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Differential reactivity of chloroplast fructose-1,6-bisphosphatase to Woodward's reagent K and diethylpyrocarbonate

Gonzalo Prat-Gay and Ricardo A. Wolosiuk

Instituto de Investigaciones Bioquímicas, Facultad de Ciencias Exactas y Naturales (U.B.A.), Buenos Aires (Argentina)

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Native chloroplast fructose-1,6-bisphosphatase (D-fructose 1,6-bisphosphate-1-phosphohydrolase, EC 3.1.3.11) was inactivated with either Woodward's reagent K or diethylpyrocarbonate. Since pseudo-first-order rate constants (k_{app}) were a hyperbolic function of inhibitor concentration, it appeared that native chloroplast fructose-1,6-bisphosphatase and either Woodward's reagent K or diethylpyrocarbonate interacted reversibly prior to formation of irreversible (inactive) complex. Protection against inactivation was afforded by preincubating the enzyme with dithiothreitol, fructose 1,6-bisphosphate, Ca^{2+} and either chloroplast thioredoxin-*f* or a chaotropic anion (trichloroacetate). Following similar incubation with an organic solvent (2-propanol), the catalytic activity remained unaltered in the presence of Woodward's reagent K but was inactivated by diethylpyrocarbonate. The enhancement of the specific activity of chloroplast fructose-1,6-bisphosphatase caused by dithiothreitol, fructose-1,6-bisphosphate, Ca^{2+} and either chloroplast thioredoxin-*f*, or chaotropic anions or cosolvents was similar. Therefore, differential reactivity to selected reagents indicated the existence of several conformations of chloroplast fructose-1,6-bisphosphatase, i.e., native and different active forms.

Introduction

In higher plants the stimulation of photosynthetic electron transport system by light causes changes in the concentration of ions, metabolites and the redox state of proteins which modify the activity of chloroplast enzymes [1–5]. A distinctive feature of light-modulated enzymes is the availability of several states with different kinetic properties; moreover, the rate of conversion between two forms is lower than the rate of catalysis (enzyme hysteresis) [6,7]. On the basis that chloroplast components influence two different processes, i.e., the modification of the enzyme and catalysis, we define two kinetic constants: $A_{0.5}$ is the concentration of modulator that yields half of the maximum specific activity, and $S_{0.5}$ is the concentration of either substrate or effector which yields half of the maximum velocity [7].

The incubation of native (inactive) chloroplast fructose-1,6-bisphosphatase with a thiol, a sugar bisphosphate and a bivalet cation generates the active form. Chloroplast thioredoxin, organic solvents or chaotropic anions contribute to the stimulation of the specific activity by lowering the $A_{0.5}$ for sugar bisphosphates, conversely, the catalytic step is not modified by chloroplast thioredoxin and is inhibited by cosolvents and chaotropic anions [8–11]. Since the quaternary structure of chloroplast fructose-1,6-bisphosphatase is not altered in the transition from the native to the active form, the reactivity of amino-acid side-chains to group-specific reagents is a sensitive technique for detecting modifications in the conformation [12–14]. In this context, if modulators determine the subsequent reaction of the enzyme with group-specific reagents, it appears that active forms of chloroplast fructose-1,6-bisphosphatase have different conformations. Therefore, in studies reported herein, we investigated the sensitivity of both native and modulator-activated chloroplast fructose-1,6-bisphosphatse to carboxyl-specific (WRK) and histidine-specific (DEPC) reagents [15–17]. Present results show that WRK and DEPC inactivate native

Abbreviations: WRK, Woodward's reagent K, *N*-ethyl-5-phenylisoxazolium-3-sulphonate; DEPC, diethylpyrocarbonate; Mops, 4-morpholinepropanesulphonic acid; Hepes 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid.

Correspondence: R.A. Wolosiuk, Fundación Campomar, Antonio Machado 151, (1405) Buenos Aires, Argentina.

chloroplast fructose-1,6-bisphosphatase. In contrast, active chloroplast fructose-1,6-bisphosphatase is slightly sensitive to WRK and DEPC, except when the enzyme is activated by organic solvents prior to treatment with DEPC. Thus, although similar specific activities of chloroplast fructose-1,6-bisphosphatase appear by the action of chloroplast thioredoxin-*f*, cosolvents, or chaotropic anions, differential reactivity to specific chemical probes indicates that modulator-generated forms have different conformations which in turn are dissimilar to the native enzyme.

Experimental procedures

Materials

WRK and DEPC, as other biochemicals, were obtained from Sigma Chemical Co. (St. Louis, MO). All other reagents were purchased from commercial sources and were analytical reagent grade. Stock solutions of WRK and DEPC were prepared freshly every day at 4° C in 10 mM HCl and absolute ethanol, respectively; the concentration of DEPC solutions was estimated spectrophotometrically with standard solutions of imidazole [17]. Fructose 1,6-bisphosphate was purified by treating concentrated solutions with Chelex-100 resin (Bio-Rad, Richmond, CA) [10]. Water was deionized and distilled prior to use in the preparation of solutions.

