# THE ROLE OF pH AND MAGNESIUM CONCENTRATION IN THE LIGHT ACTIVATION OF CHLOROPLASTIC FRUCTOSE BISPHOSPHATASE

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## 1. Introduction

Spinach chloroplastic fructose 1,6-bisphosphatase is a tetrameric enzyme which belongs to the reductive pentose phosphate cycle (Benson–Calvin cycle) in chloroplasts. This enzyme is activated by light in vivo and plays a central part in the photocontrol of $CO_2$ fixation and reduction in plants [1,2]. This activation is mimicked in vitro by dithiothreitol which reduces two strategic disulfide bridges of the enzyme [3]. Since thioredoxin greatly stimulates this activation in vitro [1,2,4] it has been tentatively considered that the same process is operative in vivo and that light activation of fructose bisphosphatase in chloroplasts is mediated by the ferredoxin–thioredoxin system [1].

On the other hand, illumination of chloroplasts results in a pH rise from 7–8 within the stroma [5] and generates an increase of free magnesium concentration from 2–5 mM [5]. One may therefore wonder whether light activation of the enzyme could not be due to these effects, rather than to disulfide reduction, since it is known that enzyme activity depends on both pH and magnesium concentration [6,7].

Moreover, upon illumination of intact chloroplasts, there is a transient of several minutes before a steady state of $CO_2$ fixation is reached. There are indeed many possible explanations for this transient phase, but one may wonder whether a likely explanation could not be a 'slow' response of fructose bisphosphatase itself, to the rise in pH generated by the illumination.

The aim of this paper is to answer these questions.

## 2. Materials and methods

Spinach leaves were purchased from a local market. Fructose 1,6-bisphosphatase was purified to homogeneity as in [8]. The enzymatic activity was followed with a coupled assay [8] by monitoring reduction of $NADP^+$ at 340 nm. The unit of enzyme activity was defined as the quantity of enzyme required to hydrolyze 1 μmol of fructose bisphosphatase/min. Steady state rate measurements were performed with a Gilford 2400 S or a Beckman M VII spectrophotometer. pH-jump experiments from 7–8, or from 8–7, were performed by adding a suitable amount of buffer in the cuvette. It was verified that over pH 7–8 the activity of the coupling enzymes (glucose phosphate isomerase and glucose phosphate dehydrogenase) does not change significantly.

Reduction of oxidized fructose bisphosphatase by dithiothreitol was carried out by incubating the enzyme with an excess of reductant for 18 hours (ratio reductant/enzyme = $6 \times 10^4$). Under these conditions reduction of the enzyme corresponds to the breaking of two disulfide bridges [3].

## 3. Results

In figure 1B the effects of a pH variation from 7–8 on the steady state rate of oxidized and reduced fructose bisphosphatase are shown. Clearly below pH 8, and for magnesium concentrations of the order of those occurring in vivo, the oxidized enzyme has no activity. Alternately, the reduced enzyme has a high activity at pH 8 but no activity at all at pH 7. In figure 1A, the effect of magnesium concentration on the activity of the reduced enzyme is shown. Clearly for a magnesium concentration of ~2 mM, the one which exists in vivo under dark conditions, the reduced enzyme has no activity. Taken together, the results of fig.1 allow the conclusion that the variation of both pH and magnesium concentration in the stroma could


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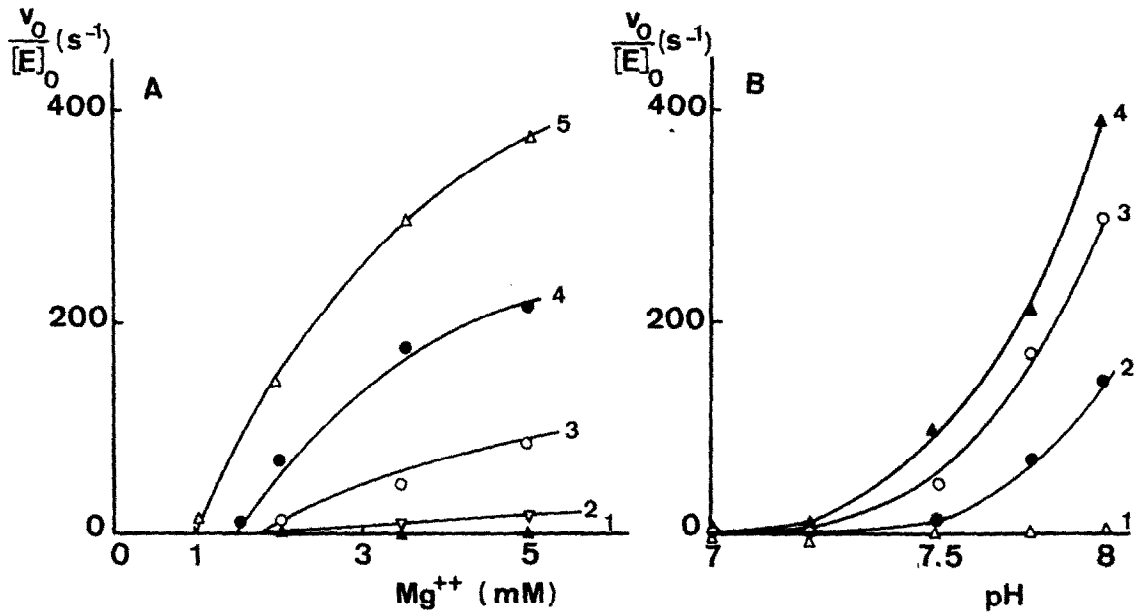


