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Regulation of phosphoribulokinase and glyceraldehyde 3-phosphate dehydrogenase by NADP(H) in a bi-enzyme complex from *Chlamydomonas reinhardtii*.

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Introduction

We have purified a bi-enzyme complex composed of two tetramers of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (EC.1.2.1.13) and two dimers of phosphoribulokinase (PRK) (EC.2.7.1.19) (Avilan *et al*, 1997). Both enzymes are involved in the CO_2 assimilation by the Benson-Calvin cycle in the chloroplast. PRK catalyzes the ATP-dependent phosphorylation of ribulose 5-phosphate (Ru5P) into ribulose 1,5-biphosphate (RuBP). Chloroplastic GAPDH catalyzes the reversible reductive dephosphorylation of 1,3-biphosphoglyceric acid (BPGA) into glyceraldehyde 3-phosphate (GAP) using NADPH or NADH as cofactors (Pawlizki and Latzko, 1974). We have also purified the isolated enzymes (not included in the complex).
The effects of NADP(H) on PRK within a PRK/CP12/GAPDH complex have been studied (Wedel and Soll, 1998). These cofactors also regulate an aggregated form of GAPDH that occurs only in higher plants and is not found in *C. reinhardtii* (Scheibe *et al*, 1996 ; Pupillo and Piccari, 1973 and 1975). We tested if the bi-enzyme complex we purify could replace this aggregated form when it was incubated with NADP(H). The effects of these cofactors on the isolated enzymes have also been studied by kinetic experiments.

We have previously shown that PRK obtained by disruption of the bi-enzyme complex, transiently adopts a metastable conformation that is highly competent for catalysis (Lebreton *et al*, 1997b). It has also been shown that after dissociation, PRK bears an imprint of GAPDH corresponding to stabilization-destabilization energies. The energy stored within the metastable PRK is then used to decrease the energy barrier of catalysis. Conversely, PRK may be expected to exert an imprinting on GAPDH. Moreover, one may wonder if the cofactors NADP(H) may alter these imprinting effects exerted between PRK and GAPDH.

Materials and methods

The purified complex or the isolated enzymes from *C. reinhardtii* (WM3-) were preincubated at 30°C with 1 mM NAD(H) or 1 mM NADP(H). Aliquots were withdrawn after specific times and placed in the assay cuvettes to test PRK, NADH- or NADPH-GAPDH activities as described earlier (Gontero *et al*, 1988 ; Baalmann *et al*, 1995). Enzyme assays were performed with a Pye Unicam UV2 spectrophotometer. Experimental data were fitted to theoretical curves using Sigma Plot 5.0.

Results

Progress curves obtained when monitoring PRK activity displayed a lag that corresponds to the conversion of the PRK inserted in the complex into a more active metastable PRK that has just been released from the complex. This curve may be fitted to :

$$Q/P_t = k_\mu.t + [(k_\mu - p(1))/p(2)].(e^{-p(2)t} - 1) \quad (1)$$

where Q is the product of the reaction catalyzed by PRK, k_μ the steady-state rate of the metastable PRK, p(1) the rate of PRK in the complex and p(2) the apparent dissociation rate constant of the complex. P_t is the total protein concentration (Lebreton *et al*, 1997a). The reaction catalyzed by the isolated PRK has no lag and the catalytic rate constant (k_σ) is lower than that of the metastable form (k_μ).

Progress curves for NADH- and NADPH-dependent activities of GAPDH were also biphasic. Yet, experiments performed with aliquots withdrawn from the NADH- and NADPH-GAPDH assay mixtures at 30 sec, 2 min and 5 min showed that, in contrast to PRK, the biphasic character of the curves was not linked to the dissociation of the complex. For NADH-GAPDH activity, the biphasic curve was linked to the approach to equilibrium and the linear part of the curve corresponded to the activity of the GAPDH that had just been released from the complex (metastable GAPDH). The observed lag for NADPH-GAPDH activity was linked to the hysteretic activation of this enzyme by its substrate, BPGA. The kinetic data obtained for NADPH-GAPDH activity may thus be fitted to equation 1, but in a different context. Q is still the observed product concentration at time t, k_μ is the steady-state rate of the BPGA-activated GAPDH (metastable form), p(1) the initial rate, p(2) the observed rate of transition between the initial state and the activated GAPDH. P_t is the total enzyme concentration (Neet and Ainslie, 1980 ; Frieden, 1979). The progress curves obtained when monitoring the NADH-dependent activity of isolated GAPDH had the same shape than that of the enzyme in the complex. On the contrary, when following the NADPH-dependent activity of the isolated GAPDH, the lag disappeared. Whatever activity is considered, the steady-state rates of the isolated forms (k_σ) were lower than those of the metastable enzymes (k_μ) (Table 1).