Methods

Preparation of chloroplast fructose-1,6-bisphosphatase and thioredoxin-f. Chloroplast fructose-1,6-bisphosphatase and thioredoxin-*f* were purified from frozen spinach leaves according to Refs. 11 and 18; all enzyme preparations used throughout this work showed a single band on sodium dodecyl sulphate polyacrylamide gel electrophoresis [19].

Treatment of chloroplast fructose-1,6-bisphosphatase with group-specific reagents. Active forms of chloroplast fructose-1,6-bisphosphatase were generated at pH 7.9 by incubation with dithiothreitol, fructose 1,6-bisphosphate, Ca^{2+} and either thioredoxin-*f*, 2-propanol, or sodium trichloroacetate; the process of activation did not occur at pH values lower than 7.4 [8–11,20]. On the other hand, the inactive form of chloroplast fructose-1,6-bisphosphatase corresponded to the native (untreated) enzyme. In the present study, chloroplast fructose-1,6-bisphosphatase was treated with WRK or DEPC either prior (native form) or subsequent (active form) to incubation with modulators.

Native chloroplast fructose-1,6-bisphosphatase (10 μg) was incubated at 23° C in 0.25 ml of 50 mM Mops-NaOH buffer (pH 7.8) containing the respective group-specific reagent. Following incubations at varying times, aliquots were withdrawn and the remaining fructose-1,6-bisphosphatase activity was assayed by one of the two following procedures. (a) The enzyme treated with group-specific reagent was injected into a solution containing (in μmol): Tris-HCl buffer (pH 7.9), 50; $MgCl_2$, 5; fructose 1,6-bisphosphate, 1.0; and EGTA, 0.05 (final volume 0.5 ml). After 5 min at 23° C, catalysis was halted by adding the reagent for the estimation of P_i released [21]. (b) Alternatively, modified chloroplast fructose-1,6-bisphosphatase was further incubated in a solution containing (in μmol): Tris-HCl buffer, (pH 7.9), 5; dithiothreitol, 0.5; fructose 1,6-bisphosphate, 0.08; $CaCl_2$ 0.015; and trichloroacetate, 15 (final volume 0.1 ml). 6.0 μmol of imidazole-HCl (pH 8.0) were introduced to this solution when DEPC was the group-specific reagent under analysis. After 30 min at 23° C. aliquots were withdrawn and injected into the assay solution that contained in a final volume of 1.0 ml (in μmol): Tris-HCl buffer (pH 7.9), 50; $MgCl_2$, 1.0; fructose 1,6-bisphosphate, 0.8; and EGTA, 0.2. Following 2 min incubation at 23° C, fructose 1,6-bisphosphate hydrolysis was measured by assaying P_i released.

On the other hand, the effect of WRK and DEPC on active forms of chloroplast fructose-1,6-bisphosphatase was examined by the following procedure. Chloroplast fructose-1,6-bisphosphatase (10 mg) was incubated for 30 min at 23° C in 0.22 ml of a solution containing (in μmol): Mops-NaOH buffer (pH 7.8), 12.5; dithiothreitol, 1.25; fructose 1,6-bisphosphate, 0.2, $CaCl_2$, 0.04; and the modulator under analysis. Subsequently, 0.03 ml of the respective group-specific reagent was added. Following incubation at varying times, the enzyme solution (0.025 ml) was withdrawn and injected into 0.075 ml of a solution containing the same modulator used in the initial incubation and the following (in μmol): Tris-HCl buffer (pH 7.9), 5.0; dithiothreitol, 0.5; fructose 1,6-bisphosphate, 0.08 and $CaCl_2$, 0.015. After 20 min at 23° C, an aliquot was withdrawn and injected into a solution containing (in μmol): Tris-HCl buffer (pH 7.9), 50; $MgCl_2$, 1.0; fructose 1,6-bisphosphate, 0.8; and EGTA, 0.2 (final volume 1.0 ml). Following 2 min incubation at 23° C, the P_i released was estimated according to Chen et al. [21].

Data analysis. Linear regression was the method chosen for finding a straight line that fitted experimental data points the best. In this respect, we analyzed only kinetic data whose correlation coefficients were higher than 0.98.

Ultraviolet differential spectrophotometry. Spectra of chloroplast fructose-1,6-bisphosphatase were obtained by using the tandem cell holder of a Hitachi 220-A double beam spectrophotometer. In the sample position, a rectangular quartz cell (pathlength: 1.0 cm), containing either modulator-treated or native enzyme, was placed in the light path with a similar cuvette filled with buffer solution; in the reference position, the cell containing the enzyme was aligned in the light path with another cell containing modulators. Following ad-

justment of the baseline with the built-in microprocessor supplied by the manufacturer, appropriate amounts of group-specific reagents were injected and successive spectra were recorded at discrete intervals.

