Temperature Dependent Conformation Changes of Ribulose-1,5-bisphosphate Carboxylase Studied by the Use of 1-Anilino-8-naphthalene Sulfonate

Günter F. Wildner and Jürgen Henkel

Lehrstuhl für Biochemie der Pflanzen, Abteilung Biologie, Ruhr-Universität Bochum, Germany

(Z. Naturforsch. **32 c**, 226–228 [1977]; received January 24, 1977)

Ribulose-1,5-bisphosphate Carboxylase, 1-Anilino-8-naphthalene Sulfonate, Cold Sensitivity, Conformation Change

The influence of temperature on the structure and enzyme activity of ribulose-1,5-bisphosphate carboxylase, isolated from *Euglena gracilis* cells, was studied. Freezing of the purified ribulose-1,5-bisphosphate carboxylase preparation causes a severe loss of enzyme activity, which can be restored again by incubation of the enzyme molecules at higher temperatures (50 °C). The titration of both enzyme samples with the fluorescence probe 1-anilino-8-naphthalene sulfonate (ANS) revealed an increase of the fluorescence emission of the low temperature form of the enzyme. Two different enzyme conformations can be assumed which differ in the number of binding sites for ANS and V_{max} values for the carboxylase reaction but show similar binding constants for ANS and the apparent K_m values for CO_2.

Introduction

Ribulose-1,5-bisphosphate carboxylase, the key enzyme of the Calvin cycle, is inactivated by freezing and reactivated by incubation at 50 °C [1–3]. The enzyme of green algae and higher plants is of a rather complex structure since it is composed of eight large and eight small subunits [4, 5].

The rearrangement of these subunits due to temperature-dependent conformation changes was studied by the use of the fluorescence probe, 1-anilino-8-naphthalene sulfonate (ANS). The enzyme was isolated and purified from *Euglena gracilis* and incubated at 0 °C and at 50 °C and subsequently titrated with ANS at 25 °C. This procedure is more advantageous than a previously applied method [6] since the temperature dependence of the ANS-protein binding constant has not to be considered.

Materials and Methods

Isolation of RuDP carboxylase

RuDP carboxylase was isolated from photoautotrophically grown [7] *Euglena gracilis* (strain z) cells. The washed cells were suspended in 100 mM Tris-sulfate buffer pH 8.0, containing 10 mM $MgCl_2$, 10 mM $NaHCO_3$ and 1 mM dithiothreitol. The chilled cell suspension was briefly sonicated and subsequently centrifuged (30 min 12000 × *g*) to remove cells debris. The enzyme was purified from the supernatant by the following methods: ammonium sulfate praecipitation (30 to 55% saturation), ultrafiltration (Amicon XM-300 membranes) and finally by centrifugation on a sucrose density gradient. The gradient consisted of 5 ml 50%, 8 ml 30%, and 8 ml 15% sucrose solution in 10 mM Tricine-NaOH buffer pH 8.0 containing 10 mM $MgCl_2$, 10 mM $NaHCO_3$ and 1 mM dithiothreitol. The enzyme was layered on the top of the gradient and centrifuged for 20 h at 65000 × *g*. Fractions were collected and analyzed for protein content, enzyme activity and homogeneity. The pooled fractions were stored below 0 °C.

Requests for reprints should be sent to Priv. Doz. Dr. G. F. Wildner, Ruhr-Universität Bochum, Abteilung Biologie, Postfach 10 21 48, *D-4630 Bochum*.

Characterization of RuDP carboxylase

The RuDP carboxylase activity was measured according to Andersen *et al.* [8] and the protein content was estimated by the biuret method. The fractions with the highest specific activity were pooled (1.4 U/mg protein). The $(CO_2)_{0.5\,V}$ value was measured and calculated to be 0.15 mM.

The fractions were also analyzed for homogeneity by disc gel electrophoresis. A 5% acrylamide monomer gel concentration was used and the electrophoresis was carried out according to Ornstein-Davis [9]. The gels were stained with Amidoblack dye and destained by repeated washings with destaining solution [10]. The fraction with the highest specific activity showed a single band.

Fluorescence assay

The fluorescence emission of the ANS-enzyme complex was measured with a Zeiss spectrophotometer (PMQII-ZMF4-M4QIII). The exciting light was passed through a narrow bandpass filter (365 nm) and the fluorescence was registered at 495 nm.

The cuvette, temperature-equilibrated at 25 °C, contained 2 ml of 50 mM Tricine-NaOH buffer pH 8.0, 10 mM $MgCl_2$, and 0.2 mg enzyme protein. The samples were titrated with ANS in 40 nmol portions.

ANS was purchased by Serva (Heidelberg), RuDP. Na_4 was synthesized enzymatically from ribose-5-phosphate.

Results and Discussion

The freezing procedure inactivates RuDP carboxylase whereas subsequent incubation at higher temperatures (50 °C) restores the carboxylase activity as shown in Fig. 1. Maximal reactivation

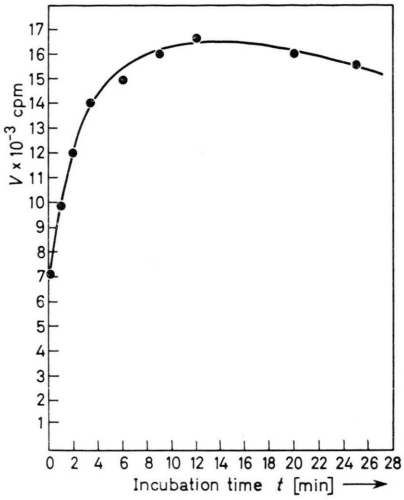

Fig. 1. Reactivation kinetics of RuDP carboxylase. The 0 °C-form of the enzyme was incubated at 50 °C and the enzyme activity was determined at 25 °C as described in Methods.