We then studied the effects of NAD(H) and NADP(H) on the PRK, NADH- and NADPH-GAPDH activities.

In the case of PRK, the complex incubated at 30°C in the absence of any cofactor (control) had a stable PRK activity. When the complex was incubated with NAD(P)(H), the initial rate p(1) remained constant and PRK activity within the complex was not affected ($15\pm3.2 s^{-1}$)(data not shown). NAD(H) had no effects on the metastable PRK (k_μ), while incubation with NADP(H) activated the metastable PRK and the steady-state rate k_μ increased from 118 ± 7 s^{-1} to 179 ± 7 s^{-1} and $389\pm 35 s^{-1}$ after incubation with NADPH and NADP (fig. 1A and B). The apparent dissociation rate constant p(2) of the control experiment decreased, which might be the consequence of the enhancement of hydrophobic forces by temperature. The complex at 30°C thus tended towards a tighter form. Incubation with NADP(H) led to an increase of p(2), which indicates that the complex underwent a conformation change towards a relaxed form that dissociated faster in the reaction mixture. NAD(H) had no effects on p(2) compared to the control.

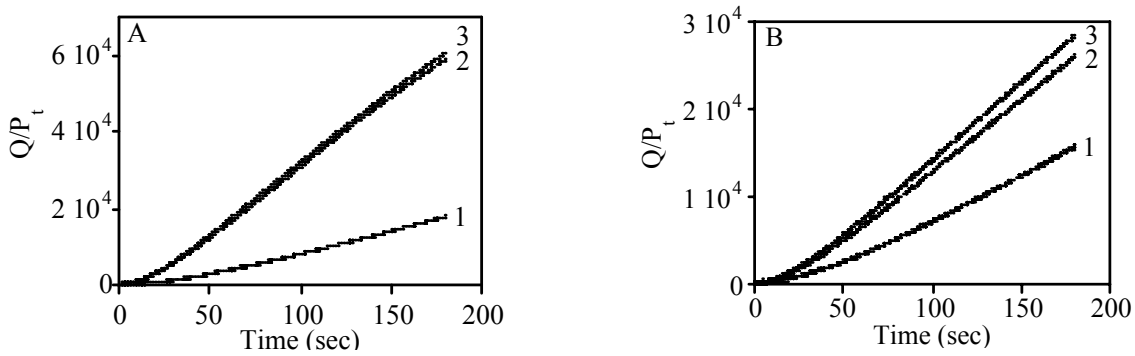

Fig. 1. Effects of NADP(H) on PRK activity. The bi-enzyme complex was incubated with 1 mM NADP (A) at 30°C and the activity measured at intervals. (1) incubation at 30°C without cofactors (control) ; (2) 35 min ; (3) 50 min incubation. The bi-enzyme complex was also incubated at 30°C with 1 mM NADPH (B). (1) control ; (2) 30 min ; (3) 60 min incubation. The final concentration of the bi-enzyme complex in the reaction mixture was 2.4 nM. Data were fitted to equation 1.

For NADH-GAPDH activity, the control had a stable activity and NAD(H) had no effects. NADP(H) inhibited NADH-GAPDH activity, NADP having the greater effect (50% inhibition). The values of the steady-state rates reached were reported in Table 1.
For NADPH-GAPDH activity, incubation of the complex at 30°C without cofactors resulted in an increased steady-state rate k_μ from 340 ± 31 s^{-1} to 435 ± 17 s^{-1}. This increase might also be due to the enhancement of hydrophobic forces described above. Taking into account this temperature effect, NADPH did not significantly alter the steady-state rate of GAPDH when monitoring the NADPH-dependent activity. Yet, upon incubation of the complex with NADP the lag disappeared and the steady-state rate increased from 435 ± 17 s^{-1} to 687 ± 73 s^{-1} (fig. 2), while NAD(H) had no effects (data not shown).

