( $1.38 \times 10^{-23} \text{ J}\cdot\text{K}^{-1}$ ) and  $h$  ( $6.63 \times 10^{-34} \text{ J}\cdot\text{s}$ ) are the Boltzmann and the Planck constants, respectively,  $k_i$  is the catalytic rate constant of the different forms,  $k_{\mu}$  is the catalytic rate constant of the metastable form released from the complex in the absence of ligand X,  $k_{\mu'}$  is the catalytic rate constant of the metastable form released from the complex incubated with ligand X, and  $k_{\sigma}$  is the catalytic rate constant of the stable form.

## RESULTS

**Determination of the Imprinting Effect of PRK on GAPDH**—Biphasic curves that could be fitted to a pseudo-first order function or to an equation including a burst were obtained when NADH-dependent activity of GAPDH within the complex was assayed (Fig. 1). Because ECL experiments showed that the complex was dissociated at the beginning of the reaction and because the form resulting from the complex dissociated by dilution in the absence of substrates also displayed a kinetic transient (data not shown), the latter could not be linked to the dissociation of the complex in the reaction mixture.

The curve being nicely fitted to a pseudo-first order function, we confirmed that the kinetic transient was correlated to an approach to equilibrium (22) by measuring the NADH-dependent activity with enzyme concentrations ranging from 1.2 to 12 nM. The time curves normalized for enzyme concentration overlapped and had the same shape (data not shown), which confirmed that the kinetic transient was due to the approach to equilibrium. The initial linear part of the curves was therefore attributed to an average steady-state rate of the dissociated form of GAPDH, the so-called metastable form originating from the complex, of  $147 \pm 5 \text{ s}^{-1}$ . This average value was much higher than that obtained with stable GAPDH ( $33 \pm 8 \text{ s}^{-1}$ ).

There was a lag on the onset of the NADPH-dependent activity of GAPDH within the complex (Fig. 2, top panel). The lag was not linked to pH or temperature jump. ECL experiments showed that the complex was not fully dissociated at the beginning of the reaction (data not shown). Moreover, the lag was still present when the complex was first dissociated in the medium without substrates (Fig. 2, bottom panel, curve 1) but was lost in the presence of BPGA (Fig. 2, bottom panel, curve 2). Therefore, the latent phase of the dissociated form is the consequence of an activation by BPGA (23) of the GAPDH, with the conversion of a less active form into a more active one. The curves may be fitted to a Neet equation (22).