Results

Effect of WRK on chloroplast fructose-1,6-bisphosphatase

Incubation of native chloroplast fructose-1,6-bisphosphatase with WRK diminished its capacity to hydrolyze fructose 1,6-bisphosphate (Fig. 1). The decay of enzyme activity followed pseudo-first-order kinetics in the first 100 s after which inactivation was halted because of the extremely rapid reagent hydrolysis [15]. The apparent rate constant of inactivation (k_{app}) – estimated from slopes of straight lines in Fig. 1A – showed a hyperbolic dependence on WRK concentration (Fig. 1B). This result is characteristic of a reversible binding process between the inhibitor and the enzyme prior to irreversible inactivation [22,23]:

$$(FBP_{ase}) + WRK \overset{K_i}{\rightleftarrows} (FBP_{ase}) \cdot WRK \overset{k_3}{\rightarrow} (FBP_{ase}) - WRK$$

where FBP_{ase} is the native form of chloroplast fructose-1,6-bisphosphatase, K_i is the dissociation constant and k_3 is the rate constant of irreversible inactivation. From the slope and the intercept of the plot in Fig. 1B, K_i and k_3 were 30 mM and $1.6 \cdot 10^{-2}$ s^{-1}, respectively [22–24]. Although not shown, similar results were obtained when remaining chloroplast fructose-1,6-bisphosphatase activity was assayed by the method (b) described under Experimental procedures.

As illustrated in Fig. 2, protection against inactivation of chloroplast fructose-1,6-bisphosphatase by WRK

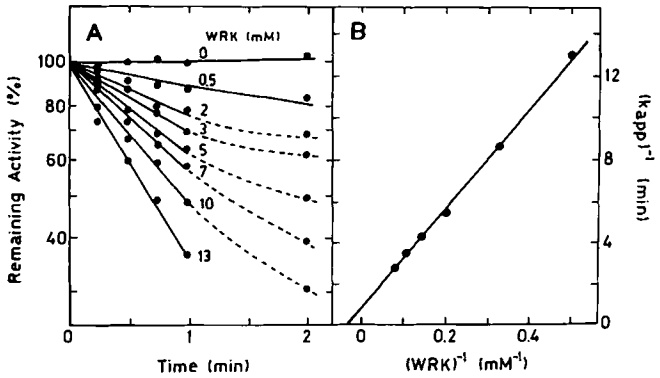


Fig. 1. Activation of native chloroplast fructose-1,6-bisphosphatase by WRK. Chloroplast fructose-1,6-bisphosphatase (10 μg) was incubated at 23°c in 0.25 ml of a solution of 0.1 M Mops-NaOH buffer (pH 7.8) containing WRK. At indicated times an aliquot (0.025 ml) was withdrawn and mixed with 0.475 ml of a solution that contained (in μmol); Tris-HCl buffer (pH 7.9), 50; $MgCl_2$, 5; fructose-1,6-bisphosphate, 1.0; and EGTA, 0.05. After 5 min at 23°C, fructose 1,6-bisphosphate hydrolysis was assayed as outlined in Methods. Control activity: 72 μmol P_i released/min per mg protein.

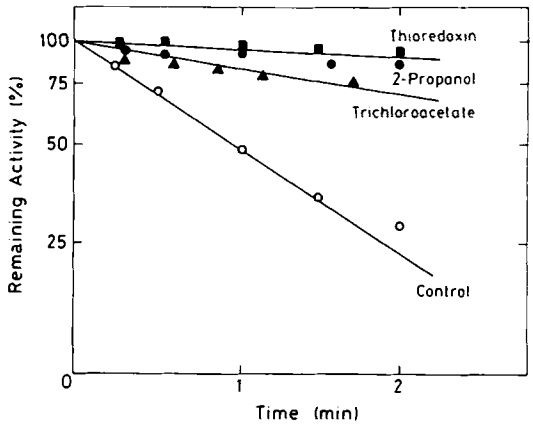


Fig. 2. Effect of the activation of chloroplast fructose-1,6-bisphosphatase with dithiothreitol, fructose 1,6-bisphosphate, Ca^{2+} and either thioredoxin-*f*, 2-propanol, or trichloroacetate on the reactivity to WRK. The enzyme (10 μg) was incubated at 23°C for 30 min in 0.22 ml of a solution containing (in μmol): Mops-NaOH buffer (pH 7.8), 12.5; dithiothreitol, 2.0; fructose 1,6-bisphosphate, 0.16; $CaCl_2$, 0.03; and, as indicated, either thioredoxin-*f*, 15 μg; 2-propanol, 493 μmol (15%, v/v); or sodium trichloroacetate, 30 μmol. Control enzyme was incubated in 0.22 ml of 0.1 M Mops-NaOH buffer (pH 7.8). WRK (0.6 μmol in 0.03 ml) was injected and the incubation was continued as indicated. 25 μl were withdrawn and mixed with 0.075 ml of the activating solution containing (in μmol): Tris-HCl buffer (pH 7.9), 5; dithiothreitol, 0.5; fructose 1,6-bisphosphate, 0.08; $CaCl_2$, 0.015; and, as indicated, either chloroplast thioredoxn-*f*, 6 μg; 2-propanol, 198 μmol (15%, v/v); sodium trichloroacetate, 15 μmol; the control enzyme was activated with the solution containing sodium trichloroacetate. After 20 min at 23°C, the enzyme activity was determined in an aliquot of the mixture as described under Methods. Control activity: 120 μmol P_i released/min per mg protein.

