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Enhancement of the reductive activation of chloroplast fructose-1,6-bisphosphatase by modulators and protein perturbants

Miguel A. BALLICORA and Ricardo A. WOLOSIUK

Instituto de Investigaciones Bioquímicas (Fundación Campomar, IIBBA-CONICET, FCEN-UBA), Buenos Aires, Argentina

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To characterize the mechanism of chloroplast fructose-1,6-bisphosphatase activation, we have examined kinetic and structural changes elicited by protein perturbants and reductants. At variance with its well-known capacity for enzyme inactivation, 150 mM sodium trichloroacetate yielded an activatable chloroplast fructose-1,6-bisphosphatase in the presence of 1.0 mM fructose 1,6-bisphosphate and 0.1 mM Ca^{2+}. Other sugar bisphosphates did not replace fructose 1,6-bisphosphate whereas Mg^{2+} and Mn^{2+} were functional in place of Ca^{2+}. Variations of the emission fluorescence of intrinsic fluorophores and a noncovalently bound extrinsic probe [2-(*p*-toluidinyl)naphthalene-6-sulfonate] indicated the presence of conformations different from the native form. A similar conclusion was drawn from the analysis of absorption spectra by means of fourth-derivative spectrophotometry. The effect of these conformational changes on the reductive process was studied by subsequently incubating the enzyme with dithiothreitol. The reaction of chloroplast fructose-1,6-bisphosphatase with dithiothreitol was accelerated 13-fold by the chaotropic anion: second-order rate constants were 48.1 $M^{-1} \cdot min^{-1}$ and 3.7 $M^{-1} \cdot min^{-1}$ in the presence and in the absence of trichloroacetate, respectively. Thus, the enhancement of the reductive activation by compounds devoid of redox activity illustrated that the modification of intramolecular noncovalent interactions of chloroplast fructose-1,6-bisphosphatase plays an essential role in the conversion of enzyme disulfide bonds to sulfhydryl groups. In consequence, a conformational change would operate concertedly with the reduction of disulfide bridges in the light-dependent activation mediated by the ferredoxin–thioredoxin system.

In chloroplasts of higher plants, reduced thioredoxin stimulates regulatory enzymes of the reductive pentose phosphate cycle [1].

$$\text{Enzyme}_{\text{inactive}} \xrightarrow{\text{Thioredoxin(SH)}_2} \text{Enzyme}_{\text{active}} .$$

The protein-disulfide reductase activity of this low-molecular-mass protein (≈12 kDa) is generally considered as the underlying mechanism because the enhancement of catalytic capacity concurs with the reduction of enzyme disulfide bonds [2]. In these studies, the extent of activation depended on the source of thioredoxin even though all of them contain the same redox sequence (-W-C-G-P-C-) [3]. An early example of this was the demonstration that the enhancement of chloroplast fructose-1,6-bisphosphatase activity by chloroplast thioredoxin-f was higher than that by chloroplast thioredoxin-m [4]. Later, site-directed mutagenesis and chemical modifications of cysteines in the active site enabled nonfunctional thioredoxins to enhance the capacity to hydrolyze fructose 1,6-bisphosphate [5, 6]. On the other hand, we found that cosolvents, chaotropic anions, and high hydrostatic pressure replace thioredoxin in the activation of key enzymes of the Benson-Calvin cycle [7–10]. Given the lack of redox activity of these protein perturbants, it appears that the role of thioredoxin in enzyme activation is not circumscribed exclusively to the reduction of disulfide bridges.

Despite extensive knowledge of the reductive activation a cogent argument for the participation of noncovalent interactions in the activation of chloroplast fructose-1,6-bisphosphatase has not yet been advanced. In this aspect, thioredoxin is not a suitable probe because (a) the reductive process overlaps with protein–protein interactions, and (b) most of its intrinsic signals are similar to those provided by the enzyme. Therefore, prior to considering mechanisms originated from the analysis of thioredoxin action, we deemed it desirable to carry out studies with low-molecular-mass compounds whose action on proteins is unique. This approach not only resolves the ambiguities concerning conformational changes and reduction of disulfide bonds but also overcomes interferences in spectroscopic studies.

On this basis, we altered intramolecular noncovalent interactions of the enzyme by a chaotropic anion (trichloroacetate) and converted its disulfide bonds to sulfhydryl groups by a reductant (dithiothreitol). Results reported herein show that trichloroacetate concertedly with fructose 1,6-bisphos-

Correspondence to R. A. Wolosiuk, Fundación Campomar, Antonio Machado 151, RA-1405 Buenos Aires, Argentina

Fax: +54 1 865 2246.

Enzyme. Chloroplast fructose-1,6-bisphosphatase (EC 3.1.3.11).

phate and Ca^{2+} stimulates the reductive activation of chloroplast fructose-1,6-bisphosphatase because it yields an enzyme conformation different from the native form.

EXPERIMENTAL PROCEDURES

Materials

Chloroplast fructose-1,6-bisphosphatase was purified to homogeneity from frozen spinach leaves as previously described [8]. Biochemicals were purchased from Sigma Chemical Co. Solutions of fructose 1,6-bisphosphate, dithiothreitol, and sodium trichloroacetate were first treated with Chelex-100 resin (BioRad Labs) to remove bi- and trivalent cations [8] and, subsequently, the pH was adjusted to 7.9.

Assay of chloroplast fructose-1,6-bisphosphatase activity at low concentrations of fructose 1,6-bisphosphate and Mg^{2+}

The enzyme was injected into a solution containing 25 µmol Tris/HCl pH 7.9, 0.4 µmol fructose 1,6-bisphosphate, 0.5 µmol $MgCl_2$ and 0.01 µmol EGTA; final volume 0.5 ml. After 10 min at 23 °C, the reaction was stopped by adding the reagent that estimates the P_i released [11].