Fig.1. Effects of magnesium concentration and pH on fructose bisphosphatase activity. (A) Variation of specific initial steady state rate as a function of magnesium concentration. Curves 1, 2, 3, 4, 5 are obtained under pH values equal to 7, 7.24, 7.54, 7.77 and 7.95, respectively. These curves are obtained with the reduced enzyme. (B) Variation of the specific initial steady state rate as a function of pH. Curve 1 is obtained with the oxidized enzyme and a magnesium concentration of 5 mM. Curves 2, 3, 4 are obtained with the reduced enzyme and magnesium concentration equal to 2, 3.5 and 5 mM, respectively. Steady state rate determinations were performed as in [8]. Concentrations of fructose 1,6-bisphosphate and $NADP^+$ are 0.5 mM and 0.3 mM, respectively. The reaction mixture contains phosphoglucose isomerase (0.7 μg/ml) and glucose 6-phosphate dehydrogenase (0.35 μg/ml). The EDTA concentration is 0.5 mM and that of Tris–HCl buffer 100 mM. Fructose bisphosphatase is fully reduced by dithiothreitol. $[E]_0$ is the total fructose bisphosphatase concentration present in the reaction mixture.

be sufficient to explain activation of fructose bisphosphatase in the light and its inactivation in the dark. Indeed this does not mean that enzyme reduction by thioredoxin does not play any role in enzyme activation, but it appears likely that light-induced pH and magnesium concentration changes are certainly an important aspect of enzyme control.

When the pH of a fructose bisphosphatase solution is raised from 7–8, the enzyme becomes slowly activated. Alternately lowering the pH from 8–7 results in a slow decrease of the reaction rate and finally a complete blocking of the reaction. Under the experimental conditions used, the activity of the coupling enzymes does not change significantly between pH 7 and 8. Therefore the variation of the overall reaction rate implies that the active enzyme concentration slowly varies after the pH-jump. Since the rate of the reaction is proportional to active enzyme concentration, it is then easy to estimate the variation of active enzyme concentration as a function of time. Within the time-scale investigated, the kinetics of this process occurs in one step only, which allows estimation of an observed rate constant. This observed rate constant decreases as a function of $H^+$ ion concentration (fig.2).

The simplest explanation of these results is to assume that between pH 7 and 8 the reduced enzyme changes its ionization state and becomes slowly activated. This tentative scheme is shown in fig.3, where $E_i$ and $E_a$ are the inactive and the active forms, $K$ the ionization constant, and $k_1$ and $k_{-1}$ the rate constants of activation and deactivation respectively. If the ionization–protonation process is very fast compared with the rate of the activation step, the overall activation process will occur in one apparent step. The rate equation of this process assumes the form

$$\ln \frac{\bar{e}_a}{\bar{e}_a - e_a} = k_{obs} t \qquad (1)$$

where $\bar{e}_a$ is the equilibrium concentration of active enzyme after the pH-jump and $e_a$ the active enzyme

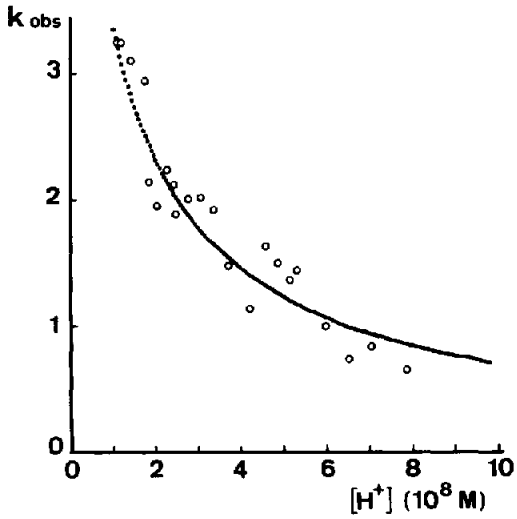


Fig.2. Variation of the observed rate constant of enzyme isomerization as a function of proton concentration. Circles are experimental values of the observed rate constant upon a pH jump from 7–8. The dotted curve is theoretical and obtained by non-linear least-square fitting of the results to equation (2). ($K = 1.16 \times 10^{-8}$ $M^{-1}$, $k_1 = 6.3$ $s^{-1}$, $k_{-1} = 0.1$ $s^{-1}$).

concentration at any time. The expression of this observed rate constant is

$$k_{obs} = \frac{K}{K + [H^+]} k_1 + k_{-1} \qquad (2)$$

The numerical values of $K$, $k_1$ and $k_{-1}$ are found to be, at pH 8, $1.16 \times 10^{-8}$ $M^{-1}$ (pK 7.93) 6.3 $s^{-1}$ and 0.1 $s^{-1}$, respectively.

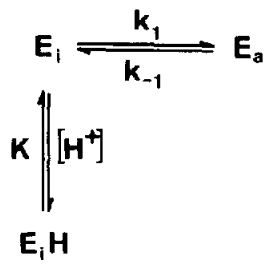


Fig.3. Tentative scheme of the slow conformational transition of fructose bisphosphatase. $E_i$, inactive enzyme; $E_a$, active form of the same enzyme.

## 4. Discussion

Although there is ample experimental evidence that reduction of fructose bisphosphatase by thioredoxin may regulate its activity in chloroplasts, the present results bring direct experimental evidence that light-induced pH and magnesium concentration changes also play an important role in this regulation process. This is in agreement with previous observations [6,7].

Fructose bisphosphatase is an oligomeric enzyme which displays sigmoidal kinetics and can be considered allosteric. In addition to this property, the enzyme slowly changes its activity, and most likely its conformation, upon a pH-jump. It therefore behaves as a 'hysteretic' enzyme [9,10]. Surprisingly the rate of this conformational transition is of the order of magnitude of the transient of $CO_2$ fixation by intact chloroplasts upon illumination. Although many possible explanations can be retained for this transient, a slow conformation change of fructose bisphosphatase is probably the simplest of them.

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