Inactive forms of wheat ribulose biphosphate carboxylase

Conversion from the slowly activating into the rapidly activating form

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Wheat ribulose biphosphate carboxylase can be converted from the slowly activating into the rapidly activating form by heat or effectors in the absence of CO_2 and Mg^{2+} . This conversion process had the same energy of activation of 95.6 kJ/mol both in the presence and in the absence of effectors, whereas the free-energy change value ranged from +2.5 kJ/mol to –3.4 kJ/mol depending on the end product involved. Far-u.v. c.d. spectra measured before and after conversion indicated that ribulose biphosphate carboxylase is an α/β -class protein and that no significant changes in gross conformation occur. Signals in the near-u.v. region suggested that the main change during conversion is re-orientation of aromatic side chains, probably near the active site; a possible site for effector binding is discussed.

Ribulose biphosphate carboxylase cannot achieve full catalytic activity until it has bound CO_2 and Mg^{2+} in an ordered reversible process normally referred to as activation (Lorimer *et al.*, 1976). This characteristic is common to enzyme from all sources so far studied, although the kinetics vary (Laing & Christeller, 1976; Christeller & Laing, 1978; Mächler *et al.*, 1980). Several compounds are positive effectors of activation (Ryan & Tolbert, 1975; Chollet & Anderson, 1976; Vater & Salnikow, 1979) and can promote the process in normally suboptimal concentrations of CO_2 or Mg^{2+} (Gutteridge *et al.*, 1982; Parry *et al.*, 1983). Most of these effectors bind at the ribulose biphosphate-binding site (Badger & Lorimer, 1981; McCurry *et al.*, 1981; Jordan *et al.*, 1983) and not at alternative sites (Buchanan & Schürmann, 1973; Chu & Bassham, 1975).

The enzyme from wheat has two inactive forms requiring different activation conditions (Gutteridge *et al.*, 1982). The rapidly activating form (E_r) can be obtained by diluting fully activated enzyme (E_{act}) into CO_2 -free buffer, so causing the activating CO_2 to dissociate (Gutteridge *et al.*, 1982); re-activation is then achieved in less than 3 min at room temperature on re-introduction of CO_2 . E_s , the slowly activating form, can be derived from E_r by incubation at 4°C in the absence of CO_2 for extended periods (Gutteridge *et al.*, 1982); full re-activation of E_s at room temperature requires more than 3 h. It is also possible to convert E_s into

E_r in the absence of CO_2 and Mg^{2+} by heating (Gutteridge *et al.*, 1982) or by incubation with effectors of activation (Parry *et al.*, 1984). The changes induced in the protein as a result of this conversion from E_s into E_r are the subject of the present paper.

Experimental

Materials

Ribulose biphosphate carboxylase from wheat was available as a freeze-dried powder (Parry *et al.*, 1983).

Bicine [*NN*-bis-(2-hydroxyethyl)glycine], the tetrasodium salt of ribulose 1,5-biphosphate, NADPH and the sodium salt of D-3-phosphoglyceric acid were supplied by Sigma Chemical Co., Poole, Dorset, U.K. Na_2HPO_4 , NaNO_3 , Na_2SO_4 , $\text{Na}_4\text{P}_2\text{O}_7$ and Tris base were from BDH Chemicals, Poole, Dorset, U.K., and NaHCO_3 and MgCl_2 were from Fisons, Loughborough, Leics., U.K. Sephadex G-25 was from Pharmacia Fine Chemicals, Uppsala, Sweden.

Methods

Conversion of E_s into E_r was studied in CO_2 -free 100 mM-Bicine/NaOH buffer, pH 8.2, at various temperatures with or without effectors (see Figure legends). E_r produced in this way was determined by adding samples to buffered 10 mM-

$\text{NaHCO}_3/20\text{mM-MgCl}_2$ at 25°C and, after 3 min, assaying for carboxylase activity (Gutteridge *et al.*, 1982). Preliminary studies (Gutteridge *et al.*, 1982) showed that E_r reached full activity in 3 min. A relative activity of 1.0 is obtained when E_s is incubated with buffered $10\text{mM-NaHCO}_3/20\text{mM-MgCl}_2$ at 40°C for 40 min before assay. Control experiments showed that activation in the presence of effectors after conversion in their absence yielded E_{act} with an activity 1.4 times that obtained when both activation and conversion were effector-free. This made it necessary for the relative equilibrium concentration of E_r derived from Fig. 1 to be adjusted downwards by a factor of 0.4 before calculation of the $\Delta G'$ values.