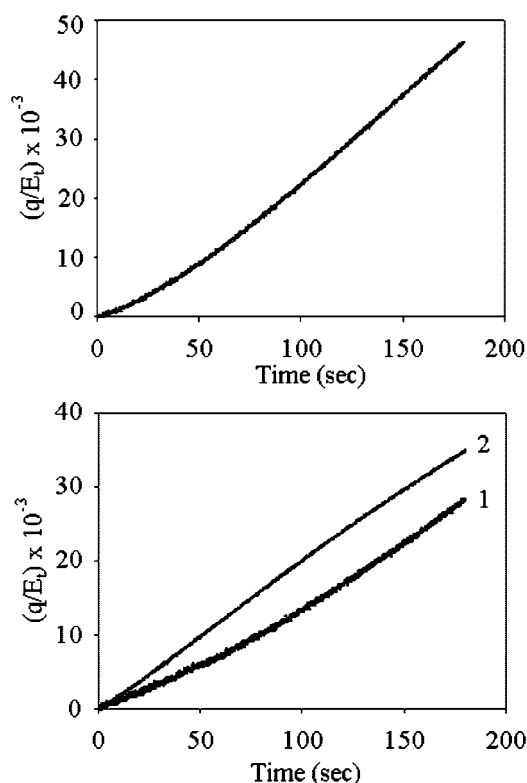


FIG. 2. **Activation by BPGA of GAPDH from the complex.** *Top*, the complex (1.2 nM) was placed in the NADPH-GAPDH reaction mixture, and the appearance of products was monitored ( $p(1) = 313.5 \pm 0.5 \text{ s}^{-1}$ ;  $p(2) = 90.8 \pm 0.9 \text{ s}^{-1}$ ;  $p(3) = 0.022 \pm 2 \times 10^{-4} \text{ s}^{-1}$ ). *Bottom*, the complex was dissociated in the reaction mixture lacking all substrates for 5 min, and the NADPH-dependent activity was monitored (*curve 1*). (These data were fitted to Equation 6:  $p(1) = 374.4 \pm 27.5 \text{ s}^{-1}$ ;  $p(2) = 100.1 \pm 0.6 \text{ s}^{-1}$ ;  $p(3) = 0.003 \pm 4 \times 10^{-4} \text{ s}^{-1}$ .) This dissociated form was incubated with BPGA ([BPGA]/[dissociated complex] =  $1.4 \times 10^5$ ) for 15 min, and the NADPH-dependent activity was measured (*curve 2*). (These data were fitted to a straight line (rate constant of  $197.5 \pm 0.1 \text{ s}^{-1}$ .) The final concentration of the complex in the assay cuvette was 2.2 nM, and that of BPGA was 0.3 mM.

$$q/E_t = p(1) \times t + [(p(1) - p(2))/p(3)] \times (e^{-p(3)t} - 1) \quad (\text{Eq. 6})$$

where  $q$  is the observed product concentration at time  $t$ ,  $p(2)$  is the rate constant at the beginning of the reaction of the less active form,  $p(1)$  is the steady-state rate constant of the BPGA-activated form,  $p(3)$  is the observed rate of the transition between the initial state and the activated state, and  $E_t$  is the total enzyme concentration.

Thus, the lag obtained with the complex was not only linked to the dissociation of the complex upon dilution but was also the consequence of an activation induced by BPGA. The linear part of the curves corresponds to the steady-state rate of a dissociated, BPGA-activated GAPDH, or metastable form. The complex was not fully dissociated at the beginning of the reaction, and the latent phase of the dissociated form was longer than the one obtained with the complex. It was thus likely that BPGA acted faster on the complex than on the dissociated form. Nonetheless, the BPGA-activated forms originating from the dissociated form or from the complex have similar activities. The average steady-state rates of these forms had a value of  $340 \pm 31 \text{ s}^{-1}$ .

In contrast to the enzyme embedded in the complex, the stable GAPDH using NADPH displayed no lag, indicating that this form was not activated by BPGA. The average rate constant of this form was about  $197 \pm 27 \text{ s}^{-1}$ , which is lower than that obtained with the BPGA-activated form released from the complex.

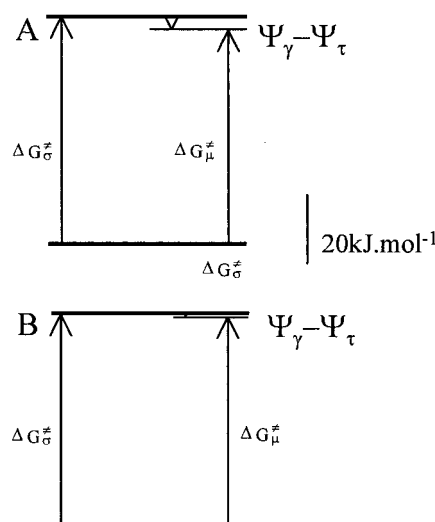


FIG. 3. **Imprinting effects of PRK on GAPDH when monitoring NADH- and NADPH-GAPDH dependent activities.** Free energies of activation obtained for the reactions catalyzed by the metastable and the stable GAPDH. *A*, NADH-dependent GAPDH activity.  $\Delta G_{\sigma}^{\ddagger} = 65.5 \pm 0.6 \text{ kJ}\cdot\text{mol}^{-1}$ ;  $\Delta G_{\mu}^{\ddagger} = 61.7 \pm 0.1 \text{ kJ}\cdot\text{mol}^{-1}$ ;  $\psi_{\gamma} - \psi_{\tau} = -3.8 \pm 0.5 \text{ kJ}\cdot\text{mol}^{-1}$ . *B*, NADPH-GAPDH activity.  $\Delta G_{\sigma}^{\ddagger} = 60.9 \pm 0.3 \text{ kJ}\cdot\text{mol}^{-1}$ ;  $\Delta G_{\mu}^{\ddagger} = 59.6 \pm 0.2 \text{ kJ}\cdot\text{mol}^{-1}$ ;  $\psi_{\gamma} - \psi_{\tau} = -1.3 \pm 0.3 \text{ kJ}\cdot\text{mol}^{-1}$ . The imprinting effect ( $\psi_{\gamma} - \psi_{\tau}$ ) corresponds to stabilization-destabilization energies.