was afforded by prior treatment with dithiothreitol (2.5 mM), fructose 1,6-bisphosphate (0.8 mM), Ca^{2+} (0.15 mM) and either thioredoxin-*f*, 2-propanol, or trichloroacetate. After 2 min incubation with 2.4 mM WRK, the specific activity of active forms decayed 20%, whereas the native form was inactivated 80%. Thus, catalytically competent forms, generated with thioredoxin-*f*, cosolvents or chaotropic anions, showed similar response to WRK.

Modification of the carboxyl groups in proteins by covalently bound WRK was followed spectrophotometrically by the increase in absorbance at both 278 nm and 343 nm [25]. As shown in Fig. 3, the spectrum of native chloroplast fructose-1,6-bisphosphatase treated with WRK revealed the presence of positive peaks at 278 and 343 nm whose ratio of absorbances ($A_{278\,nm}$: $A_{343\,nm}$) was 2.1. On the other hand, when active chloroplast fructose-1,6-bisphosphatase was treated with WRK, the ratio of absorbances was higher than 4. As mentioned previously, the A_{278}: A_{343} ratio can not be used for quantitative determinations [25]. However, on the basis of studies with other proteins, large variations in these values indicated that the extent of WRK-mediated modification of native chloroplast

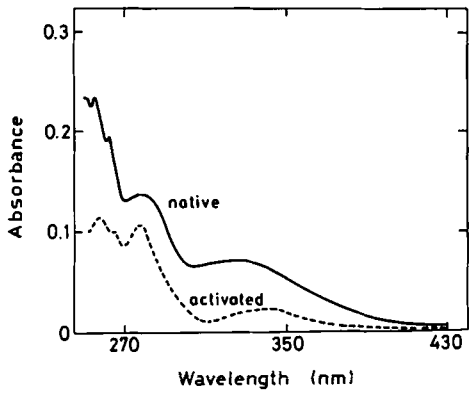


Fig. 3. Effect of WRK on the spectrum of chloroplast fructose-1,6-bisphosphatase. Chloroplast fructose-1,6-bisphosphatase (150 μg) was incubated at 23°C for 30 min in 0.2 ml of one of the following solutions, which contained (in μmol); Native enzyme: Hepes-NaOH buffer (pH 8.1), 25; or activated enzyme: Hepes-NaOH buffer (pH 8.1), 25; dithiothreitol, 1, fructose 1,6-bisphosphate, 0.16; $CaCl_2$, 0.03; and, sodium trichloroacetate, 30. Next, 1 μmol of WRK in 0.3 ml was added to each mixture and the incubation was extended for 10 min. Both solutions were subjected to gel filtration in columns of Bio-Gel P-4 (1×40 cm), and subsequently spectra of chloroplast fructose-1,6-bisphosphatase were recorded in a Hitachi 220-A double-beam spectrophotometer.

fructose-1,6-bisphosphatase was higher than in active forms [25].

Previous studies indicated that nucleophilic compounds displace WRK from proteins, whereby spectral differences originating from enol-ester intermediate are cancelled [15,25,26]. Therefore, in order to confirm the presence of activated carboxyl groups in chloroplast fructose-1,6-bisphosphatase, the native enzyme was first incubated with 6.25 mM WRK until the reaction was completed, and subsequently treated with 0.4 M glycine ethyl ester to displace bound WRK. As shown in Fig. 4, WRK-mediated increase in absorbance decayed slowly, following the addition of the nucleophile; after 3 h the absorbance of 343 nm was half of the zero-time value. Thus, as predicted for nucleophilic removal of covalently bound WRK, absorbances in the 340 nm region slowly approached those at baseline [27]. However, kinetic studies revealed that glycine ethyl-ester-dependent displacement of WRK did not restore the capacity of the enzyme for hydrolyzing fructose 1,6-bisphosphate (not shown).