Fluorescence measurements

Samples for the analysis of intrinsic fluorescence were prepared by diluting stock solutions of chloroplast fructose-1,6-bisphosphatase in 37 mM Tris/HCl pH 7.9, 1.11 mM fructose 1,6-bisphosphate, 0.11 mM Ca^{2+}. To reduce the 'inner filter' effect, the absorbance at 280 nm of enzyme solutions was in the range of 0.05−0.20. An aliquot (2.7 ml) was placed in a quartz cuvette (1.0×1.0 cm), excited at 280 nm, and the intensity of emission measured at 332 nm with a Jasco FP-770 spectrofluorometer. The excitation and emission bandwidth on monochromators were set up at 5 nm. After 30 min, 0.3 ml trichloroacetate solution was added to obtain the desired concentration. The fluorescence emission at 332 nm was acquired as above and data were corrected for dilution.

To analyze the fluorescence of an extrinsic probe, chloroplast fructose-1,6-bisphosphatase was mixed with 0.11 mM 2-(*p*-toluidinyl)naphthalene-6-sulfonate in 55 mM Tris/HCl pH 7.9. The enzyme solution (2.7 ml) was added to a quartz cell (1.0×1.0 cm) and placed in the spectrofluorometer. The exciting light was set at 366 nm and the intensity of emission was measured at 404 nm (slits 5 nm). Afterwards, small aliquots of concentrated solutions of modulators and trichloroacetate were added to the cuvette, and data of fluorescence emission were corrected for volume changes.

Fourth-derivative spectra of chloroplast fructose-1,6-bisphosphatase

Chloroplast fructose-1,6-bisphosphatase was incubated at 23 °C for 20 min in 0.8 ml 62.5 mM Tris/HCl pH 7.9. The enzyme solution was injected into a 1.0-cm path-length quartz cell and placed in a Gilford Response II spectrophotometer. Wavelengths were scanned from 270 nm to 300 nm (slit width 1.0 nm, scan rate 60 nm/min). Data were acquired by an IBM computer through a RS-232C interface. Concentrated solutions of modulators and trichloroacetate (0.1 ml) were succesively added to the cuvette. Usually 10 min elapsed between additions because an incubation of 5 min preceded the time it takes to record three spectra (5 min). Digitalized data with an interval of 0.1 nm were first corrected for volume changes and subsequently averaged. An interval of 1 nm was selected for obtaining fourth-derivative spectra by a computer program written in Pascal language (M. A. Ballicora, unpublished). Curves were smoothed by the convolution (Savitzky-Golay) method taking 51 points for averaging.

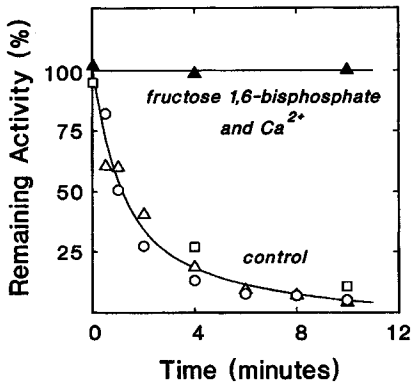


Fig. 1. The effect of fructose 1,6-bisphosphate and Ca^{2+} on the inactivation of chloroplast fructose-1,6-bisphosphatase by trichloroacetate. Chloroplast fructose-1,6-bisphosphatase (3.5 µg) was incubated in 0.5 ml 50 mM Tris/HCl pH 7.9, 150 mM sodium trichloroacetate containing, as indicated, 1.0 mM fructose 1,6-bisphosphate and 0.1 mM $CaCl_2$. When $CaCl_2$ was omitted, the solution contained 0.1 mM EGTA. (▲) Complete; (○) minus fructose 1,6-bisphosphate; (△) minus $CaCl_2$; (□) minus both fructose 1,6-bisphosphate and $CaCl_2$. At the indicated times, an aliquot (0.05 ml) was withdrawn and injected into 0.2 ml of a solution in such a way that, after mixing, the final concentration of the activation solution was 50 mM Tris/HCl pH 7.9, 5.0 mM dithiothreitol, 1.0 mM fructose 1,6-bisphosphate, 0.1 mM $CaCl_2$, 150 mM sodium trichloroacetate. After 10 min, an aliquot (0.05 ml) was injected into the solution for assaying fructose-1,6-bisphosphatase activity at low concentrations of both fructose 1,6-bisphosphate and Mg^{2+}. All incubations were carried out at 23 °C. Control activity 57 µmol P_i released · min^{-1} · mg $protein^{-1}$.

RESULTS

The effect of fructose 1,6-bisphosphate, Ca^{2+} and trichloroacetate on chloroplast fructose-1,6-bisphosphatase activity

Chaotropic anions are generally considered protein denaturants which, in the particular case of enzymes, lead to the loss of activity [12]. Fig. 1 shows that chloroplast fructose-1,6-bisphosphatase was no exception to this view because the enzyme lost 90% of its activity when it was incubated for 6 min with 150 mM sodium trichloroacetate. The rate of inactivation (k = 0.35 min^{-1}; $t_{0.5}$ = 2 min) was threefold faster than that observed with alkaline pH (k = 0.12 min^{-1}; $t_{0.5}$ = 6 min) [13]. The incorporation of either 1.0 mM fructose 1,6-bisphosphate or 0.1 mM $CaCl_2$ before or after trichloroacetate neither prevented the inactivation nor restored the hydrolytic activity, respectively. However, the enzyme retained the catalytic capacity when both modulators were present prior to the addition of trichloroacetate. Like the inactivation by alkaline pH, fructose 1,6-bisphosphate and Ca^{2+} enabled chloroplast fructose-1,6-bisphosphatase to

