Biochem. J. (1980) **185**, 689–693
Printed in Great Britain

Properties of Freshly Purified and Thiol-Treated Spinach Chloroplast Fructose Bisphosphatase

Stephen A. CHARLES and Barry HALLIWELL
Department of Biochemistry, University of London King's College, Strand, London WC2R 2LS, U.K.

(*Received* 14 *August* 1979)

Freshly purified spinach chloroplast fructose bisphosphatase is powerfully inhibited by inorganic phosphate competitively with respect to its substrate fructose 1,6-bisphosphate. The concentrations of phosphate and substrate in the chloroplast stroma are such that the enzyme in this form could not operate at a significant rate *in vivo*. Incubation of the enzyme with dithiothreitol for 24h decreases the K_m for fructose 1,6-bisphosphate from 0.8 to 0.033mM, decreases the K_m for Mg^{2+} from 9 to 2mM and substantially alleviates inhibition by inorganic phosphate. The physiological significance of thiol activation of the enzyme is discussed.

Chloroplasts contain (Smillie, 1960) an alkaline fructose bisphosphatase (EC 3.1.3.11) (Racker & Schroeder, 1958) for the specific hydrolysis of fructose 1,6-bisphosphate to fructose 6-phosphate and P_i during operation of the Calvin cycle of photosynthetic CO_2 fixation in the stroma. The purification and properties of this enzyme have been reported from several laboratories (Racker & Schroeder, 1958; Buchanan *et al.*, 1971; Preiss & Greenberg, 1971; Lazaro *et al.*, 1974; El-Badry, 1974; Baier & Latzko, 1975; Zimmermann *et al.*, 1976). There is ample evidence to suggest that fructose bisphosphatase is a metabolic control point of the cycle (for reviews see Kelly *et al.*, 1976*a*; Halliwell, 1978; Heldt *et al.*, 1978). Fructose bisphosphatase requires Mg^{2+} for its activity, and it has been suggested that light-induced changes in stromal pH and Mg^{2+} concentration are the principal means by which the enzyme is regulated *in vivo* (Kelly *et al.*, 1976*a*; Heldt *et al.*, 1978). On illumination of chloroplasts the stromal pH rises from approx. 7.0 to approx. 8.0 (Werdan *et al.*, 1975) and the Mg^{2+} concentration increases by 1–3mM (Portis & Heldt, 1976), although the basal concentration of stromal Mg^{2+} has not been established (for a review see Halliwell, 1978).

Several workers have shown that the apparent fructose bisphosphatase activity of leaves or of illuminated chloroplasts slowly increases on illumination and decreases again in the dark (Buchanan *et al.*, 1971; Kelly *et al.*, 1976*b*; Champigny & Bismuth, 1976). Activation can be mimicked by incubating leaf extracts, chloroplasts or the purified enzyme with dithiol compounds, e.g. dithiothreitol, *in vitro*. Indeed, dithiothreitol activation is a good model system for the light-activation that occurs *in vivo*, which, it has been suggested, is due to the generation of membrane-bound dithiol groups within the chloroplast (Anderson & Avron, 1976), or to production of the dithiol form of the protein thioredoxin (Buchanan *et al.*, 1979).

There have been several studies on the kinetic properties of spinach chloroplast fructose bisphosphatase, but they have been carried out under conditions that do not occur *in vivo*. For instance, non-physiological substrate concentrations have been used (Baier & Latzko, 1975; Schurmann & Wolosiuk, 1978) or Mg^{2+} concentrations lower than those present *in vivo* (Schurmann & Wolosiuk, 1978). The latter authors found that fructose bisphosphatase, assayed at 1mM-Mg^{2+} (and 6mM-fructose 1,6-bisphosphate) was activated by incubation with reduced thioredoxin, but at higher Mg^{2+} concentrations the effect of thioredoxin was much less marked.