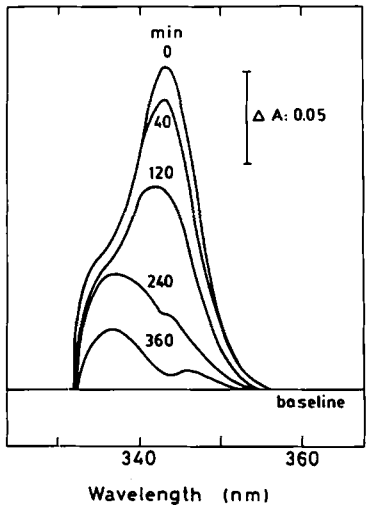


Fig. 4. Difference absorption spectra of WRK-inactivated chloroplast fructose-1,6-bisphosphatase after the addition of glycine ethyl ester. Four rectangular quartz cells (pathlength: 1.0 cm) were placed in the tandem cell holder of a Hitachi 220-A double-beam spectrophotometer containing 0.8 ml each of the following solutions: Sample position: one cuvette contained 100 μg of chloroplast fructose-1,6-bisphosphatase and the following (in μmol): Mops-NaOH buffer (pH 7.8), 100, and WRK, 5; the other cell was filled with 0.1 M Mops-NaOH buffer (pH 7.8). Reference position: 100 μg of chloroplast fructose-1,6-bisphosphatase and 5 μmol of WRK, dissolved in 0.1 M Mops-NaOH buffer (pH 7.8), were present in separated cells. Spectra were scanned every 5 min until the absorbance values were constant. Next, 0.2 ml of 2 M glycine ethyl ester were added to each cell and spectra were recorded at the times indicated.

Effect of DEPC on chloroplast fructose-1,6-bisphosphatase

To determine whether chloroplast fructose-1,6-bisphosphatase was sensitive to histidine-specific reagents, the native form of the enzyme was first incubated in Mops-NaOH buffer (pH 7.8) with DEPC, subsequently transferred to a solution of Tris-HCl buffer (pH 7.9) containing 5.0 mM dithiothreitol, 0.8 mM fructose 1,6-bisphosphate, 0.15 mM Ca^{2+}, and 0.15 M trichloroacetate; and finally, the activity at low concentrations of both fructose 1,6-bisphosphate (0.8 mM) and Mg^{2+} (1.0 mM) was assayed. As is shown in Fig. 5A the rapid loss of enzyme activity mediated by DEPC, at pH 7.8 and 23°C, was both time- and concentration-dependent. Although in the first 2 min of incubation the reaction of

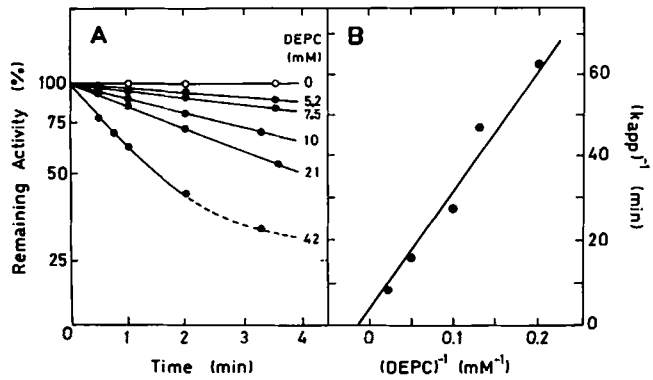


Fig. 5. Inactivation of native chloroplast fructose-1,6-bisphosphatase by DEPC. The enzyme (10 μg) was incubated at 23°C in 0.1 M Mops-NaOH buffer (pH 7.8) containing DEPC (final volume 0.25 ml). After the incubation, as indicated, 0.025 ml of the mixture were injected into 0.075 ml of a solution that contained (in μmol): Tris-HCl buffer (pH 7.9), 5; dithiothreitol, 0.5; fructose 1,6-bisphosphate, 0.08; $CaCl_2$, 0.015; trichloroacetate, 15; and, imidazole-HCl (pH 8.0), 2. Following 20 min incubation at 23°C, 0.9 ml of the solution for the assay of enzyme activity was added and fructose 1,6-bisphosphate hydrolysis was tested as described under Methods. Control activity: 60 μmol P_i released/min per mg protein.

native chloroplast fructose-1,6-bisphosphatase with DEPC followed pseudo-first-order kinetics, longer times caused an apparent deviation from linearity attributed to the instability of DEPC at pH 7.8. In contrast, specific activity remained unaltered for at least 20 min when DEPC was omitted. The plot of the reciprocal of k_{app} – obtained from Fig. 5A as indicated above – versus the reciprocal of DEPC concentrations was linear and it yielded a finite intercept on the y-axis (Fig. 5B). On the basis of previous analysis, it appeared that a molecule of chloroplast fructose-1,6-bisphosphatase interacted reversibly with one molecule of DEPC producing a complex whose K_i was 50 mM^{-1}; the rate constant (k_3) for the subsequent formation of the inactive complex was $4.2 \cdot 10^{-3}$ s^{-1} [22–24].