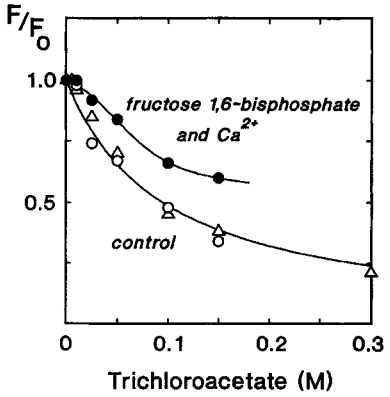


Fig. 2. The effect of sodium trichloroacetate on the intrinsic fluorescence of chloroplast fructose-1,6-bisphosphatase. In a spectrofluorometric cuvette (1.0×1.0 cm), chloroplast fructose-1,6-bisphosphatase (10 μg) was incubated at 23 °C in 2.7 ml of a solution containing 100 μmol Tris/HCl pH 7.9, and, as indicated, 3 μmol fructose 1,6-bisphosphate and 0.3 μmol $CaCl_2$. (○) Control; (●) plus fructose 1,6-bisphosphate and Ca^{2+}. After 30 min, a solution of sodium trichloroacetate (0.3 ml) was added to give the final concentration indicated on the *x* axis. Following 5 min of incubation, the sample was excited at 280 nm and the emission fluorescence was measured at 332 nm (slit: 5.0 nm). F_o represents the emission fluorescence of fructose-1,6-bisphosphatase solution in the absence of sodium trichloroacetate; therefore, after the addition of trichloroacetate, all data were corrected for volume changes. The fluorescence emission at 350 nm of 5.4 mg *N*-acetyl-L-tryptophan ethyl ester in 2.7 ml 50 mM Tris/HCl pH 7.9 (△) was measured by exciting at 280 nm (slit: 5.0 nm). Data from both enzyme and *N*-acetyl-L-tryptophan ethyl ester were normalized for comparison.

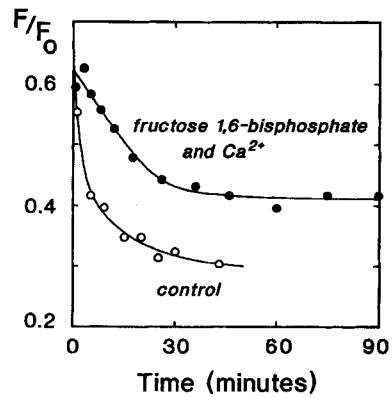


Fig. 3. Time progress of trichloroacetate effect on the intrinsic fluorescence of chloroplast fructose-1,6-bisphosphatase. The enzyme (12.0 μg) was dissolved in 2.16 ml of a solution containing 100 μmol Tris/HCl pH 7.9 and, as indicated, 2.4 μmol fructose 1,6-bisphosphate and 0.24 μmol $CaCl_2$. At zero time, 0.24 ml 1.5 M sodium trichloroacetate was added and the emission fluorescence of the solution was recorded successively. Excitation wavelength was 280 nm and emission wavelength 332 nm (slit 5.0 nm). All incubations were performed at 23 °C. F_o represents the emission fluorescence of the solution before the addition of sodium trichloroacetate; therefore, subsequent data were corrected for volume changes.

withstand the irreversible loss of activity originated from the alteration of noncovalent interactions.

Although not shown, we found that fructose 1,6-bisphosphate was unique in protecting the enzyme from the inactivation by trichloroacetate whereas an alternative substrate (sedoheptulose 1,7-bisphosphate), an inhibitor (fructose 2,6-bisphosphate) or nonfunctional sugar bisphosphates (ribulose 1,5-bisphosphate, glucose 1,6-bisphosphate) were totally ineffective. In contrast to sugar bisphosphates, the requirement of the bivalent metal was not specific because either low concentrations of Mn^{2+} or high concentrations of Mg^{2+} were also functional in preventing the loss of activity. In another complementary experiment, the removal of Ca^{2+} by chelation immediately lowered the capacity to hydrolyze fructose 1,6-bisphosphate; the subsequent addition of $CaCl_2$ (0.4 mM) did not restore the original activity even though the incubation was prolonged for 1 h (data not shown).

The action of trichloroacetate on the intrinsic fluorescence of chloroplast fructose-1,6-bisphosphatase