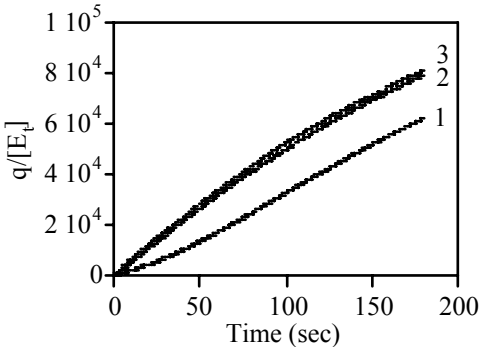

Fig. 2. Time curves of the reaction catalyzed by NADPH-GAPDH after incubation with 1mM NADP at 30°C. (1) control experiment (incubation at 30°C without NADP), (2) 30 min and (3) 60 min incubation. The final concentration of the complex in the reaction mixture was 1.1 nM.

We performed the same incubation experiments on isolated GAPDH and PRK and these isolated enzymes were not regulated by either of these cofactors.

We also compared the ratios of the NADH- and NADPH-dependent activities under the different conditions described above (Table 1).

Table 1. Catalytic rate constants of the metastable and stable enzymes in the different conditions studied. Comparison of the NADPH- and NADH-dependent activities of GAPDH.

	No incubation	Incubation with NADPH	Incubation with NADP
$(k_\sigma)_{NADH\text{-}GAPDH}$ (s^{-1})	33 ± 8	33 ± 8	33 ± 8
$(k_\sigma)_{NADPH\text{-}GAPDH}$ (s^{-1})	197 ± 27	197 ±27	197 ±27
$(k_\sigma)_{NADPH\text{-}GAPDH}/(k_\sigma)_{NADH\text{-}GAPDH}$	6.2 ± 1.5	6.2 ± 1.5	6.2 ± 1.5
$(k_\mu)_{NADH\text{-}GAPDH}$ (s^{-1})	147 ± 5	117 ± 2	73 ± 5
$(k_\mu)_{NADPH\text{-}GAPDH}$ (s^{-1})	340 ± 31 435 ± 17	435 ± 17	687 ± 73
$(k_\mu)_{NADPH\text{-}GAPDH}/(k_\mu)_{NADH\text{-}GAPDH}$	2.3 ± 0.2 3.0 ± 0.1	3.7 ± 0.1	9.4 ± 1.0

From the catalytic rate constants given in Table 1, one may calculate the free energies of activation of the reactions catalyzed by GAPDH using NADH or NADPH, under its metastable (μ) or its isolated (σ) state. These free energies of activation are :

$$\Delta G^{\neq}_{(\mu)} = RT \ln(k_B T/(h k_\mu)) \quad (2)$$

and

$$\Delta G^{\neq}_{(\sigma)} = RT \ln(k_B T/(h k_\sigma)) \quad (3)$$

where k_B and h are the Boltzmann and the Planck constants respectively.

The difference between these two energies represents the fraction of energy transferred from PRK to GAPDH that is stored even after the dissociation of the complex. This energy corresponds to the imprinting effect and it is then used to alter the rate of the catalysis performed by GAPDH using NADPH or NADH as cofactors.
These calculations show that the association of the two enzymes resulted in a decrease of the free energies of activation of the reactions catalyzed by GAPDH using NADH as cofactor (-3.8 kJ/mol) or NADPH (-1.3 kJ/mol). These effects are depicted in the thermodynamic diagrams of fig. 3.

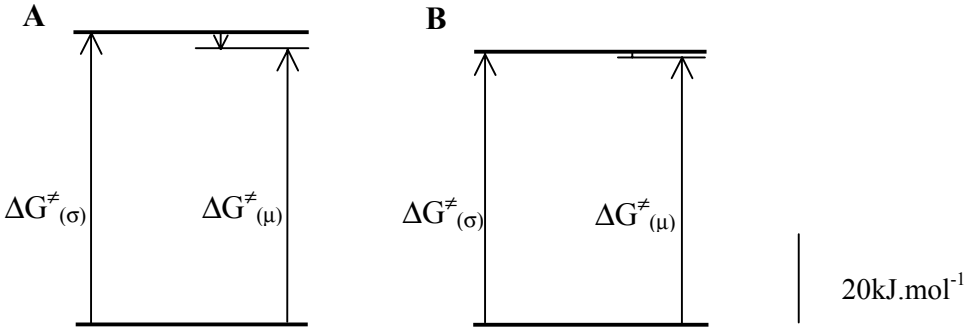

Fig.3. Imprinting effects after dissociation of the bi-enzyme complex. The imprinting effect exerted upon GAPDH by PRK alters the rate of catalysis whatever NADH (A) or NADPH (B) is used as cofactor.