It seemed to us that the physiological significance of thiol activation could not be adequately assessed without a detailed comparison of the properties of freshly purified and thiol-treated fructose bisphosphatase under the conditions likely to exist *in vivo*. For example, the stromal concentration of fructose 1,6-bisphosphate in illuminated chloroplasts is 0.3–0.4mM (Lilley *et al.*, 1977; Heldt & Chon, 1978; Kaiser & Bassham, 1979) and does not change significantly in the dark [in their model experiments Baier & Latzko (1975) assumed concentrations of 1.0mM in the light and 0.2mM in the dark]. Estimates of the stromal P_i concentration range from 10 to 138mM (Hall, 1976), although more recent

0306-3275/80/030689-05 $1.50/1

evidence suggests that values of 5–10 mM in the light are more representative (Lilley *et al.*, 1977; Heldt & Chon, 1978; Kaiser & Bassham, 1979). Clearly, P_i should be included in assay mixtures of fructose bisphosphatase if physiological conditions are to be approximated.

The present paper reports a kinetic study of the freshly purified and thiol-treated forms of fructose bisphosphatase, with particular reference to the effect of P_i.

Materials and Methods

Materials

All reagents were purchased from Sigma Chemical Co., Poole, Dorset, U.K. Spinach (*Spinacia oleracea*) was purchased locally.

Determination of fructose bisphosphatase activity

Reaction mixtures contained, in a total volume of 2 ml, 100 mM-Tris/HCl buffer, pH 8.0, 0.5 mM-EDTA, 10 mM-$MgCl_2$, 0.3 mM-$NADP^+$, 3 units (μmol/min) of phosphoglucose isomerase (EC 5.3.1.9), 1 unit (μmol/min) of glucose 6-phosphate dehydrogenase (EC 1.1.1.49) and about 0.02 unit (μmol/min) of fructose bisphosphatase. The reaction was started by the addition of 1 mM-fructose 1,6-bisphosphate and followed by the change in absorbance at 340 nm in a Pye Unicam SP.8-100 u.v. spectrophotometer. The initial rate of change in absorbance was proportional to the amount of fructose bisphosphatase added over the range used in this study. Dithiothreitol or P_i at the concentrations used did not affect the coupled enzyme assay. The amount of enzyme required to hydrolyse 1 μmol of fructose 1,6-bisphosphate/min at 25°C was defined as 1 unit.

Purification of spinach chloroplast fructose bisphosphatase

The purification was essentially similar to that described by Zimmermann *et al.* (1976), except for the following differences: the cation K^+ replaced Na^+ in all buffers and in the salt gradient used in the ion-exchange step; the DEAE-Sephadex A-50 ion-exchange column was 56 cm × 5.3 cm^2.

The enzyme purified by this method catalysed the hydrolysis of fructose 1,6-bisphosphate with a specific activity of 101 units/mg of protein under the standard conditions of assay. Protein was determined by the method of Lowry *et al.* (1951), with dry bovine serum albumin as standard.

Treatment of fructose bisphosphatase with dithiothreitol

Freshly purified fructose bisphosphatase (40 μg) was incubated with 20 mM-dithiothreitol as described by Zimmermann *et al.* (1976).

Results

Properties of freshly purified fructose bisphosphatase

P_i at concentrations comparable with those in the chloroplast stroma was found to inhibit freshly purified spinach chloroplast fructose bisphosphatase severely (Fig. 1). At pH 8.0 over 50% inhibition

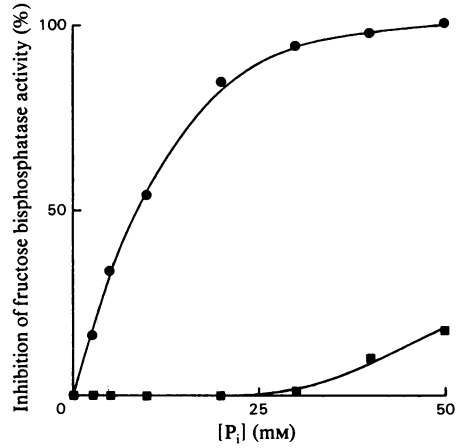

Fig. 1. *Effect of P_i on the activity of fructose bisphosphatase*
Enzyme activity was determined as described in the Materials and Methods section except that P_i was added and the concentrations were varied as shown. ●, Freshly purified fructose bisphosphatase; ■, thiol-treated fructose bisphosphatase.

