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

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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 ± 0.5 and 1.3 ± 0.3 kJ mol⁻¹. 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 $K_{d5\text{NADPP}}$ of 0.68 ± 0.16 mm and a $K_{d5\text{NADPH}}$ of 2.93 ± 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 ± 0.2 kJ mol⁻¹ and decreases that of the reactions catalyzed by phosphoribulokinase and NADPH-glyceraldehyde 3-phosphate dehydrogenase by 3 ± 0.2 and 1.2 ± 0.3 kJ mol⁻¹, 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 CO₂ assimilation in plants (2–5). The dissociation of enzymes catalyzing consecutive reactions may 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-biphosphate, the CO₂ acceptor in photosynthetic organisms; the dehydrogenase catalyzes the reversible reduction and dephosphorylation of 1,3-biphosphoglyceric acid (BPGA)¹ 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-biphosphate 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 GDPH (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 GDPH, 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 GDPH in spinach (16, 17), and this form is absent from *C. reinhardtii*. Thus, one may wonder (i) if the GDPH 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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¹ The abbreviations used are: BPGA, 1,3 biphosphoglyceric acid; PRK, phosphoribulokinase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.
EXPERIMENTAL PROCEDURES

Enzyme Purification—The complex was purified to apparent homogeneity from C. reinhardtii (WM3) 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 added 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 NADP/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 NADP/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 Amsher Biosciences Phastsystem apparatus. The proteins were transferred to nitrocellulose filters (0.45 μm; Schleicher & Schuell) 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.

\[ \Delta G_{\text{m}}^* = \Delta G_{\text{m}} + (\Psi - \Psi_1) \]

where \( \Delta G_{\text{m}}^* \) and \( \Delta G_{\text{m}} \) are the free energies of activation of a given process performed by the metastable (m) and stable (s) forms of the same enzyme. The difference \( (\Psi - \Psi_1) \) is the fraction of energy that is stored after the dissociation of the supramolecular edifice, where \( \Psi \) and \( \Psi_1 \) 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 \( \Delta G_{\text{m}}^* \) of a given chemical process performed by this metastable form (\( \mu \)) and the free energy of activation \( \Delta G_{\text{m}}^\mu \) of the metastable form (\( \mu \)) obtained in the absence of the ligand X may be compared.

\[ \Delta G_{\text{m}}^\mu = \Delta G_{\text{m}}^* + \Delta X \]

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 (\( h_{\text{m}} \)).

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

\[ \Delta G_{\text{m}}^\mu = R T \ln \frac{k_{\text{m}} T}{h_{\text{m}}} \]

where \( k_{\text{m}} \) is the catalytic rate constant of the metastable form released from the complex incubated with ligand X, and \( h_{\text{m}} \) 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 overlap 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 \( 147 \pm 5 \ s^{-1} \). This average value was much higher than that obtained with stable GAPDH (\( 33 \pm 8 \ s^{-1} \)).

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).
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 $\Delta G^\neq_A$ and of the stable form $\Delta G^\neq$ 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 NAD(P) 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 NAD(P) decreased the steady-state reaction rates monotonically with time (Fig. 4). These data were thus fitted to the equation.

$$y = y_0 + A \times (e^{-\lambda t} - 1)$$

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 $147 \pm 5$ 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 ± 4 × 10$^{-3}$s$^{-1}$, corresponding to a half-time of inhibition of about 4 min, whatever the cofactor used: NADP or NADPH. 1 mM NADP gave 50% inhibition and a final average rate constant of 73 ± 5 s$^{-1}$, whereas incubation with 1 mM NADPH gave 20% inhibition and a final average rate of 117 ± 2 s$^{-1}$.

When following NADP-dependent activity of GAPDH for the complex incubated at 30 °C, in the absence of any cofactor, the activity increased from 340 ± 31 s$^{-1}$ to 435 ± 17 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, 1 mM NADP did not significantly modify the rate of the metastable GAPDH. Only 1 mM NADP increased this reaction rate to an average value of 687 ± 73 s$^{-1}$.