C.d. measurements were made with a Jasco J40 CS scanning spectropolarimeter. Bicine, present with the enzyme for freeze-drying, was replaced with 10mM-Tris/HCl buffer pH 8.0, by gel filtration of the enzyme solution through Sephadex G-25 previously equilibrated with this latter buffer. For thermal conversion the cuvette holder of the spectropolarimeter was equilibrated at 35°C and the enzyme was incubated *in situ*; effector-mediated conversion was carried out at room temperature.

In the absence of suitable information on the amino acid sequence of the wheat enzyme large subunit it is assumed, where this is discussed, that it is identical with that of the spinach enzyme. The

numbering of residues is based on the information of Zurawski *et al.* (1981).

Results

Rate of conversion in the absence and in the presence of effectors

In the absence of effectors (Fig. 1a) the rate and extent of conversion is markedly increased as temperature increases. At 25°C the amount of E_r at equilibrium gives a relative activity of 0.27 compared with 0.58 at 37°C and 0.65 at 40°C (last result not shown).

Effectors enhance the rate and extent of conversion at 25°C (Figs. 1a and 1b). The maximum relative activity obtained in the presence of 2mM-NADPH or $3\text{-phosphoglycerate}$ is 1.0, and that in the presence of the oxyanions SO_4^{2-} , PO_4^{3-} and $\text{P}_2\text{O}_7^{4-}$ is 1.1–1.2. These values must be adjusted downwards by 0.4, however, to allow for increases arising solely during activation (see the Experimental section); thus NADPH and $3\text{-phosphoglycerate}$ would give a relative activity of 0.6 and the oxyanions 0.7–0.8 at equilibrium.

Thermodynamic parameters of the conversion of E_s into E_r

The initial rates of conversion at various temperatures in the presence and in the absence of

Fig. 1. Time courses for the conversion of wheat ribulose biphosphate carboxylase from the slowly activating into the rapidly activating form at 25°C or 35°C in the absence of effectors and at 25°C in the presence of effectors

A solution of enzyme ($20\text{--}30\text{mg/ml}$ in CO_2 -free 100mM-Bicine buffer, pH 8.2) was incubated at 25°C or 35°C in the presence and in the absence of effectors (see below). Samples ($10\mu\text{l}$) were taken at the times indicated, mixed with $10\mu\text{l}$ of Bicine buffer, pH 8.2, containing 20mM-NaHCO_3 and 40mM-MgCl_2 , and incubated at 25°C for a further 3 min before assay (see the Experimental section). The results are expressed relative to the activity obtained from a similar enzyme sample incubated in the same solution (lacking effectors) at 40°C for 40 min before assay. (a) $\square$, 2mM-NADPH ; $\circ$, $2\text{mM-3-phosphoglycerate}$; $\triangle$, no effectors, 25°C ; $\blacktriangle$, no effectors, 35°C . (b) $\square$, $10\text{mM-P}_2\text{O}_7^{4-}$; $\circ$, 10mM-SO_4^{2-} ; $\triangle$, 10mM-PO_4^{3-} ; $\bullet$, 10mM-NO_3^- .