If inactivation of chloroplast fructose-1,6-bisphosphatase arose from reaction of DEPC with groups essential for the conversion to active forms, then it should be expected that prior incubations with modulators retard the decay of activity. Therefore, chloroplast fructose-1,6-bisphosphatase was (i) activated in a soluution of Mops-NaOH buffer at pH 7.8, with dithiothreitol, fructose 1,6-bisphosphate, Ca^{2+}, and either thioredoxin-*f*, trichloroacetate, or 2-propanol, (ii) treated with DEPC, (iii) incubated again in a solution of Tris-HCl buffer (pH 7.9) containing dithiothreitol, fructose 1,6-bisphosphate, Ca^{2+} and the modulator used in step (i), and, finally, (iv) assayed at low concentrations of both fructose 1,6-bisphosphate and Mg^{2+}. Fig. 6 depicts that following incubation with either thioredoxin-*f* or trichloroacetate, the activity of chloroplast fructose-1,6-bisphosphatase decayed 50% by the action of DEPC in 6 min; similar inactivation for chloroplast fructose-1,6-bisphosphatase activated with 2-propanol or the native form was achieved in 2 min. This difference in DEPC-mediated inactivation suggested that active forms of chloroplast fructose-1,6-bisphosphatase had different conformations. On the other hand, although chloroplast fructose-1,6-bisphosphatase was also inactivated by DEPC at pH 7.0, neither thioredoxin-*f* nor trichloroacetate retarded the inactivation (not shown). Since at pH 7.0, modulators did not stimulate chloroplast fructose-1,6-bisphosphatase activity, it appeared that only thioredoxin- and chaotrope-mediated changes which gave rise to active forms of the enzyme prevented the inactivation by DEPC.

N-Carbethoxylation of histidine residues in proteins increased the absorbance in the wavelength range of 240 to 245 nm whereas *O*-carbethoxylation of tyrosine groups lowered the absorbance in the region of 280–290 nm [17]. Moreover, as control for our studies we established that *N*-carbethoxylation of free histidine was not impaired by thioredoxin-*f*, 2-propanol or trichloroacetate (not shown). Ultraviolet-difference spectra of native chloroplast fructose-1,6-bisphosphatase treated with DEPC at pH 7.8 showed that absorbances were

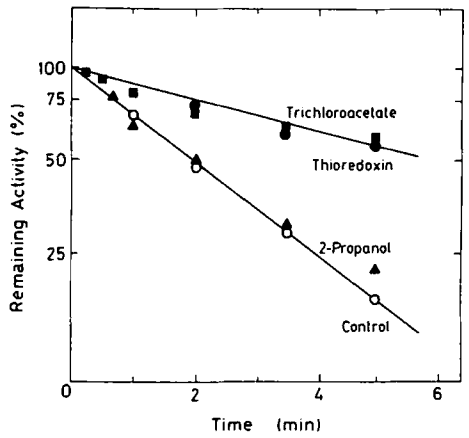


Fig. 6. Effect of the activation of chloroplast fructose-1,6-bisphosphatase with dithiothreitol, fructose 1,6-bisphosphate, Ca^{2+} and either thioredoxin-*f* 2-propanol or trichloroacetate on the reactivity to DEPC. Chloroplast fructose-1,6-bisphosphatase (10 μg) was incubated at 23° C for 30 min in 0.22 ml of a solution containing (in μmol): Mops-NaOH buffer (pH 7.8), 12.5; dithiothreitol, 2.0; fructose 1,6-bisphosphate, 0.16; $CaCl_2$, 0.03; and, as indicated, either chloroplast thioredoxin-*f*, 15 μg; 2-propanol, 490 μmol (15%, v/v); or sodium trichloroacetate, 30 μmol. Control enzyme was incubated in 0.22 ml of 0.1 M Mops-NaOH buffer (pH 7.8). DEPC (2.0 μmol in 0.03 ml) was added and the incubation was extended, as indicated. An aliquot (0.025 ml), was withdrawn and injected into 0.075 ml of the activating solution containing (in μmol): Tris-HCl buffer (pH 7.9), 5; dithiothreitol, 0.5; fructose 1,6-bisphosphate, 0.08; $CaCl_2$, 0.015; and, as indicated, either chloroplast thioredoxin-*f*, 6.0 μg; 2-propanol, 198 μmol (15%, v/v); or, sodium trichloroacetate, 15 μmol; the specific activity of the control enzyme was stimulated in the presence of sodium trichloroacetate. Following 20 min incubation at 23° C, the catalytic capacity was assayed in an aliquot of the mixture as described under Methods. Control activity: 96 μmol P_i released/min per mg protein.

positive from 240 to 245 nm, whereas at 282 and 287 nm they were negative (Fig. 7). On the basis of known molar extinction coefficients for carbethoxyl derivatives of histidine ($\varepsilon_{242\,nm} = 3200\ M^{-1} \cdot cm^{-1}$) and tyrosine ($\varepsilon_{275\,nm} = 1310\ M^{-1} \cdot cm^{-1}$), our results suggested that 7 mol of histidine residues and 16 mol of tyrosine residues per mol of native chloroplast fructose-1,6-bisphosphatase were modified by DEPC [17]. In parallel experiments carried out at pH 7.0 we observed that ultraviolet-difference spectra of native chloroplast fructose-1,6-bisphosphatase displayed only positive absorbance at 242 nm (not shown). Thus, our spectrophotometric analysis of DEPC-treated enzyme supported the view that at pH 7.8 chloroplast fructose-1,6-bisphosphatase has a different conformation with respect to pH 7.0. On the other hand, in trichloroacetate-activated chloroplast fructose-1,6-bisphosphatase, 11 and 18 mol of histidine and tyrosine groups, respectively, reacted with DEPC. Although these values could be higher because of the negative contributions of *O*-carbethoxylated tyrosine on the spectra of *N*-carbethoxylated histidine, and vice-versa, they clearly established differences in the reactiv-

