THE pH-INDUCED DISSOCIATION OF FRUCTOSE 1,6-BISPHOSPHATASE OF SPINACH CHLOROPLASTS

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Received 29 January 1980
Revised version received 14 March 1980

1. Introduction

Fructose 1,6-bisphosphatase from higher plants is receiving considerable attention [1–4]. The reason for this interest rests on the belief that this enzyme plays a major role in the control by light of the Calvin cycle [5,6]. Buchanan and his colleagues have suggested [7,8] that thioredoxin, reduced by electrons from photosystem I, reacts with fructose 1,6-bisphosphatase, thus reducing disulfide bonds of this enzyme and leading to a conformation change itself associated with a modulation of its activity.

Fructose 1,6-bisphosphatase from spinach chloroplasts is a tetramer with apparently identical subunits and a MW of 160 000. Raising the pH from 7.5 to 8.8 results in the dissociation of the enzyme in two equal halves of MW 80 000 [2]. Whereas the tetramer is active the dimer is almost devoid of activity. Since it is widely accepted that the pH of the chloroplast stroma changes upon illumination [9], it is of interest to study the kinetics of a conformational transition associated with the dissociation reaction and to relate this process to a possible change in the accessibility of sulfhydryl groups, for these groups are believed to play a key role in the activation-deactivation process of the enzyme [2]. This is the aim of the present paper.

2. Material and methods

Spinach leaves were purchased from a local market. Chloroplast fructose 1,6-bisphosphatase was purified according [2]. The enzyme was homogeneous to polyacrylamide gel electrophoresis (at pH 7.5, 7% polyacrylamide). The purified enzyme contains 8 tryptophan residues as determined by the method of PAJOT [10]. Enzyme activity was estimated with a coupled assay [2] by monitoring the rate of $NADP^+$ reduction.

Difference absorbance spectra were recorded with a Beckman Acta M VII spectrophotometer. A Gilford 2400 S spectrophotometer was routinely used to determine enzyme activity. Fluorescence emission measurements were made with a double-beam Fica 55 spectrofluorimeter. The fluorescence of the tryptophan residues, excited at 287 nm, was followed at 320 nm. Alternatively, for low levels of fluorescence change, the following device was used: excitation at 287 nm was effected with a xenon lamp (150 W) and a monochromator (10 nm band-pass); the emission spectrum was recorded with a photomultiplier tube (EMI 96 52B) and a potentiometric recorder (Sefram); stray light rejection was obtained with a cut-off filter (below 400 nm) in front of the photomultiplier.

Available sulfhydryl content was determined by reaction with 5′,5′-dithiobis-(2-nitrobenzoic acid) as described in [11]. Total sulfhydryl content was estimated with the same technique after denaturing the protein with 2% sodium dodecylsulfate. Protein was determined according to [12], with bovine serum albumin as a standard.

A computer BASIC program was written to normalize and fit the data to an exponential. Fitting was effected with a Wang 2200 mini-computer.

NADP, glucose-6-phosphate dehydrogenase, phosphoglucoisomerase and fructose bisphosphate were purchased from Boehringer, 5′,5′-dithiobis-(2-nitrobenzoic acid) from Sigma, and all other chemicals from Merck.

3. Results

Upon raising the pH from 7.5 up to 8.8, one observes, together with the dissociation of the enzyme, significant changes of the absorbance and fluorescence spectra (fig.1). These changes occur slowly and suggest that the environment of one or several tryptophan residues is being changed. The kinetics of the absorbance changes can be easily monitored at 287 nm. In the time scale investigated, this process occurs in one detectable step (fig.2A). If the pH is lowered from 8.8 to 7.5 no spectral change is observed (fig.2B). The time constant of the exponential (fig.2C) has a value of 2×10^{-3} s^{-1} at 35°C.

The kinetics of this conformational transition may also be followed by monitoring a slow quenching of the fluorescence of tryptophan residues (fig.2B). As previously, this process occurs in one step only and has the same time constant as the one followed by spectrophotometry (fig.2C).

This change of a spectroscopic signal (absorbance or fluorescence) is correlated with a decrease of activity of the enzyme. From the decay of the activity, which is exponential, one can estimate a time constant which has roughly the same value (2.10×10^{-3} s^{-1}) as the one calculated from spectroscopic data (fig.4). After the new equilibrium is reached, the remaining activity is close to zero, and a pH-jump from 8.8 to 7.5 does not restore any activity. Together with data of fig.2B, these results suggest that the dissociation process is irreversible.