The above results suggested that perhaps fructose 1,6-bisphosphate and Ca^{2+} mediated the modification of the tertiary structure whereby the enzyme overcame inactivation by the chaotropic anion. Spinach chloroplast fructose-1,6-bisphosphatase is a tetramer that contains two tryptophan residues per subunit [14]. This amino acid residue constituted a potential intrinsic probe for sensing structural changes because the fluorescence emission is highly sensitive to surroundings. As shown in Fig. 2, the emission at 332 nm declined when chloroplast fructose-1,6-bisphosphatase was excited at 280 nm in the presence of increasing concentrations of trichloroacetate. However, the effect could not be ascribed to intramolecular motions of tryptophan residues because the maximum of fluorescence emission remained at 332 nm whereas for the enzyme treated with 8.0 M urea (i.e. denatured) it was 350 nm [15]. Furthermore, the decay of fluorescence was similar to the trichloroacetate-mediated quenching of *N*-acetyl-tryptophan ethyl ester. In both cases, the linear dependence of fluorescence yield ratios (F_o/F) on trichloroacetate concentration was described by $F_o/F = 1+K_{sv} \cdot$ [trichloroacetate], in which the Stern-Volmer quenching constant (K_{sv}) was 11.8 M^{-1}. Apparently, intrinsic fluorophores of chloroplast fructose-1,6-bisphosphatase were all equally accessible to the quencher. At variance, 1.0 mM fructose 1,6-bisphosphate and 0.1 mM $CaCl_2$ alleviated the quenching of tryptophan residues observed with trichloroacetate. This gave rise to a Stern-Volmer plot curved downwards. In principle, if one population of tryptophan residues were accessible to the quencher whereas the other were inaccessible, it should be expected that the total fluorescence (F) was comprised of the intensity of the former fraction $\{F_{oa} \cdot (1+K_{sv} \cdot$ [trichloroacetate]$)^{-1}\}$ and the intensity of the latter fraction (F_{ob}) [16]. As a corollary, $F_o = F_{oa}+F_{ob}$ when the quencher was absent. Linear secondary plots of $F_o \cdot (F_o-F)^{-1}$ versus [trichloroacetate]$^{-1}$ suggested that half of tryptophan residues $[F_{oa} \cdot (F_{oa}+F_{ob})^{-1} = 0.5]$ were accessible to trichloroacetate when chloroplast fructose-1,6-bisphosphatase was treated with fructose 1,6-bisphosphate and Ca^{2+}.

The temporal variation of emission intensity was consistent with the idea that these modulators modified the exposure of intrinsic fluorophores. As depicted in Fig. 3, when native chloroplast fructose-1,6-bisphosphatase was excited at 280 nm in the presence of 150 mM trichloroacetate, the fast decay of emission at 332 nm ($t_{0.5}$ = 2 min) was followed by a slow decrease until extinction. The initial rate of quenching was similar to the loss of activity (cf. Fig. 1). Conversely, when the enzyme was incubated with 1.0 mM fructose 1,6-bisphosphate and 0.1 mM Ca^{2+} prior to trichloroacetate, the fluorescence emission decayed slowly ($t_{0.5}$ = 15 min) to F/F_o = 0.66 and remained constant afterwards. While these results allowed the unambiguous assignment of the effect of

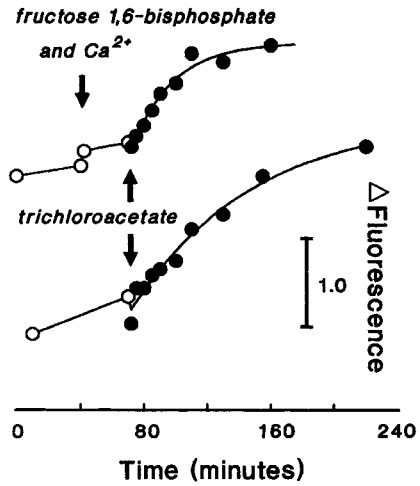


Fig. 4. The effect of sodium trichloroacetate on the fluorescence of 2-(*p*-toluidinyl)naphthalene-6-sulfonate in the presence of chloroplast fructose-1,6-bisphosphatase. The enzyme (15.0 μg) was dissolved in 2.7 ml of a solution containing 150 μmol Tris/HCl pH 7.9, 0.30 μmol 2-(*p*-toluidinyl)naphthalene-6-sulfonate. The sample was excited at 366 nm and the emission fluorescence was measured at 404 nm (excitation and emission bandwidth on monochromators: 5.0 nm); 3 μmol fructose 1,6-bisphosphate and 0.3 μmol $CaCl_2$ were injected, as indicated (total volume 3.0 ml) and, subsequently, 0.45 μmol sodium trichloroacetate was added (final volume 3.3 ml). The emission fluorescence at 404 nm (excitation: 366 nm) was continuously monitored: fluorescence intensity (○) before and (●) after the incorporation of trichloroacetate. All incubations were at 23 °C.

fructose 1,6-bisphosphate and Ca^{2+} to conformational rearrangements of chloroplast fructose-1,6-bisphosphatase, more studies are necessary to establish how and which tryptophan residues became inaccessible to trichloroacetate.

The effect of trichloroacetate on 2-(*p*-toluidinyl)naphthalene-6-sulfonate fluorescence

To confirm the structural effect of fructose 1,6-bisphosphate and Ca^{2+}, we investigated the interaction of chloroplast fructose-1,6-bisphosphatase with reporter compounds whose fluorescence emission is low in aqueous solutions and high in nonpolar solvents. A solution of 0.03 μM chloroplast fructose-1,6-bisphosphatase containing 86 μM 2-(*p*-toluidinyl)-naphthalene-6-sulfonate was excited at 366 nm and the emission intensity was monitored at 404 nm. As shown in Fig. 4, 140 mM trichloroacetate slowly enhanced the emission of the extrinsic probe (k = 0.02 min^{-1}; $t_{0.5}$ = 40 min). It was interesting to observe that, in total contrast with these results, the chaotropic anion quenched the low (basal) emission when the enzyme was absent. Apparently, the slow incorporation of 2-(*p*-toluidinyl)naphthalene-6-sulfonate into chloroplast fructose-1,6-bisphosphatase was the slowest step, because the fluorescence increase was not modified by changing the order in which the fluorophore and the perturbant were added. Subsequently, in order to assess the effect of fructose 1,6-bisphosphate (1.0 mM) and $CaCl_2$ (0.1 mM), we incubated the enzyme with these modulators prior to the addition of trichloroacetate (140 mM). In Fig. 4, the moderate increase of 2-(*p*-toluidinyl)naphthalene-6-sulfonate fluorescence by fructose 1,6-bisphosphate and Ca^{2+} was similar to that reported by Chardot et al. [17]. However, the concerted action of these modulators increased the rate by which trichloroacetate enhanced the emission of 2-(*p*-toluidinyl)naphthalene-6-sulfonate (k = 0.04 min^{-1}; $t_{0.5}$ = 15 min).