The above theory was used to describe thermodynamically how protein-protein interactions act via imprinting to modify the rate constant of a chemical process. Equation 5 can be used to calculate the free energies of activation of the metastable form  $\Delta G_{\mu}^{\ddagger}$  and of the stable form  $\Delta G_{\sigma}^{\ddagger}$  for the NADH- and NADPH-dependent GAPDH activities. The imprinting effects of PRK on GAPDH were quantified with Equations 1 and 2. The energy diagrams are reported in Fig. 3.

*Impact of NADP(H) on the Imprinting Effect of PRK on GAPDH*—The complex was incubated with 1 mM NAD(P)(H), and NADH-dependent GAPDH activity was monitored. Similarly to the control experiment (complex alone incubated at 30 °C), the reaction rates were not affected by incubation with 1 mM NAD(H) (data not shown). However, incubation with 1 mM NADP(H) decreased the steady-state reaction rates mono-exponentially with time (Fig. 4). These data were thus fitted to the equation.

$$y = y_0 + A \times (e^{-\lambda \times t} - 1) \quad (\text{Eq. 7})$$

where  $A$  and  $\lambda$  are the amplitude and the time constant of the inhibition process, respectively, and  $y_0$  is the steady-state rate (average value of  $147 \pm 5 \text{ s}^{-1}$ ) of the metastable NADH-GAPDH before incubation of the complex.

The reaction rate constants decreased with a mean time constant of  $0.003 \pm 4 \times 10^{-4} \text{ s}^{-1}$ , corresponding to a half-time of inhibition of about 4 min, whatever the cofactor used: NADP or NADPH. 1mM NADP gave 50% inhibition and a final average rate constant of  $73 \pm 5 \text{ s}^{-1}$ , whereas incubation with 1mM NADPH gave 20% inhibition and a final average rate of  $117 \pm 2 \text{ s}^{-1}$ .

When following NADPH-dependent activity of GAPDH for the complex incubated at 30 °C, in the absence of any cofactor, the activity increased from  $340 \pm 31 \text{ s}^{-1}$  to  $435 \pm 17 \text{ s}^{-1}$ . Because hydrophobic forces increase with temperature (24), this activation might be linked to the enhancement of hydrophobic forces in the complex. Taking into account this temperature effect, 1mM NADPH did not significantly modify the rate of the metastable GAPDH. Only 1mM NADP increased this reaction rate to an average value of  $687 \pm 73 \text{ s}^{-1}$ .

To determine for both NADH- and NADPH-dependent



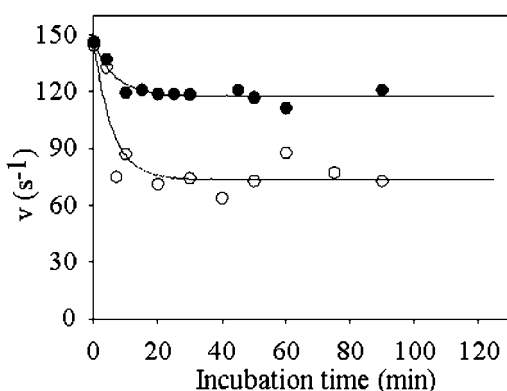


FIG. 4. Changes in the steady-state rate constant for NADH-GAPDH with incubation time. The steady-state rates ( $v$ ) were obtained for the enzyme incubated at 30 °C with 1 mM NADP (○) or 1 mM NADPH (●) by fitting the linear part of the curves of the reaction catalyzed by NADH-GAPDH to straight lines. The complex concentration used was 1.8 nM. The data were fitted to Equation 7 in the main text (after incubation with NADP:  $y_0 = 149.5 \pm 12.3 \text{ s}^{-1}$ ,  $A = 76.1 \pm 13.0 \text{ s}^{-1}$ ,  $\lambda = 0.003 \pm 0.001 \text{ s}^{-1}$ ; after incubation with NADPH:  $y_0 = 147.4 \pm 3.3 \text{ s}^{-1}$ ,  $A = 29.9 \pm 3.5 \text{ s}^{-1}$ ,  $\lambda = 0.003 \pm 7 \times 10^{-4} \text{ s}^{-1}$ ).