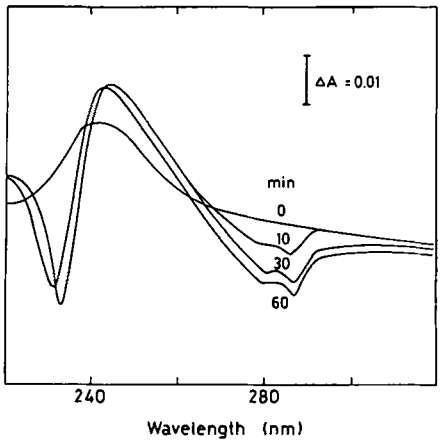


Fig. 7. Ultraviolet-difference spectra of chloroplast fructose-1,6-bisphosphatase after treatment with DEPC. In the tandem cell holder of a Hitachi 220-A double-beam spectrophotometer were placed four rectangular quartz-cells (pathlength: 1.0 cm) containing 1.0 ml each of the following solutions: Sample position: one cell contained 100 μg of chloroplast fructose-1,6-bisphosphatase and the following (in μmol): Mops-NaOH buffer (pH 7.8), 100; and, DEPC, 7; the other cuvette was filled with 0.1 M Mops-NaOH buffer (pH 7.8). Reference position: in 0.1 M Mops-NaOH were dissolved 100 μg of chloroplast fructose-1,6-bisphosphatase in one cuvette and 7 μmol of DEPC in the accompanying cuvette. Successive spectra were recorded at the intervals indicated.

ity of these amino-acid residues in native and active chloroplast fructose-1,6-bisphosphatase [17].

To correlate structural modifications with loss of enzyme activity, the reaction of DEPC with both histidine and tyrosine residues of chloroplast fructose-1,6-bisphosphatase was analyzed by following the absorbance changes at 242 and 280 nm, respectively; simultaneously, aliquots were withdrawn and assayed for remaining fructose-1,6-bisphosphatase activity. As

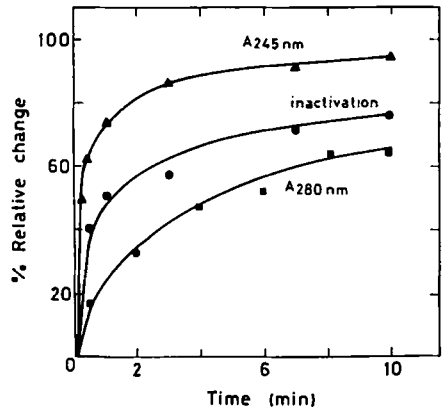


Fig. 8. Time-course of the DEPC effect on native chloroplast fructose-1,6-bisphosphatase. Spectrophotometric analysis: two cuvettes containing chloroplast fructose-1,6-bisphosphatase (100 μg) in 0.1 M Mops-NaOH buffer (pH 7.8) were placed in the reference and sample position of a Hitachi 220-A double-beam spectrophotometer. After 2 min incubation at 23° C, DEPC (11 mM) was added to the cuvette in the sample position and absorbances at 245 and 280 nm were recorded at definite intervals. Assay of activity was carried out as described in Fig. 5.

shown in Fig. 8, in the presence of 11 mM DEPC, *N*-carbethoxylation of histidine ($t_{0.5} = 0.4$ min) was faster than enzyme inactivation ($t_{0.5} = 1.0$ min), whereas *O*-carbethoxylation of tyrosine constituted the slowest process ($t_{0.5} = 5.0$ min).

Studies with other proteins showed that NH_2OH removes the carbethoxyl group from modified histidine and tyrosine residues [17]. Irrespective of pH, spectra of DEPC-treated chloroplast fructose-1,6-bisphosphatase approached the baseline following incubation with 0.5 M NH_2OH. However, treatment with NH_2OH did not restore the catalytic capacity of chloroplast fructose-1,6-bisphosphatase at pH 7.8 whereas it was partially recovered at pH 7.0. The absence of complete reactivation was attributed to side-effects of NH_2OH because, after incubation with 0.5 M NH_2OH in the absence of DEPC, chloroplast fructose-1,6-bisphosphatase slowly lost the capacity to hydrolyze fructose 1,6-bisphosphate.