Although these data were congruent with the enhancement of fluorescence when anilino-naphthalene derivatives are placed into a nonpolar environment [18], a clear mechanism was not so apparent for the response to trichloroacetate. In control experiments 150 mM trichloroacetate quenched the fluorescence of 2-(*p*-toluidinyl)naphthalene-6-sulfonate buried into the small hydrophobic pocket of β-cyclodextrin [19]. From this result we reasoned that trichloroacetate changed the conformation of chloroplast fructose-1,6-bisphosphatase in a way that facilitated the slow incorporation of 2-(*p*-toluidinyl)naphthalene-6-sulfonate into a hydrophobic domain away from the bulk solvent; i.e. the access of trichloroacetate to the probe for quenching was severely restricted. In this context, the interaction of fructose 1,6-bisphosphate and Ca^{2+} with the enzyme undoubtedly contributed to an acceleration of the structural modification mediated by trichloroacetate.

Modification of fourth-derivative spectra of chloroplast fructose-1,6-bisphosphatase by fructose 1,6-bisphosphate, Ca^{2+} and trichloroacetate

We reported previously that the activation of chloroplast fructose-1,6-bisphosphatase was accompanied by modifications of difference spectra [8]. The main disadvantage of this analysis is that rotational and vibrational transitions broaden absorption peaks of proteins in conventional ultraviolet spectra and consequently mask the information about aromatic amino acids. However, fourth-derivative spectrophotometry unravels constituent bands in a position similar to the original spectrum [20]. Using this approach, Fig. 5 shows that two main peaks of native chloroplast fructose-1,6-bisphosphatase at 279.5 nm and at 287.5 nm (curve 1) were not modified by either 1.0 mM fructose 1,6-bisphosphate (curve 2) or 0.1 mM $CaCl_2$. Nevertheless, the simultaneous presence of these modulators displaced maxima intensity to 278 nm and 288 nm (curve 3). Hence, aromatic amino acids in the enzyme molecule sensed the modification mediated by the concerted action of fructose 1,6-bisphosphate and Ca^{2+}. The fourth-derivative spectrum returned to that observed with the native enzyme (curve 4) when EGTA (0.24 mM) was incorporated. At this stage, the addition of trichloroacetate (150 mM) yielded three well defined peaks located at 277 nm, 284 nm and 290.5 nm (curve 5). However, this profile remained when Ca^{2+} (0.4 mM) was added to prevail over the chelating effect of EGTA. Taking together these results and kinetic studies, we inferred that chloroplast fructose-1,6-bisphosphatase was irreversibly denatured by trichloroacetate. Accordingly, we observed a spectrum similar to curve 5 after the native enzyme was incubated with trichloroacetate in the absence of modulators. In contrast, the incorporation of trichloroacetate to a solution of chloroplast fructose-1,6-bisphosphatase containing 1.0 mM fructose 1,6-bisphosphate and 0.1 mM $CaCl_2$ yielded a radically different fourth-derivative spectrum: two maxima peaks were located at 277.5 nm and at 285.5 nm (curve 6). These data not only showed conformational differences between native chloroplast fructose-1,6-bisphosphatase and the enzyme treated with modulators and protein perturbants but, more important, illustrated the requirement of a strict sequence of additions to elicit a given tertiary structure. In conclusion, only the ordered addition of fructose 1,6-bisphosphate, Ca^{2+} and trichloroacetate gener-

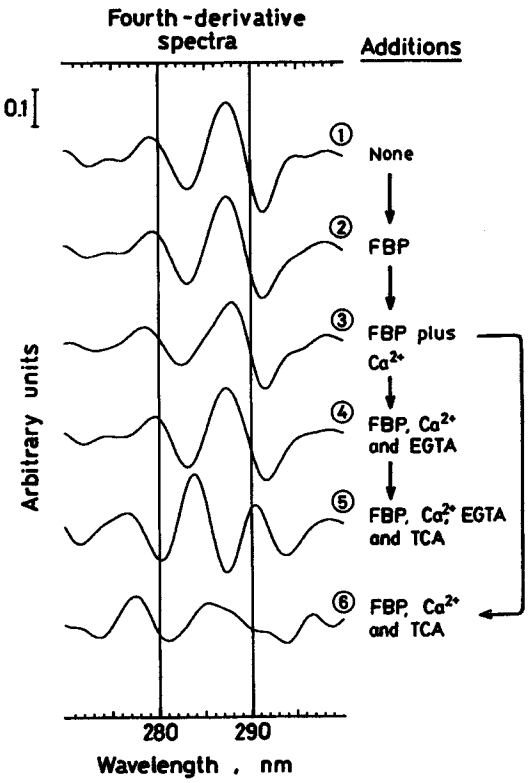


Fig. 5. Fourth-derivative analysis of ultraviolet spectra of chloroplast fructose-1,6-bisphosphatase. The enzyme (140 μg) was incubated at 23 °C for 20 min in 0.8 ml of a solution containing 50 μmol Tris/HCl pH 7.9 and the ultraviolet spectrum was scanned three times (slit 1.0 nm; scan speed 60 nm/min). Data were transferred to an IBM computer through a RS-232C interface, and the fourth-derivative was calculated from the average of three spectra by a program developed in our laboratory. Successively, 0.1 ml 10 mM fructose 1,6-bisphosphate (FBP); 0.1 ml 1.0 mM $CaCl_2$, 0.2 ml 10 mM EGTA and 0.1 ml 1.5 M sodium trichloroacetate (TCA) were added. After each addition, spectra were determined after an incubation of 5 min. Digitalized data (interval 0.1 nm) were first stored in the computer, subsequently corrected for volume changes, and finally processed to obtain the fourth-derivative of spectra.

ated activatable conformations; otherwise, the enzyme was irreversibly inactivated.