One may also derive the free energies of activation of the process catalyzed by GAPDH and PRK released from the complex that has been incubated with the different cofactors, using the catalytic rate constants given in Table 1 and results obtained from fig. 1. The impact of the cofactors on the imprinting of GAPDH on PRK and *vice versa* has been quantified. NADPH decreases the energy barrier of the reaction catalyzed by PRK by 1.1 kJ/mol and increases the energy barrier of the reaction catalyzed by the metastable GAPDH using NADH as cofactor, by 0.6 kJ/mol. It has no effect on the energy barrier of the reaction catalyzed by the metastable GAPDH using NADPH as cofactor. NADP produces a decrease (1.2 kJ/mol) in the energy barrier of the reaction catalyzed by the metastable GAPDH using NADPH as cofactor. It also decreases the energy barrier of the reaction catalyzed by PRK by 3 kJ/mol. Opposite effects have been observed when the NADH-dependent activity of GAPDH was monitored. NADP in that case increases the height of the energy barrier of this reaction by about 1.8 kJ/mol.

Discussion

There is now considerable evidence that multi-enzyme complexes are involved in the Benson-Calvin cycle (Müller, 1972 ; Süss *et al*, 1995 ; Anderson *et al*, 1995 ; Clasper *et al* , 1991 ; Sainis *et al*, 1994). Some of these complexes catalyze consecutive reactions (Gontero *et al*, 1988 ; Hosur *et al*, 1993) but others do not. Functional advantage, if any, for these structures may reside in the modifications of the kinetic properties of the enzymes involved in these edifices. This is precisely the case for the bi-enzyme complex we have purified from *C. reinhardtii*. Indeed, we have previously shown that association within this bi-enzyme complex results in a modification of the apparent substrate binding constants and of the catalytic rate constant of PRK (Lebreton *et al*, 1997b). Moreover, thioredoxin may act on the metastable PRK or on the enzyme embedded in the complex, but the time required to activate the enzyme in the complex is shorter (about 20 sec) (Avilan *et al*, 2000).
It has also been previously shown that the metastable PRK had different kinetic properties than the isolated enzyme. The higher catalytic rate constants obtained for the metastable PRK compared to the isolated enzyme were linked to an imprinting or memory effect of GAPDH on PRK (Lebreton *et al*, 1997b). We show here that PRK also exerts an imprinting on GAPDH, resulting in higher catalytic rate constants for the metastable GAPDH compared to the isolated enzyme. This imprinting is quantified by calculating and comparing the free energies of activation of the isolated and metastable GAPDH. Our results show that the imprinting effect of PRK on GAPDH is smaller than that of GAPDH on PRK (Lebreton *et al*, 1997b), but this imprinting always results in higher catalytic rate constants for the metastable forms.

The isolated and metastable forms of PRK and GAPDH also behave differently as to their regulation by NADP(H). The isolated PRK and GAPDH are not regulated by these two metabolites, while PRK and NADPH-dependent activities are increased after incubation of the complex with NADP(H). On the contrary, NADH-GAPDH dependent activity is inhibited. Regulation by NADP or NADPH is only possible when GAPDH interacts with PRK, showing that protein-protein interactions play a major role in the emergence of new regulatory properties for the enzymes embedded in the complex. As previously, using thermodynamics, the impacts of NADP and NADPH on the imprinting effects between the two enzymes have been determined and NADP has the greater effect.
The dual specificity towards NADH or NADPH is an intrinsic property of chloroplastic GAPDH. Factors determining the use of one cofactor instead of the other are of capital importance. We show here that whatever form of GAPDH is considered, NADPH-dependent activity is always higher than the NADH-dependent activity. Yet, the ratio NADPH-GAPDH activity/NADH-GAPDH activity of the metastable GAPDH is decreased by a factor 2.7

compared to the isolated enzyme. Moreover, incubation of the complex with NADP(H) shifts the activity towards the NADPH-dependent one, whereas incubation of the isolated GAPDH does not modify the ratio of activities. Hence, protein-protein interactions enable the modulation of two different activities in a single enzyme.

Probably, the model (PRK-GAPDH) studied here is a simplified scheme of the situation *in vivo* because it is likely that in the cell, the interactions involve more than two enzymes. Nonetheless, it is a good tool to study how protein-protein interactions may result in different kinetic properties and how physiological advantage might arise from these heterologous interactions.

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