Estimation of the values of the rate constant of the conformational transition may be effected at different temperatures and an Arrhenius plot may be constructed (fig.3). From this plot one can estimate the values of thermodynamic parameters associated with the rate constant of the conformational transition (table 1). It is worth noting the negative value of the entropy of activation $\Delta S^{\neq}$.

Since it is believed that –SH groups play an important role in the control of the active conformation of fructose 1,6-bisphosphatase it is of interest to determine the number of these groups, in either the dimeric or the tetrameric states. At pH 8.8, in the dimeric state,

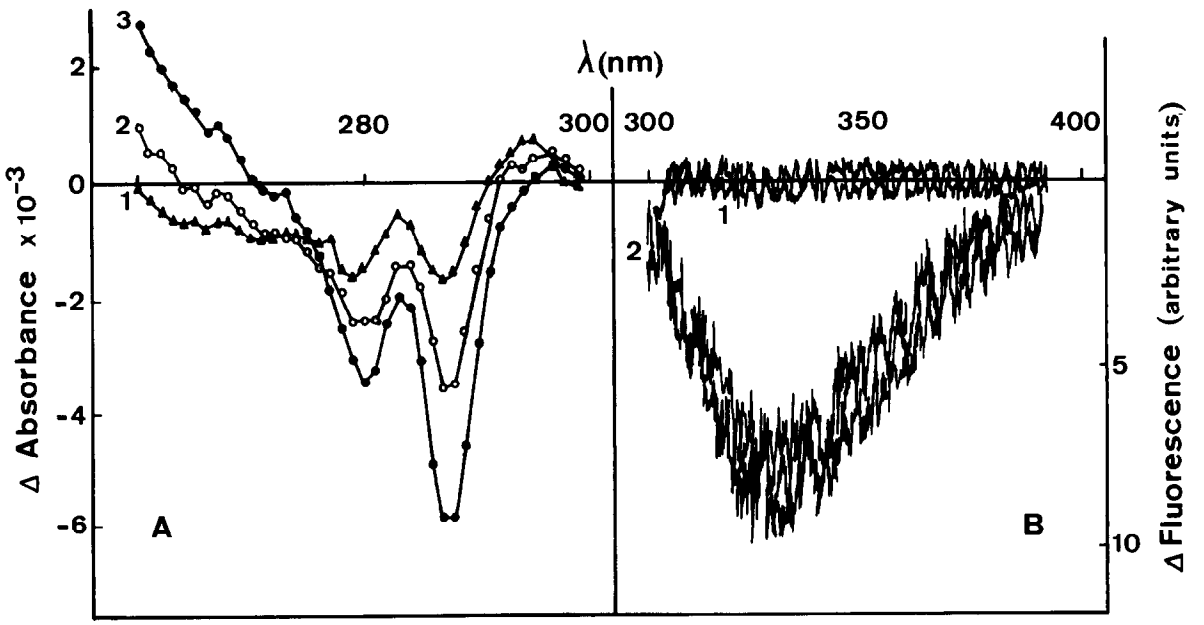

Fig.1. Spectral changes associated with the conformational transition of fructose 1,6-bisphosphatase induced by a pH-jump from 7.5 to 8.8. (A) Absorbance changes of the enzyme (difference spectra) at different times (1 : 5 min, 2 : 15 min, 3 : 60 min) after the pH-jump. The maximum value of $\Delta A/A$ at 287 nm is 0.03. (B) Intrinsic fluorescence changes of the enzyme (difference spectrum). Trace 1 is the baseline monitored before the pH-jump. Trace 2 is monitored 90 min after the pH-jump, and corresponds to the difference spectrum between the dimeric and the tetrameric states of the enzyme. The maximum value (90 min of the pH-jump) of $\Delta F/F$ at 320 nm is 0.20. Excitation is effected at 287 nm.