The effect of trichloroacetate on the reductive activation of chloroplast fructose-1,6-bisphosphatase

Although dithiols (and monothiols to lesser extent) enhance the activity of chloroplast fructose-1,6-bisphosphatase [21], we performed the above experiments in the absence of reductants because our initial purpose was to delineate kinetic and structural effects of modulators and protein perturbants that do not transform covalent bonds. Clearly, the modification of intramolecular noncovalent interactions provoked the appearance of two functional entities: one originated from the action of fructose 1,6-bisphosphate and Ca^{2+} whereas the other depended on the additional presence of trichloroacetate. On this basis, it was tempting to speculate that conformational rearrangements would condition the posterior activation of chloroplast fructose-1,6-bisphosphatase by reductants. To study this issue, we treated chloroplast fructose-1,6-bisphosphatase for 15 min with 1.25 mM fructose 1,6-bisphosphate and 0.125 mM Ca^{2+} in the presence of

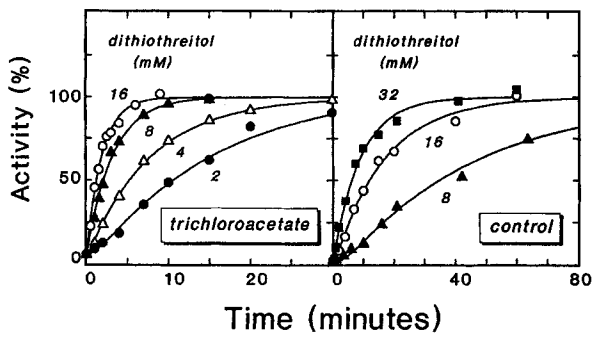


Fig. 6. Effect of dithiothreitol on the activation of chloroplast fructose-1,6-bisphosphatase mediated by fructose 1,6-bisphosphate, Ca^{2+}, and sodium trichloroacetate. The enzyme (1.8 μg) was incubated at 23 °C for 15 min in 0.4 ml of a solution containing 50 μmol Tris/HCl pH 7.9, 0.5 μmol fructose 1,6-bisphosphate, 0.05 μmol $CaCl_2$, and (left only) 75 μmol sodium trichloroacetate. Dithiothreitol was added to the indicated concentrations (final volume: 0.5 ml). At varying times, an aliquot (0.04 ml) was withdrawn and injected into the solution for assaying fructose-1,6-bisphosphatase activity at low concentrations of both fructose 1,6-bisphosphate and Mg^{2+}. Control activity: 51.0 μmol Pi released · min^{-1} · mg · $protein^{-1}$.

150 mM trichloroacetate and, after an incubation with dithiothreitol, we assayed the catalytic activity. As depicted in Fig. 6, the chaotropic anion curtailed the time for the reductive activation of the enzyme. At 2.5 mM dithiothreitol, the $t_{0.5}$ was 8 min in the presence and 2 h in the absence of trichloroacetate. Germane to these data is the observation of Soulie et al. [22] that, with similar concentration of dithiothreitol but in absence of modulators, 4 h elapsed before the half-activation of chloroplast fructose-1,6-bisphosphatase. Although the variation in the rate of the activation was less than 10% with different enzyme preparations, the reductive process always followed the trend described above.

The capacity of thiol-bearing compounds for reducing disulfide bonds in proteins made plausible the following scheme:

Oxidized enzyme (inactive) — Dithiothreitol — Reduced enzyme (active) — 1,4dithiane

Thus, the rate of chloroplast fructose-1,6-bisphosphatase reduction ($d[ES_2]/dt$) was given by $k \cdot [ES_2] \cdot$ [dithiothreitol] where k = second-order rate constant, and ES_2 = the oxidized enzyme. In our studies, the concentration of dithiothreitol (>2 mM) always exceeded the concentration of chloroplast fructose-1,6-bisphosphatase (<10 μM); therefore, the reaction described in Eqn (1) should follow pseudo-first-order kinetics: $-d[ES_2]/dt = k' \cdot [ES_2]$, in which $k' = k \cdot$ [dithiothreitol]. With the integration of the rate of enzyme conversion in mind [$\ln 2 = k' \cdot (t_{0.5})$], we obtained the corresponding $t_{0.5}$ from data in Fig. 6 by using the non-linear regression method of Gauss-Newton [23]. Taking into account that $k' = \ln 2 \cdot (t_{0.5})^{-1} = k \cdot$ [dithiothreitol], in Fig. 7 we plotted the inverse of $t_{0.5}$ versus the concentration of dithiothreitol. This secondary plot illustrates that trichloroacetate increased 13-fold the second-order rate constant of dithiothreitol-mediated activation: $k = 48.1\ M^{-1} \cdot min^{-1}$ in the