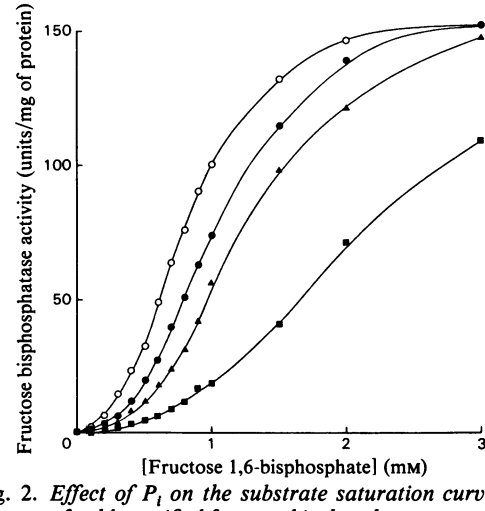

Fig. 2. *Effect of P_i on the substrate saturation curve of freshly purified fructose bisphosphatase*
Enzyme activity was determined as described in the Materials and Methods section except that the concentration of fructose 1,6-bisphosphate was varied and P_i was added as shown. O, Control; ●, +5 mM-P_i; ▲, +10 mM-P_i; ■, +20 mM-P_i.

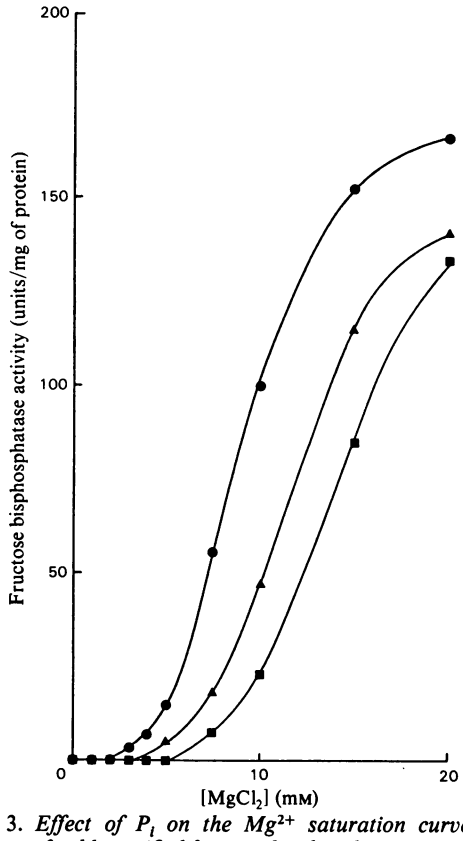

Fig. 3. *Effect of P_i on the Mg^{2+} saturation curve of freshly purified fructose bisphosphatase*
Enzyme activity was determined as described in the Materials and Methods section except that EDTA was omitted, the concentration of $MgCl_2$ was varied and P_i was added as shown. ●, Control; ▲, +10mM-P_i; ■, +20mM-P_i.

was seen with 10mM-P_i, and 50mM-P_i caused complete inhibition.

Fig. 2 shows the substrate saturation curve for the enzyme and the effect of three different concentrations of P_i. Although the kinetics are sigmoidal, it may be seen that half-maximal velocity is achieved at a substrate concentration of 0.8mM. Higher fructose 1,6-bisphosphate concentrations decrease the inhibition caused by P_i, which suggests that the latter is a competitive inhibitor with respect to substrate.

Fig. 3 shows the Mg^{2+} saturation curve for the enzyme. Half-maximal velocity at pH 8.0 is achieved at an Mg^{2+} concentration of 9mM, and it appears that elevated concentrations of Mg^{2+} are unable to relieve the inhibition caused by P_i.

Properties of thiol-treated fructose bisphosphatase

Freshly purified fructose bisphosphatase was incubated with dithiothreitol as described in the Materials and Methods section. In agreement with Zimmermann *et al.* (1976), we found that the thiol-treated enzyme did not rapidly revert to its original form on removal of the dithiothreitol; in fact, it was stable for several weeks. When assayed at 1mM-fructose 1,6-bisphosphate and pH 8.0 the thiol-treated enzyme was not inhibited by up to 20mM-P_i (Fig. 1). More than 80% of the activity remained in the presence of 50 mM-P_i.