GAPDH activities whether the amplitude of the effects was dependent on the concentration of the cofactor used, we repeated the same experiments at NADP concentrations ranging from 0.5 to 5 mM and at NADPH concentrations ranging from 1 to 10 mM (Fig. 5). Increasing NADPH in the incubation mixture results in an increase of NADPH concentration in the reaction mixture. As a consequence, NADPH-dependent activity interferes, whereas measuring GAPDH activity using NADH and the effects of NADPH concentrations higher than 1 mM cannot be studied on NADH-dependent activity of GAPDH. On the contrary, the activation of NADPH-dependent activity of GAPDH may be followed.

When increasing NADP concentration in the incubation mixture, the percentage of inhibition of the NADH-dependent GAPDH activity increased and reached a maximal value of  $81 \pm 6$  with a  $\bar{K}_{0.5}$  of  $0.65 \pm 0.18 \text{ mM}$ , whereas the percentage of activation of the NADPH-dependent GAPDH activity increased and reached a maximal value of  $121 \pm 1$  with a  $\bar{K}_{0.5}$  of  $0.65 \pm 0.02 \text{ mM}$ . The percentage of activation by NADPH of the NADPH-dependent GAPDH activity increased and reached a maximal value of  $169 \pm 7$  with a  $\bar{K}_{0.5}$  of  $2.9 \pm 0.9 \text{ mM}$ . When possible, to check whether the absence of effects observed after incubation with 1 mM NAD(H) was linked to the concentration used, the complex was also incubated with 5 mM NAD(H), and no effect was observed.

The changes in the GAPDH catalytic rate constants upon incubation of the complex with NADP(H) were used to determine the effects of these cofactors on the imprinting of PRK on GAPDH. All of the calculations were done with 1 mM NADP(H), because it was possible to quantify and compare the effects of all of the cofactors at this concentration. The free energies of activation obtained for the metastable GAPDH released from the complex incubated at 30 °C without cofactors ( $\Delta G_{\mu}^{\ddagger}$ ) or from the metastable GAPDH released from the complex incubated with NADP(H) ( $\Delta G_{\mu}^{\ddagger}$ ) were obtained from Equation 5. The impact  $\Delta X$  of NADP(H) on the imprinting effect was then determined using Equations 3 and 4 (Table I). Interestingly,  $\Delta G_{\sigma}^{\ddagger}$  remained constant, because none of these cofactors affected the rate constants of stable GAPDH.

When possible,  $\Delta X$  was also calculated for the maximum percentage of inhibition or activation obtained when varying the concentration of cofactors in the incubation mixture. With NADP, the  $\Delta X$  was multiplied by 2.3 and 1.7 for NADH- and NADPH-dependent activities, respectively. For NADPH-

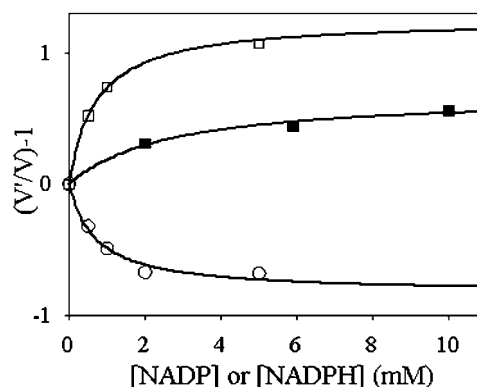


FIG. 5. Effect of different concentrations of NADP(H) on GAPDH. The complex was incubated with concentrations of NADP ranging from 0.5 to 5 mM. At each concentration, the activity was measured at different incubation times and plotted as a function of time. The curves obtained for each concentration were then fitted to monoexponential curves to obtain the maximal reaction rate  $V'$ . The ratio  $V'/V - 1$  was reported, where  $V$  is the reaction rate before incubation. □, NADPH-dependent activity; ○, NADH-dependent activity. The same experiments were performed with NADPH ranging from 1 to 10 mM, and the same ratio for NADPH-dependent activity of GAPDH (■) was reported.