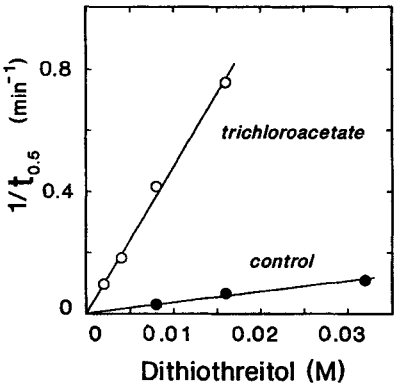
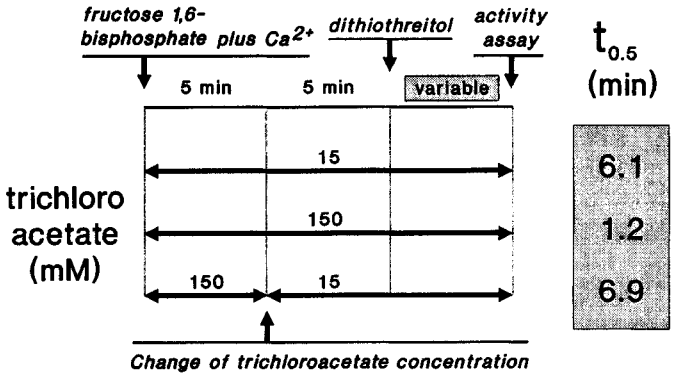


Fig. 7. Effect of sodium trichloroacetate on the reductive activation of chloroplast fructose-1,6-bisphosphatase. $t_{0.5}$ for the activation process were determined by non-linear regression fits (Gauss-Newton method) of data depicted in Fig. 6. The concentration of sodium trichloroacetate in the conversion of inactive chloroplast fructose-1,6-bisphosphatase to an active form was 150 mM.



Scheme 1. Reversibility of the trichloroacetate action on fructose 1,6-bisphosphate/Ca^{2+}-treated chloroplast fructose-1,6-bisphosphatase. The enzyme (2.5 μg) was incubated in 0.05 ml 0.1 M Tris/HCl pH 7.9, 1.0 mM fructose 1,6-bisphosphate, 0.1 mM $CaCl_2$ containing sodium trichloroacetate, as indicated. After 5 min, 0.45 ml of the same solution containing 15 mM, 150 mM or 0 M sodium trichloroacetate was added, and the incubation was continued for another 5 min. Following the addition of 0.025 ml 0.1 M dithiothreitol, the enzyme was first incubated at varying times, and subsequently, the fructose-1,6-bisphosphatase activity was tested in an 0.05-ml aliquot at low concentrations of both fructose 1,6-bisphosphate and Mg^{2+}, as described under Experimental Procedures. Time for reaching half of the maximal fructose-1,6-bisphosphatase activity ($t_{0.5}$) was obtained from data of activity/time by the non-linear regression method of Gauss-Newton. All incubations were carried out at 23 °C.

presence and 3.7 $M^{-1} \cdot min^{-1}$ in absence of 150 mM trichloroacetate. The linear dependence of $(t_{0.5})^{-1}$ on the concentration of dithiothreitol validated the above considerations for the reaction described in Eqn (1). If conformational changes of oxidized chloroplast fructose-1,6-bisphosphatase conditioned the reductive process, in the range of dithiothreitol concentration used (2−32 mM), then plots in Fig. 7 would be curved downwards. Although not shown, an independent experiment was consistent with the slowness of the reductive process relative to modifications of noncovalent interactions. The $t_{0.5}$ was 2.5 min when chloroplast fructose-1,6-bisphosphatase was treated for 15 min or 0 min with fructose 1,6-bisphosphate (1.25 mM), $CaCl_2$ (0.125 mM), and trichloroacetate (150 mM), prior to the incubation with dithiothreitol (8.0 mM).

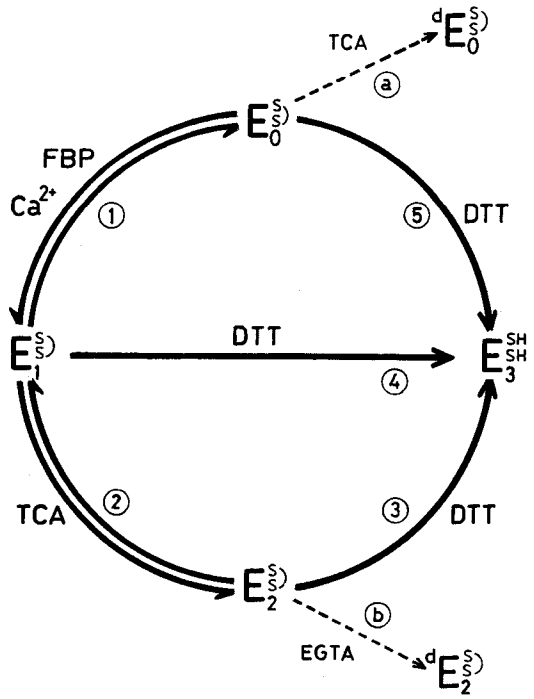
To prove that the requirement of the chaotropic anion during the process of reductive activation was not transient, a solution of chloroplast fructose-1,6-bisphosphatase containing 1.0 mM fructose 1,6-bisphosphate and 0.1 mM Ca^{2+} was successively incubated with (a) 150 mM trichloroacetate for 5 min; (b) 15.0 mM trichloroacetate for 5 min; and (c) 5.0 mM dithiothreitol for various times; finally, (d) the activity of fructose-1,6-bisphosphatase was assayed. On this basis, longer $t_{0.5}$ values were indicative of lower response of chloroplast fructose-1,6-bisphosphatase to the activation by dithiothreitol. As depicted in Scheme I, chloroplast fructose-1,6-bisphosphatase treated continuously with 15.0 mM trichloroacetate and the enzyme incubated alternatively with high and low concentrations, increased slowly the activity upon addition of dithiothreitol; conversely, the time elapsed for obtaining the highest specific activity was shortened by the permanent presence of 150 mM trichloroacetate.