To determine for both NADH-and NADPH-dependent
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 NADP 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 NADPH-dependent GAPDH activity increased and reached a maximal value of 81 ± 6 with a \( K_{0.5} \) of 0.65 ± 0.18 mM, whereas the percentage of activation of the NADP-dependent GAPDH activity increased and reached a maximal value of 121 ± 1 with a \( K_{0.5} \) of 0.65 ± 0.02 mM. The percentage of activation by NADPH of the NADP-dependent GAPDH activity increased and reached a maximal value of 169 ± 7 with a \( K_{0.5} \) of 2.9 ± 0.9 mM. When possible, to check whether the absence of effects observed after incubation with 1 mM NADH (linked to the concentration used, the complex was also incubated with 5 mM NADH), 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 effect of PRK on GAPDH. All of the calculations were done with 1 mM NADPH, 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 (\( \Delta G'_{\text{p}} \)) or from the metastable GAPDH released from the complex incubated with NADP(H) (\( \Delta G'_{\text{p}} \)) 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, \( \Delta G'_{\text{p}} \) 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-dependent activity, the effect of 1 mM NADP 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 PRK on GAPDH**

<table>
<thead>
<tr>
<th>NADP-GAPDH</th>
<th>( \Delta G'_{\text{p}} ) (kJ mol(^{-1}))</th>
<th>( \Delta G'_{\text{p}} ) (kJ mol(^{-1}))</th>
<th>( \Delta X_{\text{NADP}} ) (kJ mol(^{-1}))</th>
<th>( \Delta G'_{\text{p}} ) (kJ mol(^{-1}))</th>
<th>( \Delta G'_{\text{p}} ) (kJ mol(^{-1}))</th>
<th>( \Delta X_{\text{NADPH}} ) (kJ mol(^{-1}))</th>
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<tbody>
<tr>
<td>NADPH-GAPDH</td>
<td>61.7 ± 0.1</td>
<td>63.5 ± 0.2</td>
<td>1.8 ± 0.2</td>
<td>62.3 ± 0.05</td>
<td>0.8 ± 0.1</td>
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<tr>
<td>NADP-GAPDH</td>
<td>59.6 ± 0.2</td>
<td>59.0 ± 0.1</td>
<td>57.8 ± 0.3</td>
<td>57.8 ± 0.3</td>
<td>1.2 ± 0.3</td>
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**Impact of NADP(H) on the Imprint 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 monoenzymatically as a consequence of enhanced hydrophobic forces. Incubation with 1 mM NADH 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 ± 3.2 s\(^{-1}\)) (data not shown). On the contrary, the average steady-state rates \( p(1) \) of the metastable PRK increased from 118 ± 7 s\(^{-1}\) without incubation to 179 ± 7 s\(^{-1}\) after incubation with 1 mM NADPH and 389 ± 35 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.

\[
y = y_0 + A \times (1 - e^{-\Delta X \times t})
\] (Eq. 8)
Impact of NADP(H) on the imprinting effect of GAPDH on PRK

<table>
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<tr>
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<th>PRK</th>
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<tbody>
<tr>
<td>ΔG°_PRK</td>
<td>62.3 ± 0.1</td>
</tr>
<tr>
<td>ΔG°_NADP</td>
<td>58.3 ± 0.2</td>
</tr>
<tr>
<td>ΔG°_NADPH</td>
<td>-3 ± 0.2</td>
</tr>
<tr>
<td>ΔG°_NADPH</td>
<td>61.2 ± 0.1</td>
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<tr>
<td>ΔX_0</td>
<td>-1.1 ± 0.1</td>
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where y₀ is the initial rate constant of the metastable PRK before incubation 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 NADP(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 untreated complex (ΔG°_PRK) and the free energy of activation of the reaction catalyzed by the metastable PRK released from the incubated complex (ΔG°_PRK) 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 [ΔX] 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 ± 37 with a K₀,5 of 0.75 ± 0.25 mm. After incubation with NADPH, the maximum percentage of activation was 274 ± 40 with a K₀,5 of 2.9 ± 1 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 (ΔG°_NADPH = 0.8 ± 0.5 kJ mol⁻¹) or NADPH-dependent activity (ΔG°_NADPH = 1.3 ± 0.3 kJ 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 NAD(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 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 NADP(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).

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We find that NAD(H) has no effects on GAPDH when it is in the complex, whereas incubation of the complex with NAD(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
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 ± 0.16 m). 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 ± 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 (ΔX_{NADP} of −1.2 ± 0.3 kJ mol⁻¹) and by PRK released from the incubated complex (ΔX_{NADP} of −3 ± 0.2 kJ mol⁻¹). This effect is even greater at higher NADP (or NADPH) concentrations and reaches a ΔX_{NADP} of −2 kJ mol⁻¹ (or −1.3 kJ mol⁻¹) for NADP(H)-dependent activity of GAPDH and −3.9 kJ mol⁻¹ (or −3.3 kJ mol⁻¹) 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 (ΔX_{NADP} of +1.8 ± 0.2 kJ mol⁻¹). This effect also increases with higher concentrations of NADP, and the ΔX_{NADP} reaches a value of +4.2 kJ mol⁻¹.

The effects 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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