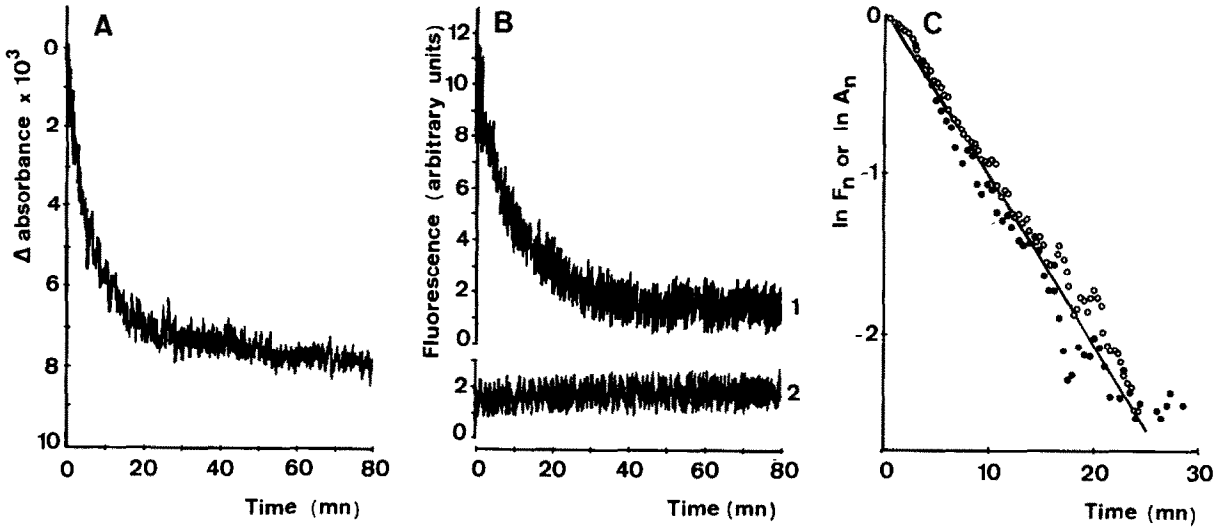

Fig.2. Slow conformational transition of fructose 1,6-bisphosphatase from spinach chloroplasts. (A) Absorbance change at 287 nm after a pH-jump from 7.5 to 8.8. (B) Intrinsic fluorescence change at 320 nm after a pH-jump from 7.5 to 8.8 (trace 1) or from 8.8 to 7.5 (trace 2). (C) Semi-log plot of the above normalized fluorescence (F_n) and absorbance (A_n) data after a pH-jump from 7.5 to 8.8. Closed and open circles represent absorbance and fluorescence data, respectively. Enzyme concentration is 0.01 mg/ml. The buffer used was a Tris–EDTA buffer and the temperature was 35°C.

6 (±0.31) sulfhydryl groups can be titrated. Denaturing the enzyme by sodium dodecylsulfate does not increase the number of available sulfhydryl groups. At pH 7.5, in the tetrameric state, no –SH group is directly available. However, after denaturing the protein by sodium dodecylsulfate, 12 (±0.27) –SH groups are titrated. When the enzyme passes from the tetrameric to the dimeric state, the accessibility of sulfhydryl groups per monomer increases from zero to three (fig.4). However, the kinetics of this change in availability is slower than the kinetics of inactivation and of spectral changes.

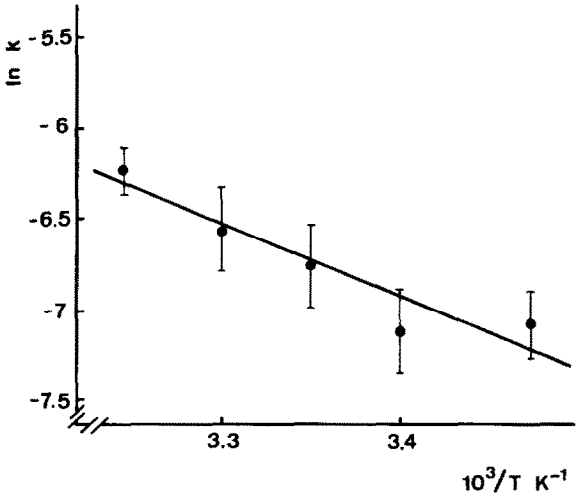

Fig.3. Arrhenius plot of the rate constant for 1,6-bisphosphatase conformational transition. k values are obtained by following the kinetics of the fluorescence change.