Fig. 4 shows the substrate saturation curve of the thiol-treated enzyme. It is hyperbolic, as opposed to sigmoidal for the untreated enzyme (Fig. 2), and maximum activity is achieved at physiological concentrations of fructose 1,6-bisphosphate. The data were plotted according to the method of

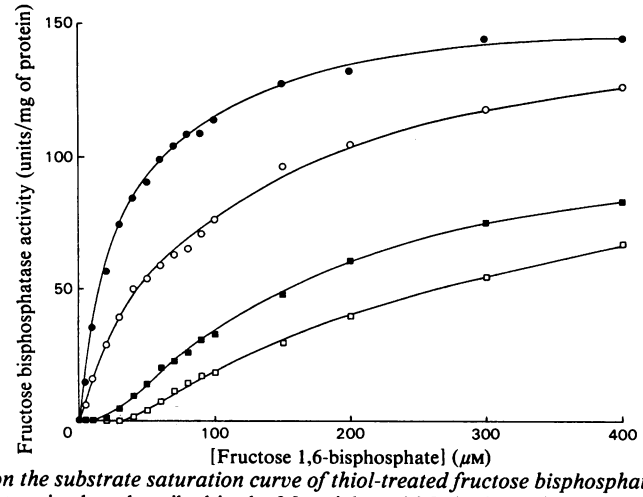

Fig. 4. *Effect of P_i on the substrate saturation curve of thiol-treated fructose bisphosphatase at two pH values*
Enzyme activity was determined as described in the Materials and Methods section except that the concentration of fructose 1,6-bisphosphate and the pH were varied and P_i was added as shown. pH 7.5 was obtained by using 100mM-imidazole/HCl buffer in place of 100mM-Tris/HCl buffer. ●, pH 8.0; ○, pH 8.0 + 20mM-P_i; ■, pH 7.5; □, pH 7.5 + 20mM-P_i.

Lineweaver & Burk (1934), and $V_{max.}$ was found to be similar to that of the freshly purified enzyme (Fig. 5*a*). However, the K_m was decreased to 33 μM. Fig. 5(*a*) also illustrates that inhibition of the thiol-treated enzyme by P_i is competitive with respect to substrate and the K_i for P_i is 12 mM (Fig. 5*b*).

The Mg^{2+} saturation curve of the thiol-treated fructose bisphosphatase at pH 8.0 (Fig. 6) also shows a decrease in sigmoidicity, with the result that the K_m for Mg^{2+} is decreased to 2 mM.

Figs. 4 and 6 show the substrate and Mg^{2+} saturation curves for the thiol-treated enzyme at pH 7.5. Both the $V_{max.}$ and the affinity for substrate and cofactor are substantially lower than at pH 8.0. Fig. 6 also shows the effect of Mg^{2+} on the thiol-treated enzyme at pH 7.0. $V_{max.}$ is lowered still

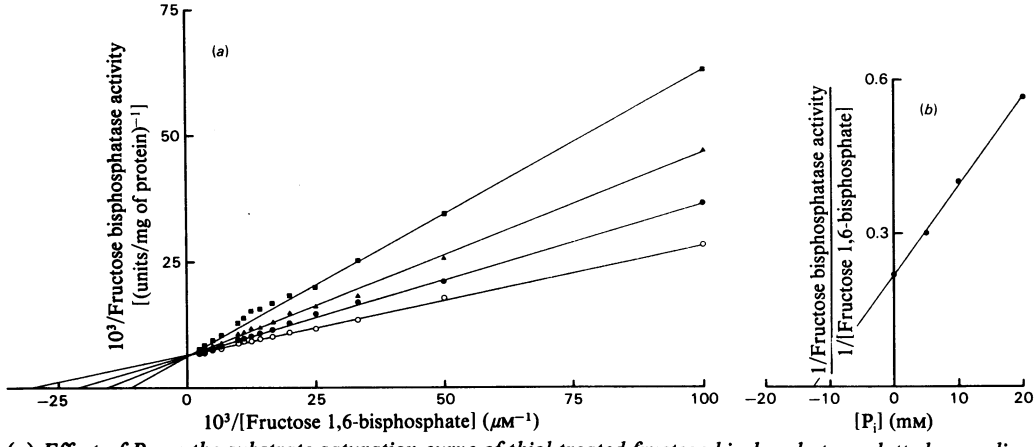

Fig. 5. (*a*) *Effect of P_i on the substrate saturation curve of thiol-treated fructose bisphosphatase plotted according to the method of Lineweaver & Burk (1934) and (b) determination of K_i for P_i*