Fig. 2. Arrhenius plot showing the energy of activation for the conversion of wheat ribulose biphosphate carboxylase from the slowly activating into the rapidly activating form in the presence of effectors

Initial rates of conversion at various temperatures were determined by incubating 10 μl of enzyme solution (20–30 mg/ml in CO_2 -free 100 mM-Bicine buffer, pH 8.2) with or without effectors for 5 min. After this time the sample was activated and assayed as previously (see the legend to Fig. 1). ●, Heat only; ○, 10 mM- PO_4^{3-} ; □, 2 mM-3-phosphoglycerate.

effectors were used to construct Fig. 2. Differences in these initial rates between thermal, 3-phosphoglycerate-mediated and PO_4^{3-} -mediated conversion have caused the lines obtained to be separated, but the slopes of the plots of $\log v$ versus $1/T$ are identical. This value is the activation energy (E_A) for the conversion of E_s into E_r and is 95.6 kJ/mol.

The free-energy change ($\Delta G'$) for the conversion can be derived from the relative activities at equilibrium, adjusted where necessary, in Fig. 1. Thus $\Delta G'$ for thermal conversion at 25°C and pH 8.2 is 2.5 kJ/mol, but 3-phosphoglycerate lowers this value to -1.0 kJ/mol and PO_4^{3-} lowers it to -3.4 kJ/mol.

Effects of thermal conversion on the near-u.v. c.d. spectrum

Fig. 3(a) shows baseline-corrected spectra measured before and after incubation of the enzyme at 35°C. Spectra in this region are primarily associated with the aromatic amino acid

side chains and disulphide bonds; ribulose biphosphate carboxylase contains many of the former and, potentially, also the latter [for information on the amino acid composition of enzymes from various sources see Lorimer (1981) and Shinozaki *et al.* (1983)].

The pre- and post-incubation spectra differ from each other in several areas. Most notable are the 2 nm red-shift, the enhancement of the minima at 262, 279 and 287 nm and the additional peak at 282 nm. The region between 225 and 280 nm is most changed, and the signals here may be attributable to phenylalanine side chains. The additional peak is probably due to the $^1\text{L}_b$ 0-0 transition of a tryptophan side chain.

Effects of PO_4^{3-} - and 3-phosphoglycerate-mediated conversion on the near-u.v. c.d. spectrum

Fig. 3(a) shows the spectra obtained before and after incubation in the presence of 10 mM- PO_4^{3-} or 2 mM-3-phosphoglycerate. The post-incubation spectra are extensively modified and show large increases in the principal minima. The increases at 280 nm and above are most likely due to restriction of the configurational freedom of tryptophan to the 0-0 and 0+850 vibronic transitions of the $^1\text{L}_b$ electronic transition states. Increases at 268 and 276 nm are probably due to restriction of a phenylalanine to the $^1\text{L}_b$ state transitions also, although that at the higher wavelength may be due to tyrosine.

The small additional peak at 282 nm observed on thermal conversion is greatly enhanced and can be seen as a shoulder to the main 287 nm minimum.

The differences in signal strength between the two effectors can be attributed to the slightly different ways in which they interact with the enzyme; the overall interaction with 3-phosphoglycerate must be stronger than with PO_4^{3-} , as the signal is increased quite uniformly. Notable differences are the more pronounced shoulders at 282 and 262 nm with 3-phosphoglycerate.

Effects of heat, 3-phosphoglycerate or PO_4^{3-} on the far-u.v. c.d. spectrum

The far-u.v. c.d. spectra obtained before and after conversion by heat or effectors are shown in Fig. 3(b). In this region the signals originate principally from the peptide bonds of the protein, so that the gross conformation is reflected. The pre-incubation spectrum is typical of a protein belonging to the α/β -conformational class (Levitt & Chothia, 1976; Manavalan & Johnson, 1983) and does not show any marked post-conversion changes: this suggests that the secondary structure is mainly conserved throughout the conversion process.

Fig. 3. Baseline-corrected c.d. spectra of wheat ribulose biphosphate carboxylase before and after conversion in the presence or in the absence of effectors

The c.d. of enzyme solutions (in 10 mM-Tris/HCl buffer, pH 8.0) was measured before and after incubation at room temperature for 120 min in the presence of effectors, and at 35°C in the absence of effectors. (a) Near-u.v. region (255–320 nm); 1.0 mg of enzyme/ml; 1 cm path-length. Results as expressed as $[\theta]$, assuming an M_r value of 70000 for the large/small subunit dimer. Noise levels were less than ± 650 . (b) Far-u.v. region (195–260 nm); 0.3 mg of enzyme/ml; 0.5 mm path-length. The results are expressed in terms of $[\theta]_{mrw}$, assuming an average monomer weight of 110. Above 195 nm noise levels were less than ± 150 .