Discussion

The specific activity of chloroplast fructose-1,6-bisphosphatase was enhanced by dithiothreitol, fructose 1,6-bisphosphate, Ca^{2+} and either chloroplast thioredoxin-*f*, cosolvents or chaotropic anions [8–11]. Thus, protein perturbants – not involved in redox reactions – exerted on chloroplast fructose-1,6-bisphosphatase a stimulatory action similar to a protein whose primary function was attributed to -S-S-/-SH cycling [29]. Therefore, two questions were of particular relevance: (i) did active chloroplast fructose-1,6-bisphosphatase have a conformation that differed significantly from the native form? and (ii) were active forms of the enzyme conformationally similar? In order to approach the first question we tested the effect of group-specific reagents before and after chloroplast fructose-1,6-bisphosphatase was converted to a catalytically competent form. With regard to the second question we compared the action of group-specific reagents on active forms generated by incubations with both physiological (thioredoxin-*f*) and nonphysiological (cosolvents, chaotropic anions) modulators.

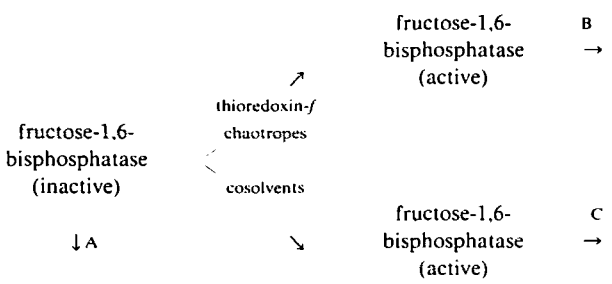
Following reaction with WRK and DEPC, native chloroplast fructose-1,6-bisphosphatase was inactivated. Neither incubating the enzyme with modulators nor performing the assay in the presence of high concentrations of both fructose 1,6-bisphosphate and Mg^{2+} restored the catalytic capacity. Subsequent spectrophotometric studies on the native enzyme indicated that WRK-mediated inactivation arose from chemical modifications of carboxyl residues. On the other hand, although spectral variations gave indications that both histidine and tyrosine residues reacted with DEPC, kinetic studies showed that *N*-carbethoxylation of histidine preceded the inactivation process, whereas *O*-carbethoxylation of tyrosine was a subsequent reaction.

Consequently, both carboxyl and histidyl groups were essential for the stimulation of the specific activity and the catalytic process of chloroplast fructose-1,6-bisphosphatase. Requirement of histidine residues for the activity of chloroplast fructose-1,6-bisphosphatase was also inferred from amino-acid sequence data which showed that two histidines residues were conserved in several known fructose-1,6-bisphosphatases [30].

Inactivation by WRK and DEPC was reduced by prior incubation of chloroplast fructose-1,6-bisphosphatase with either chloroplast thioredoxin-*f* or trichloroacetate. Since neither thioredoxin-*f* nor chaotropic anions hampered the capacity of WRK and DEPC to react with low molecular weight carboxyl- and histidine-bearing compounds, respectively, it appeared that different exposure of these groups in active chloroplast fructose-1,6-bisphosphatase accounted for slow activity loss. On the other hand, chloroplast fructose-1,6-bisphosphatase preincubated with 2-propanol was not inactivated by WRK, whereas DEPC abolished the catalytic capacity. Thus, although the response of active chloroplast fructose-1,6-bisophosphatase to WRK was independent of the modulator, the reactivity ot DEPC was largely dependent on the process of activation. These results indicated that in the active chloroplast fructose-1,6-bisphosphatase modulators protected carboxyl groups necessary for catalysis, whereas they conditioned the exposure of essential histidine residues. In contrast, when the activation process did not take place, i.e., at pH 7.0, modulators did not prevent the inactivation by either WRK or DEPC. On this basis, prior conversion to active forms was required to preserve the enzyme against inactivation by these group-specific reagents.

In relation to this analysis, we found that the reaction of active chloroplast fructose-1,6-bisphosphatase with iodoacetamide was independent of modulators (Stein, M., unpublished data), whereas they conditioned the response to dansyl chloride (Labate, H., unpublished data). In summary, although (i) similar specific activities were obtained in the presence of either chloroplast thioredoxin-*f* or nonphysiological perturbants, and (ii) the reactivity of active chloroplast fructose-1,6-bisphosphatase to WRK and iodoacetamide was not dependent on the modulator used, contrasting effects obtained with DEPC and dansyl chloride indicated that different conformations of active enzyme arose from the action of modulators. On the basis of these results, the following scheme was designed to show the effect of group-specific reagents on both native and active chloroplast fructose-1,6-bisphosphatase (Scheme I).

Like fructose-1,6-bisphosphatase, the specific activity of other chloroplast enzymes (NADP-glyceraldehyde-3-*P* dehydrogenase, NADP-malate dehydrogenase, phosphoribulokinase) was stimulated by either thioredoxin, cosolvents, or chaotropic anions [31–34]. Therefore, additional studies are needed to establish whether flexibility in the structure of chloroplast fructose-1,6-bisphosphatase – with retention of catalytic activity – is a characteristic of other light-activated enzymes of the reductive pentose cycle.



Compound	Effect of group-specific reagents on		
	A	B	C
WRK	inactivation	partial inactivation	partial inactivation
DEPC	inactivation	partial inactivation	inactivation

Scheme I. Interactions of chloroplast fructose-1,6-bisphosphatase with group-specific reagents.

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