(*a*) Enzyme activity was determined as described in the Materials and Methods section except that the concentration of fructose 1,6-bisphosphate was varied and P_i was added as shown. ○, Control; ●, +5 mM-P_i; ▲, +10 mM-P_i; ■, +20 mM-P_i. $V_{max.}$ was found to be 154 units/mg of protein and the K_m values were as follows: control, 33 μM; +5 mM-P_i, 45 μM; +10 mM-P_i, 63 μM; +20 mM-P_i, 83 μM. From (*b*) the K_i value was found to be 12 mM.

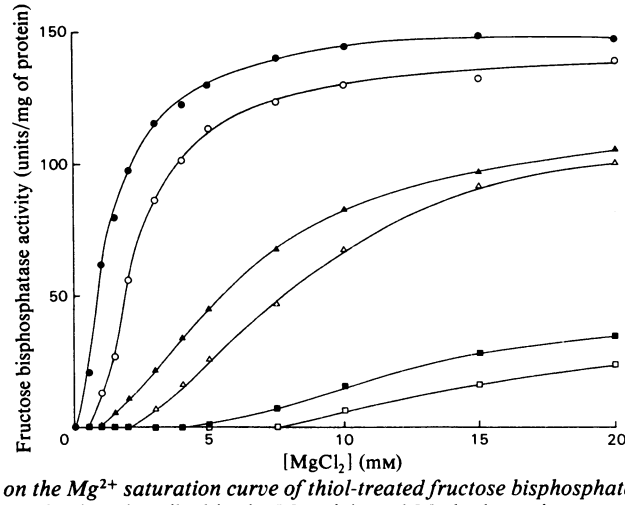

Fig. 6. *Effect of P_i on the Mg^{2+} saturation curve of thiol-treated fructose bisphosphatase at three pH values*

Enzyme activity was determined as described in the Materials and Methods section except that EDTA was omitted, the concentration of $MgCl_2$ and the pH were varied and P_i was added as shown. pH 7.5 and 7.0 were obtained by using 100 mM-imidazole/HCl buffer in place of 100 mM-Tris/HCl buffer. The concentration of fructose 1,6-bisphosphate was 0.4 mM. ●, pH 8.0; ○, pH 8.0 + 20 mM-P_i; ▲, pH 7.5; △, pH 7.5 + 20 mM-P_i; ■, pH 7.0; □, pH 7.0 + 20 mM-P_i.

further and the enzyme is completely inactive at 4mM-Mg^{2+}. In the presence of 20mM-P_i the enzyme is completely inactive at Mg^{2+} concentrations as high as 7.5mM.

Discussion

At the pH (8.0) and fructose 1,6-bisphosphate concentration (0.3–0.4mM) present in the stroma of the illuminated chloroplast, freshly purified spinach chloroplast fructose bisphosphatase would be almost inactive because of its low affinity for substrate (K_m 0.8mM) and inhibition by P_i. Hence the form of the enzyme obtained by Zimmermann *et al.* (1976) and by ourselves is unlikely to be the form that functions in the chloroplast *in vivo*.

Since the enzyme *in vivo* is acted on in the light by the dithiol protein thioredoxin and by membrane-bound dithiol groups, we examined the effect of incubation with dithiothreitol on its properties. Dithiothreitol is widely used as a model for the effect of dithiol groups *in vivo* (Anderson & Avron, 1976; Buchanan *et al.*, 1979). At pH8.0, the enzyme is obviously altered in some way by the dithiol because the kinetics change from sigmoid to hyperbolic and the K_m values for substrate and Mg^{2+} are brought within the physiological range. The $V_{max.}$ of the enzyme is unchanged, so the effect of dithiothreitol is not strictly an activation process, but at the concentration of substrate present *in vivo* an increase in activity will be achieved because of the decrease in K_m. Also, the virtually complete inhibition by stromal P_i will be largely alleviated. The thiol-treated enzyme therefore seems well adapted to a role *in vivo*, and the function of the dithiol systems *in vivo* would seem to be that of generating this form.