4. Discussion

Takabe et al. [4] have shown a slow increase of the fluorescence of toluidinyl naphthalene sulfonate

Table 1
Thermodynamic parameters of the dissociation of fructose 1,6-bisphosphatase from spinach chloroplasts

	Kcal (J) mol^{-1}
Energy of activation E_a	7.9 (33 000)
Enthalpy of activation $\Delta H^{\neq}$ (308 K)	7.3 (31 000)
	cal (J) mol^{-1} K^{-1}
Entropy of activation $\Delta S^{\neq}$ (308 K)	–47 (–200)
	Kcal (J) mol^{-1}
Free energy of activation $\Delta G^{\neq}$ (308 K)	22 (92 000)

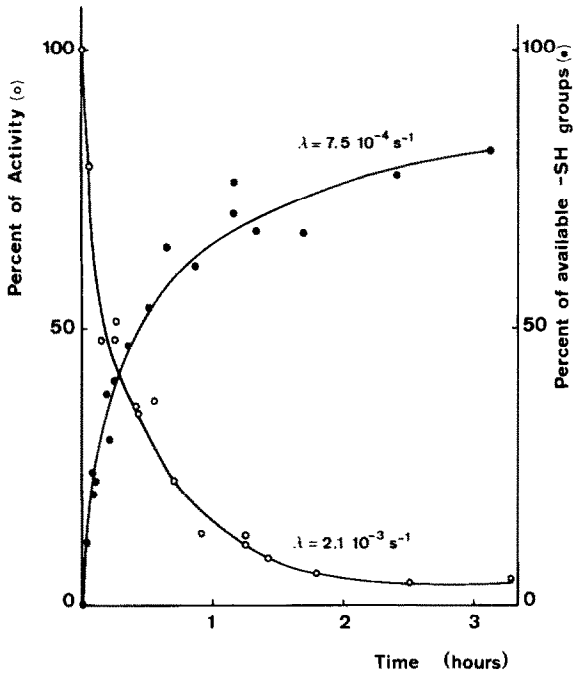

Fig.4. Loss of activity and availability of –SH groups during the conformational transition of fructose 1,6-bisphosphatase. (A) Progressive loss of activity after a pH-jump from 7.5 to 8.8. Data are normalized as percent of initial activity. (B) Progressive availability of –SH groups after a pH-jump from 7.5 to 8.8. Data are normalized as percent of total content of sulfhydryl groups. The buffer used was a Tris–EDTA buffer and the temperature was 35°C.

bound to fructose 1,6-bisphosphatase upon a pH-jump from 8 to 9. This result strongly suggests that a conformation change is occurring. However, one may wonder whether it is not the binding of the fluorescent probe to the protein which triggers its conformation change. Results presented in this paper prove it is not so, since both the intrinsic absorbance and fluorescence of the protein slowly change upon raising the pH.

The entropic term $-T\Delta S^{\neq}$ plays an important part in the value of free energy of activation $\Delta G^{\neq}$. The negative value of $\Delta S^{\neq}$, which makes the term $-T\Delta S^{\neq}$ positive, may imply an ordering of solvent molecules, or alternatively a decrease of degree of freedom of polypeptide chains in the transition state, or both.

There is a slight discrepancy between the number of –SH groups determined by Zimmermann et al. [2] and our present results. We have found twelve –SH groups for the tetrameric fructose 1,6-bisphosphatase, whereas Zimmermann et al. [2] have titrated ten –SH groups. All the –SH groups we have titrated were unavailable in the absence of sodium dodecylsulfate, whereas Zimmermann et al. [2] have found four of these –SH groups directly accessible in the native enzyme. The reason for this discrepancy is unknown. It is worth noting however, that the finding that the dimer has six –SH groups is quite consistent with the result that the native tetrameric enzyme contains twelve –SH groups.

The kinetics of the appearance of available –SH groups, during the dissociation process, is slower than the kinetics of spectral changes, and therefore slower than the progressive loss of activity. This result is not surprising for a change of activity, or a change of a spectral property, may be indicative of more subtle conformation changes, than the increase of accessibility of sulfhydryl groups that were initially buried in the protein molecule and thus inaccessible to the –SH reagent.

It would be interesting to know whether fructose 1,6-bisphosphate and Mg^{2+} bind independently to the dimeric or to the tetrameric state of the enzyme and whether they modify the rate of the dissociation process. This work is in progress.

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