TABLE I  
Impact of NADP(H) on the imprinting effect of PRK on GAPDH

	NADH-GAPDH
$\Delta G_{\mu}^{\ddagger}$ (kJ·mol <sup>-1</sup> )	$61.7 \pm 0.1$
$(\Delta G_{\mu}^{\ddagger})_{\text{NADP}}$ (kJ·mol <sup>-1</sup> )	$63.5 \pm 0.2$
$(\Delta X)_{\text{NADP}}$ (kJ·mol <sup>-1</sup> )	$1.8 \pm 0.2$
$(\Delta G_{\mu}^{\ddagger})_{\text{NADPH}}$ (kJ·mol <sup>-1</sup> )	$62.3 \pm 0.05$
$(\Delta X)_{\text{NADPH}}$ (kJ·mol <sup>-1</sup> )	$0.6 \pm 0.1$
	NADPH-GAPDH
$\Delta G_{\mu}^{\ddagger}$ (kJ·mol <sup>-1</sup> )	$59.6 \pm 0.2$
$(\Delta G_{\mu}^{\ddagger})_{\text{temperature}}$ (kJ·mol <sup>-1</sup> )	$59.0 \pm 0.1$
$(\Delta G_{\mu}^{\ddagger})_{\text{NADP}}$ (kJ·mol <sup>-1</sup> )	$57.8 \pm 0.3$
$(\Delta X)_{\text{NADP}}$ (kJ·mol <sup>-1</sup> )	$-1.2 \pm 0.3$

dependent activity, the effect of 1 mM NADPH on the imprinting was negligible, but it had a value of  $-1.3 \text{ kJ} \cdot \text{mol}^{-1}$  for the estimated maximum percentage of activation.

*Impact of NADP(H) on the Imprinting Effect of GAPDH on PRK*—The time course of PRK activity within the complex showed a lag, which has been previously linked to the conversion of the PRK in the complex to a dissociated metastable form (10). This curve may also be fitted to Equation 6. The parameter  $p(3)$  is the apparent dissociation rate constant of the complex,  $p(1)$  is the steady-state rate of the metastable form of PRK, and  $p(2)$  is the rate of the PRK within the complex.

The reaction rates of PRK embedded in the complex and of the metastable form after incubation at 30 °C in the absence of any cofactor were constant, whereas the apparent dissociation rate constants tended to decrease monoexponentially as a consequence of enhanced hydrophobic forces. Incubation with 1 mM NAD(H) had no effect. The lag was still present, although affected, when the complex was incubated with 1 mM NADP(H). The initial rate ( $p(2)$ ) remained constant, and PRK activity within the complex was not greatly affected (mean =  $15 \pm 3.2 \text{ s}^{-1}$ ) (data not shown). On the contrary, the average steady-state rates  $p(1)$  of the metastable PRK increased from  $118 \pm 7 \text{ s}^{-1}$  without incubation to  $179 \pm 7 \text{ s}^{-1}$  after incubation with 1 mM NADPH and  $389 \pm 35 \text{ s}^{-1}$  after incubation with 1 mM NADP. The experimental data  $p(1)$  derived from Equation 6 were plotted against incubation time with the cofactors (Fig. 6A). These data may be fitted to the following equation.

$$y = y_0 + A \times (1 - e^{-\lambda x}) \quad (\text{Eq. 8})$$

TABLE II  
Impact of NADP(H) on the imprinting effect of GAPDH on PRK

	PRK
$\Delta G_{\mu}^{\ddagger}$ (kJ·mol <sup>-1</sup> )	62.3 ± 0.1
$(\Delta G_{\mu}^{\ddagger})_{\text{NADP}}$ (kJ·mol <sup>-1</sup> )	59.3 ± 0.2
$(\Delta X)_{\text{NADP}}$ (kJ·mol <sup>-1</sup> )	-3 ± 0.2
$(\Delta G_{\mu}^{\ddagger})_{\text{NADPH}}$ (kJ·mol <sup>-1</sup> )	61.2 ± 0.1
$(\Delta X)_{\text{NADPH}}$ (kJ·mol <sup>-1</sup> )	-1.1 ± 0.1

where  $y_0$  is the initial rate constant of the metastable PRK before incubation of the complex and  $A$  and  $\lambda$  are the amplitude and the time constant of the activation process, respectively. The mean time constant of activation by NADP or NADPH,  $\lambda$ , was  $0.003 \pm 8 \times 10^{-4} \text{ s}^{-1}$ , corresponding to a half-time of activation of about 4 min.