Discussion

The activation energy, E_A , does not vary, suggesting that the reaction route of E_s to E_r is the same whichever conditions prevail in the surrounding medium. However, the value of $\Delta G'$ varies from +2.3 kJ/mol to -3.4 kJ/mol, indicating that the end point of the reaction is not the same in all cases. The value obtained for thermal conversion represents the difference in energy between E_s and E_r , whereas the other values are the difference between E_s and $E_r \cdot 3$ -phosphoglycerate or $E_r \cdot PO_4^{3-}$: the E_r effector (E_{rF}) complex is therefore a lower energy state than E_r . Binding of effectors stabilizes E_r , and this may be similar to the stabilization of E_{act} by effectors (Gutteridge *et al.*, 1982; Jordan *et al.*, 1983).

Formation of E_{rF} forces the $E_s \rightarrow E_r$ reaction to the right by effectively removing E_r . A similar situation probably arises during activation, as the probable route is $E_s \rightarrow E_r \rightarrow E_{act}$, so that formation of E_{act} pulls the reaction to the right; this is supported by the finding that a relative activity of 1.0 is obtained by heating at 40°C with optimal concentrations of activators, whereas incubation at this temperature in the absence of activators yields a relative activity of 0.65 after activation.

Since the far-u.v. spectra from E_r , E_{rF} and E_s are very similar, it can be concluded that there is no change in the gross conformation of wheat ribulose biphosphate carboxylase during conversion or binding of effectors to E_r . The main clues to the nature of the changes that do occur come from the near-u.v. spectra. These suggest that E_r differs from E_s by a slight re-orientation of the aromatic amino acid side chains, especially of phenylalanine. If it is assumed that changes involved in E_r formation render the enzyme more easily activated, then it may be concluded that one or more phenylalanine side chains become re-oriented in order to open the activation site.

The lysine residue that becomes carbamoylated during activation (lysine-201; Lorimer & Mizioro, 1980; Lorimer, 1981) lies in a region of the protein that has a high proportion of hydrophobic residues and many potential β -turn sequences (as judged by the method of Chou & Fasman, 1978). Because of this, and the relative flexibility of such a region, it is possible that lysine-201 is inaccessible to solvent in E_s but not so in E_r ; the $\Delta G'$ value for thermal conversion indicates the possibility of a weak hydrophobic interaction being broken. Changes in the near-u.v. c.d. spectrum suggest that thermal conversion affects phenylalanine, and it is

possible that this represents exposure of one or more phenylalanine residues to solvent.

Differences in the near-u.v. spectra of E_s and E_{rF} are probably related to effector binding. The large increases in the minima due to several different aromatic side chains indicate that the effectors must bind to a region rich in these residues. Effectors bind at the ribulose biphosphate-binding site (Badger & Lorimer, 1981; McCurry *et al.*, 1981; Jordan *et al.*, 1983), and this is proposed to be in the region of cysteine-173 and cysteine-461 in the inactive enzyme (Schloss *et al.*, 1979). However, neither of these regions is particularly rich in aromatic side chains, and it is possible that other regions within the active site interact with effectors.

One such region is near the carbamoylation site, between residues 210 and 220, and contains three arginine residues (residues 213, 215 and 217) as well as four aromatic residues (211, 218 and 220 are phenylalanine, 214 is tryptophan). Arginine residues are known to interact with tetragonal oxyanions such as PO_4^{3-} , $P_2O_7^{4-}$ and SO_4^{2-} (Ichimura *et al.*, 1978), and it is possible that the complex phosphorylated effectors also interact here. Such interactions may decrease the hydrophobicity, and so stabilize E_r and E_{act} in preference to E_s . Further work with arginine-specific and active-site-directed reagents should yield more detailed information on these interactions.

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