DISCUSSION

The present study attempts to assess the contribution of conformational changes to the reductive activation of chloroplast fructose-1,6-bisphosphatase. Our hypothesis, therefore, is that low-molecular-mass compounds, which have an unique effect on proteins, would disclose unambiguously modifications of the tertiary structure and the reductive process. To test this idea, we rely upon an anion without redox activity (trichloroacetate) and a dithiol (dithiothreitol) as perturbant of noncovalent interactions and reductant of disulfide bonds, respectively. The major conclusion of the present analysis is that the rate of the reductive activation of chloroplast fructose-1,6-bisphosphatase is strongly accelerated by specific conformational changes of the native form.

From the foregoing kinetic and spectroscopic data, Scheme II comes to the fore. As expected from a protein denaturant, trichloroacetate inactivates native chloroplast fructose-1,6-bisphosphatase (E_o), and concurrently generates a different profile of the fourth-derivative spectrum (^dE_o; reaction a). Based largely on the observation that neither the basal activity nor the original spectrum are recovered upon removal of the chaotropic anion, we infer that chloroplast fructose-1,6-bisphosphatase is irreversibly denaturated. On the other hand, another fourth-derivative spectrum which does not resemble that of native enzyme, (E_1), parallels the stimulation of activity by fructose 1,6-bisphosphate and Ca^{2+} (reaction 1). If the alteration of the tertiary structure is the mechanism through which the modulation of fructose 1,6-bisphosphate and Ca^{2+} is achieved, then it follows that other events linked to the structure of chloroplast fructose-1,6-bisphosphatase would also be altered. In fact, the modification of two processes is in line with this expectation: fructose 1,6-bisphosphate and Ca^{2+}, on the one hand, partially prevent the quenching of intrinsic fluorescence by trichloroacetate whereas, on the other hand, they increase the emission of an extrinsic fluorophore. From this evidence, not unexpectedly the preincubation of chloroplast fructose-1,6-bisphosphatase with fructose 1,6-bisphosphate and Ca^{2+} also (a) prevents the inactivation triggered by alkaline pH [13], and (b) drastically changes the kinetic of denaturation by urea (M. A. Ballicora, PhD thesis).

These results are analogous to changes mediated by fructose 1,6-bisphosphate and Ca^{2+} in circular dichroic spectra of chloroplast fructose-1,6-bisphosphatase [17]. However,



Scheme 2. Mechanism of trichloroacetate action on chloroplast fructose-1,6-bisphosphatase. E, chloroplast fructose-1,6-bisphosphatase; FBP, fructose 1,6-bisphosphate; TCA, trichloroacetate; DTT, dithiothreitol.

the striking and counter-intuitive effect of these modulators is that the subsequent incorporation of trichloroacetate not only leads to a radically different spectrum (reaction 2) but also facilitates the reversible formation of a catalytically competent form (E_2). At this stage, the removal of Ca^{2+} yields both a catalytically inactive enzyme and a new spectrum ($^dE_2$) (reaction b). Given that neither ^dE_o nor $^dE_2$ recover the catalytic capacity, the important aspect of Scheme II is the requirement of a precise order in the addition of modulators for obtaining functional states. This important concept becomes evident in present studies because reversible steps (reactions 1 and 2) effectively prevail over irreversible processes (reactions a and b) in the competition for (activatable) enzyme forms.

Dithiothreitol does not require any modulator for the stimulation of chloroplast fructose-1,6-bisphosphatase activity; nevertheless, reaction 5 is extremely slow [22]. Although fructose 1,6-bisphosphate and Ca^{2+} accelerate the enzyme activation (reaction 4) [24], the remarkable feature is that trichloroacetate greatly shortens the time for the reductive process (reaction 3). Relevant to the fast activation by the concerted action of modulators, are our previous results in which dithiothreitol and either (a) fructose 1,6-bisphosphate, Ca^{2+}, and thioredoxin or (b) urea, guanidinium thiocyanate or sodium dodecylsulfate were found to be necessary for the *S*-carboxyamidomethylation of chloroplast fructose-1,6-bisphosphatase [25]. In consequence, disulfide bonds are not reduced to a significant extent unless the conformation of the oxidized enzyme is modified by modulators in (a) or by protein perturbants in (b). Perhaps one limitation of Scheme II, that could be overcome by structural studies, is whether E_3 represents one or various states of reduced chloroplast fructose-1,6-bisphosphatase.

A basic tenet in the modulation of chloroplast fructose-1,6-bisphosphatase by light is that the reduction of disulfide bonds by the ferredoxin–thioredoxin system elicits the catalytically competent form [1, 2]. Nevertheless, present results cannot be reconciled with the notion that thioredoxin mediates only the conversion of disulfides to sulfhydryl groups. If the conformational change of chloroplast fructose-1,6-bisphosphatase contributes to accelerate the reduction of disulfide bonds, we infer that chloroplast thioredoxin-f speeds up the reaction that converts the native enzyme (E_o) to the active form (E_3) because it modifies intramolecular noncovalent interactions of the enzyme. Consistent with this, we recently found that the activation of chloroplast fructose-1,6-bisphosphatase by bacterial thioredoxins is similar to chloroplast thioredoxin-f when nonphysiological modulators devoid of redox activity are present (S. Mora Garcia, unpublished results). Moreover, site-directed mutagenesis of amino acid residues located outside the redox center enables *Escherichia coli* thioredoxin to activate chloroplast fructose-1,6-bisphosphatase [5, 6]. It is well known that some thioredoxin-dependent events are not linked to the redox exchange reaction [26, 27]. In this context, our results suggest the complementation of conformational changes with the reductive process for the activation of fructose-1,6-bisphosphatase by the ferredoxin–thioredoxin system.

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