The increase in the reaction rates  $p(1)$  as a function of incubation time with 1 mM NADP(H) was concomitant to an increase in the apparent dissociation rate constant  $p(3)$  of the complex. Thus, the complex probably underwent a conformation change leading to faster dissociation (Fig. 6B). This effect was confirmed by immunoblots of the complex mixed with 1 mM NAD(P)(H) for 1 h. The complex was slightly dissociated after incubation with 1 mM NAD(H), as was the control. There was greater dissociation after incubation with 1 mM NADP(H), although the complex was still detected. This dissociation may be due to weaker protein-protein interactions within the complex after incubation with NADP(H) (Fig. 6C).

The imprinting effects of GAPDH on PRK have been established (10). The estimated rate constants for the metastable PRK released from the complex incubated with NADP(H) were used to determine the impact of these cofactors on the imprinting. The free energy of activation of the reaction catalyzed by the metastable PRK released from the incubated complex ( $\Delta G_{\mu}^{\ddagger}$ ) and the free energy of activation of the reaction catalyzed by the metastable PRK released from an untreated complex ( $\Delta G_{\mu}^{\ddagger}$ ) were calculated (Equations 1 and 2). We then determined from Equations 3 and 4 the fraction of energy attributed to the presence of NADP(H) that is responsible for altering the imprinting effect (Table II).

As for GAPDH, the amplitude of the effects observed on the metastable PRK was dependent on the concentration of NADP(H) used in the incubation mixture (data not shown). The maximum  $|\Delta X|$  reached were 1.3- and 3-fold higher after incubation with different concentrations of NADP and NADPH, respectively. The maximum percentage of activation reached with NADP was  $367 \pm 37$  with a  $\bar{K}_{0.5}$  of  $0.75 \pm 0.25 \text{ mM}$ . After incubation with NADPH, the maximum percentage of activation was  $274 \pm 40$  with a  $\bar{K}_{0.5}$  of  $2.9 \pm 1 \text{ mM}$ . Interestingly, these same cofactors had no effect on PRK activity when the stable enzyme was incubated (data not shown).

#### DISCUSSION

We demonstrate here that PRK has an imprinting effect on GAPDH. We calculated and compared the free energies of activation of the reactions catalyzed by the metastable GAPDH and the stable enzyme to quantify the imprinting effect of PRK on GAPDH. We conclude that protein-protein interactions are responsible for decreasing the energy barrier of the reactions catalyzed by the metastable GAPDH when NADH-dependent activity ( $-3.8 \pm 0.5 \text{ kJ}\cdot\text{mol}^{-1}$ ) or NADPH-dependent activity ( $-1.3 \pm 0.3 \text{ kJ}\cdot\text{mol}^{-1}$ ) is monitored. This decrease is reminiscent of the one observed with PRK (10, 14, 25).

We find that NAD(H) has no effects on GAPDH when it is in the complex, whereas incubation of the complex with NADP(H) leads to activation of the metastable GAPDH when monitoring NADPH-dependent activity and to inhibition of the metastable GAPDH using NADH. None of these cofactors has an effect on