When the light is turned off, the reduced enzyme does not rapidly revert to its 'low-affinity' state *in vivo* (Kelly *et al.*, 1976*b*; Champigny & Bismuth, 1976). Similarly, the dithiothreitol-treated enzyme is quite stable *in vitro*. It therefore seems that changes in the reduction state of the enzyme are too slow to account for 'fine control' of the enzyme activity. In the dark the stromal pH falls from approx. 8.0 to approx. 7.0 and the Mg^{2+} concentration by 1–3mM (Krause, 1977). Figs. 4 and 6 show that the pH fall substantially decreases the $V_{max.}$ of the enzyme and also its affinity for substrate and Mg^{2+}. It seems that these changes would be sufficient to regulate the 'thiol-treated form' of the enzyme *in vivo*.

We are grateful to Professor M. C. Scrutton and Dr. G. F. Betts for advice, to the Wellcome Foundation and the Rank Prize Funds for financial support and to Miss Bernice Archard for excellent assistance in preparation of the manuscript. S. A. C. thanks the Science Research Council for a Research Studentship.

References

Anderson, L. E. & Avron, M. (1976) *Plant Physiol.* **57**, 209–213

Baier, D. & Latzko, E. (1975) *Biochim. Biophys. Acta* **396**, 141–148

Buchanan, B. B., Schurmann, P. & Kalberer, P. P. (1971) *J. Biol. Chem.* **246**, 5952–5959

Buchanan, B. B., Wolosiuk, R. A. & Schurmann, P. (1979) *Trends Biochem. Sci.* **4**, 93–95

Champigny, M. L. & Bismuth, E. (1976) *Physiol. Plant.* **36**, 95–100

El-Badry, A. M. (1974) *Biochim. Biophys. Acta* **333**, 366–377

Hall, D. O. (1976) in *The Intact Chloroplast*, vol. 1 (Barber, J., ed.), pp. 135–170, Elsevier, Amsterdam

Halliwell, B. (1978) *Prog. Biophys. Mol. Biol.* **33**, 1–54

Heldt, H. W. & Chon, C. J. (1978) *FEBS Lett.* **92**, 234–240

Heldt, H. W., Chon, C. J., Lilley, R. McC. & Portis, A. R. (1978) *Proc. Congr. Photosynth. 4th* 469–478

Kaiser, W. M. & Bassham, J. A. (1979) *Plant Physiol.* **63**, 105–108

Kelly, G. J., Latzko, E. & Gibbs, M. (1976*a*) *Annu. Rev. Plant Physiol.* **27**, 181–205

Kelly, G. J., Zimmermann, G. & Latzko, E. (1976*b*) *Biochem. Biophys. Res. Commun.* **70**, 193–199

Krause, G. H. (1977) *Biochim. Biophys. Acta* **466**, 500–510

Lazaro, J. J., Gorge, J. L. & Mayor, F. (1974) *Phytochemistry* **13**, 2455–2461

Lilley, R. McC., Chon, C. J., Mosbach, A. & Heldt, H. W. (1977) *Biochim. Biophys. Acta* **460**, 259–272

Lineweaver, H. & Burk, D. (1934) *J. Am. Chem. Soc.* **56**, 658–666

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275

Portis, A. R. & Heldt, H. W. (1976) *Biochim. Biophys. Acta* **449**, 434–446

Preiss, J. & Greenberg, E. (1971) *Methods Enzymol.* **23**, 691–696

Racker, E. & Schroeder, E. A. R. (1958) *Arch. Biochem. Biophys.* **74**, 326–344

Schurmann, P. & Wolosiuk, R. A. (1978) *Biochim. Biophys. Acta* **522**, 130–138

Smillie, R. (1960) *Nature (London)* **187**, 1024–1025

Werdan, K., Heldt, H. W. & Milovancev, M. (1975) *Biochim. Biophys. Acta* **396**, 276–292

Zimmermann, G., Kelly, G. J. & Latzko, E. (1976) *Eur. J. Biochem.* **70**, 361–367