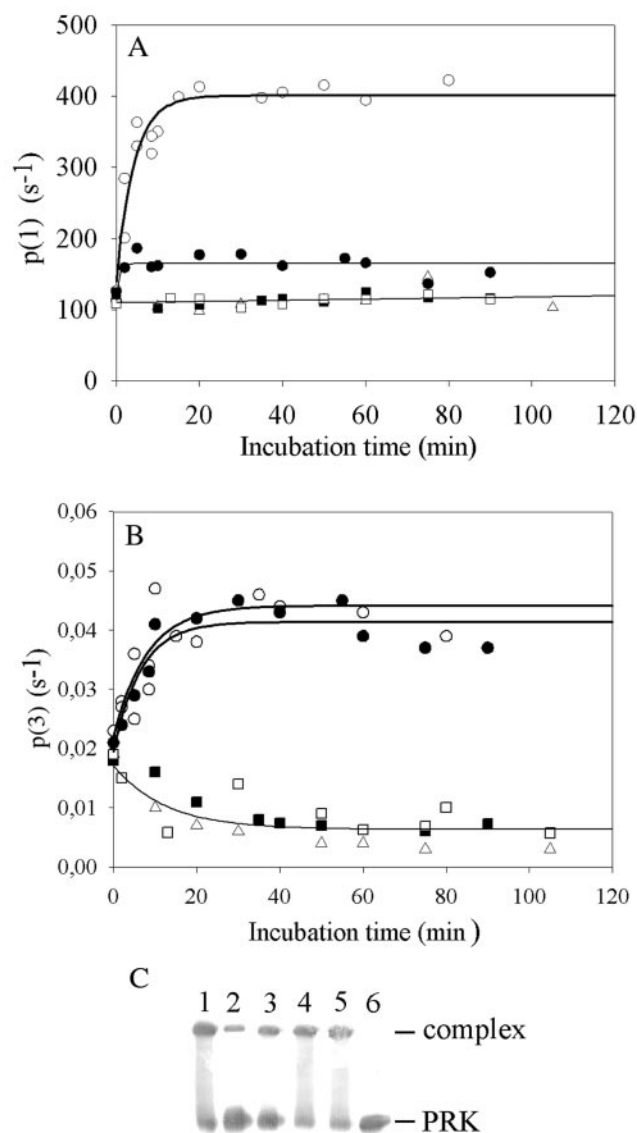


FIG. 6. Changes in the steady-state rates  $p(1)$  of metastable PRK and in the apparent dissociation rate constants  $p(3)$  of the complex with incubation time.

The values of  $p(1)$  and  $p(3)$  were obtained by fitting the time curves obtained with the complex incubated without cofactors or with 1 mM NAD(P)(H) at 30 °C to Equation 6 in the main text.  $\Delta$ , control experiment;  $\circ$ , incubation with NADP;  $\bullet$ , incubation with NADPH;  $\square$ , incubation with NAD;  $\blacksquare$ , incubation with NADH. A, changes in the steady-state rate of metastable PRK as a function of incubation time. The data were fitted to Equation 8 in the main text except for the  $p(1)$  derived from the control experiment or incubation in the presence of NAD(H) where the data were fitted to a straight line (for incubation with NADPH:  $y_0 = 112.3 \pm 6.9 \text{ s}^{-1}$ ,  $A = 57.9 \pm 7.5 \text{ s}^{-1}$ ,  $\lambda = 0.004 \pm 1.5 \times 10^{-3} \text{ s}^{-1}$ ; for incubation with NADP:  $y_0 = 140.1 \pm 25.4 \text{ s}^{-1}$ ,  $A = 263.0 \pm 26.3 \text{ s}^{-1}$ ,  $\lambda = 0.004 \pm 0.001 \text{ s}^{-1}$ ). B, changes in the apparent dissociation rate constant of the complex after different incubation times of the complex with NAD(P)(H). The data were fitted to Equation 8 in the main text for  $p(3)$  derived from experiments done with NADP(H) (after incubation with NADPH:  $y_0 = 0.020 \pm 0.003 \text{ s}^{-1}$ ,  $A = 0.022 \pm 0.003 \text{ s}^{-1}$ ,  $\lambda = 0.003 \pm 9 \times 10^{-4} \text{ s}^{-1}$ ; after incubation with NADP:  $y_0 = 0.023 \pm 0.004 \text{ s}^{-1}$ ,  $A = 0.02 \pm 0.004 \text{ s}^{-1}$ ,  $\lambda = 0.002 \pm 9 \times 10^{-4} \text{ s}^{-1}$ ) and to Equation 7 in the main text for the control experiment ( $y_0 = 0.019 \pm 6 \times 10^{-4} \text{ s}^{-1}$ ,  $A = 0.015 \pm 7 \times 10^{-4} \text{ s}^{-1}$ ,  $\lambda = 0.001 \pm 1 \times 10^{-4} \text{ s}^{-1}$ ). C, correlation between the observed changes in the apparent dissociation rate constant and the strength of protein-protein interactions within the complex. The complex (500 ng) with no incubation (lane 1) or after incubation with NADP (lane 2), NADPH (lane 3), NAD (lane 4), or NADH (lane 5) for 1 h was electrophoresed under nonreducing conditions (4–15% PAGE), transferred to nitrocellulose, and probed with anti-PRK antibodies. Lane 6 corresponds to a complex fully dissociated in the presence of 20 mM dithiothreitol.

the stable enzyme. Furthermore, the regulation of GAPDH within this complex and its activation by BPGA are very similar to that of the 600-kDa form of GAPDH in higher plants (17, 18). Thus, the complex can replace this 600-kDa form. This complex is all the more important because the independent stable GAPDH purified from *C. reinhardtii* is neither regulated by NAD(P)(H) nor activated by BPGA.

We show that NAD(H) has no effect on the metastable PRK, whereas NADP(H) has a positive effect. The stable PRK is not regulated by either of these metabolites. The half-maximum activation of PRK or of NADPH-dependent activity or the half-maximum inhibition of NADH-dependent activity of GAPDH is obtained for the same NADP concentration (mean value of  $0.68 \pm 0.16$  mM). The time required to obtain 50% of the effect of NADP on the metastable PRK or on the NADH-dependent activity of GAPDH is also the same (4 min). The half-maximum of activation for PRK and NADPH-dependent activity of GAPDH is obtained for a mean value of  $2.93 \pm 0.87$  mM NADPH.

The effects of 1 mM NADP(H) on the imprinting were quantified. These two cofactors significantly decrease the energy barrier of the reactions catalyzed by the metastable GAPDH using NADPH as cofactor ( $\Delta X_{\text{NADP}}$  of  $-1.2 \pm 0.3$  kJ·mol<sup>-1</sup>) and by PRK released from the incubated complex ( $\Delta X_{\text{NADP}}$  of  $-3 \pm 0.2$  kJ·mol<sup>-1</sup>). This effect is even greater at higher NADP (or NADPH) concentrations and reaches a  $\Delta X_{\text{max}}$  of  $-2$  kJ·mol<sup>-1</sup> (or  $-1.3$  kJ·mol<sup>-1</sup>) for NADPH-dependent activity of GAPDH and  $-3.9$  kJ·mol<sup>-1</sup> (or  $-3.3$  kJ·mol<sup>-1</sup>) for PRK. On the contrary, the energy barrier of the reaction catalyzed by the NADH-GAPDH released from an incubated complex is higher than the energy barrier of the reaction catalyzed by GAPDH released from an untreated complex ( $\Delta X_{\text{NADP}}$  of  $+1.8 \pm 0.2$  kJ·mol<sup>-1</sup>). This effect also increases with higher concentrations of NADP, and the  $\Delta X_{\text{max}}$  reaches a value of  $+4.2$  kJ·mol<sup>-1</sup>.

The results obtained with GAPDH and PRK demonstrate how important protein-protein interactions are for the emergence of new regulatory properties of the enzymes involved in multi-enzyme complexes. First, the higher activities of the metastable enzymes compared with those of the stable enzymes are the consequence of information transfer within the complex. In this way, part of the energy stored during association between the enzymes is used to increase the rate of catalysis after disruption via imprinting. This information transfer corresponds to stabilization-destabilization energies that come from a conformation change in the enzymes within the complex. Second, the information transfer may also be modulated by the binding of a ligand, such as NADP or NADPH. The signal triggered by these two cofactors may thus have two targets within a single "control unit." The extra 2'-phosphate present on NADP(H) compared with NAD(H) is probably responsible for the induced conformation change and the subsequent effect on enzyme activity. The extra positive charge on the nicotinamide ring of NADP might also be responsible for the greater efficiency of this cofactor compared with its reduced form, NADPH.

Imprinting has also been demonstrated for copolymers and their ligands (26, 27). Some enzymes may also retain for a

while the conformation they had before the release of the reaction product (28–34). How different conformational states can be dictated by the "history" of sample preparation has been widely described for lipase (35), which retains an active conformation induced by high detergent concentration. Thus, there is no doubt that imprinting and memory effects are widespread in cell biology and that some of the functional properties of enzymes are due to these effects. Nonetheless, the physical nature of the forces involved in these processes (electrostatic, hydrophobic, and Van der Waal's interactions) remains to be elucidated.

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**Thermodynamic Analysis of the Emergence of New Regulatory Properties in a Phosphoribulokinase-Glyceraldehyde 3-Phosphate Dehydrogenase Complex**

Emmanuelle Graciet, Sandrine Lebreton, Jean-Michel Camadro and Brigitte Gontero

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