(230%) was observed after 14 min incubation at 50 °C. Both samples were used for fluorescence analysis with the probe ANS. The titration curves are presented in Fig. 2. The significant differences in the maximal fluorescence emission of the ANS-protein complex reflects a change of this complex. The increase of the fluorescence emission (see Fig. 2) can be attributed either to an increase of the number of enzyme-bound ANS molecules or by a change of the fluorescence quantum yield of the ANS-enzyme complex indicating structural alterations due to changes of the hydrophobic areas of the enzyme molecules and the environment of the bound ANS molecules.

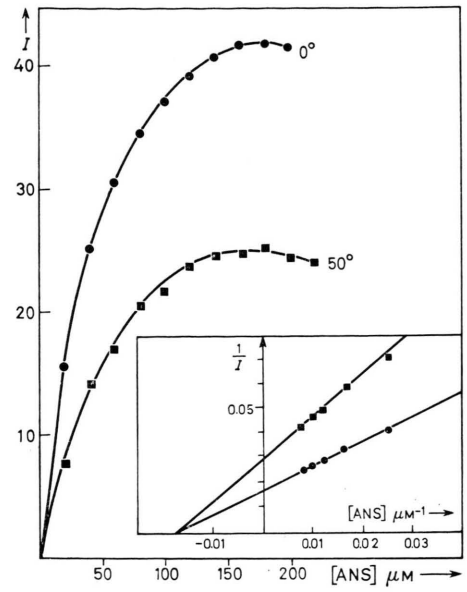

Fig. 2. Fluorescence analysis. The 0 °C and 50 °C treated enzyme samples were titrated with ANS and fluorescence emission was recorded, the values were corrected for the intrinsic fluorescence emission of ANS. (For details see Methods.)

The presence of 1 mM RuDP during the heat treatment at 50 °C prevented the conformational change and kept the enzyme molecule in the low temperature form (Table I). The observations by Kwok and Wildman [11] and Chu and Bassham [12, 13] suggest that RuDP changes the binding sites of the enzyme and decreases its activity by negative allosteric cooperation.

The experiments in the presence of 10 mM $NaHCO_3$ demonstrated that this substrate had no influence on the temperature dependent alteration of the enzyme (Table I).

Table I. Influence of the substrate on the fluorescence emission.

Additions	0 °C	50 °C
control	100 *	60
plus RuDP	100	98
plus $NaHCO_3$	96	57

* The fluorescence intensity was arbitrarly set as 100%.

The Lineweaver-Burk plots of the ANS-titration (insert of Fig. 2) shows that ANS was bound in both samples with an $(ANS)_{0.5\,I}$ value of 0.05 mM. This result suggests that both conformations of the

enzyme have similar binding affinity to ANS molecules. Furthermore, the straight lines indicated that no cooperative effects of ANS could be observed in the concentration range of 20 – 150 μM ANS.

As a further method for the study of the temperature dependence of conformational changes of the RuDP carboxylase molecules, the equilibrium dialysis was employed. The dialysis was carried out with 16 nmol of enzyme protein in 50 mM Tricine buffer pH 8.0 containing 10 mM $MgCl_2$ and 0.15 mM ANS at two different temperatures: 0 and 25 °C. The reactivation process occured at 50 °C. The optical densities of the protein samples (dialysates) and of the dialysis buffers were measured and the content of the bound ANS was estimated by using an extinction coefficient (360 nm) of 5000 $cm^2 \cdot mmol^{-1}$. The results of these experiments showed that at 0 °C 14 molecules of ANS were bound to one molecule of enzyme, whereas at 25 °C the value decreased to 7. These observations support the results of the fluorescence analysis studies.

The $(CO_2)_{0.5\,V}$ values have been estimated at 25 °C for both enzyme samples (0 and 50 °C treatment) and the results are shown in Fig. 3. The apparent $(CO_2)_{0.5\,V}$ were identical in both samples and equivalent to 150 μM. This value was also estimated after the last step of the isolation procedure. The apparent V_{max}-values were different, the 0 °C conformation was 67.5% less active as the 50 °C form of the enzyme.

The assumption of two temperature dependent conformations of RuDP carboxylase [1–3] could be confirmed by the use of the fluorescence probe ANS. Both methods, fluorescence analysis and equilibrium

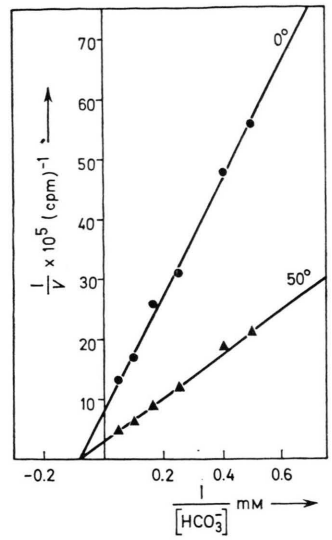

Fig. 3. Kinetic analysis of the two temperature dependent enzyme conformation. The activities of the cold inactivated (0 °C) and heat reactivated (50 °C) enzyme samples were measured at 25 °C. The assay mixture contained in 200 μl: 10 μmol Tricine-NaOH buffer pH 8.0, 2 μmol $MgCl_2$, 0.2 μmol RuDP, $NaH^{14}CO_3$ (1 Ci/mol) in the range of 0.2 to 4 μmol and enzyme equivalent to 0.05 mg protein. The reaction was stopped after 2 min by the addition of 50 μl glacial acid and the radioactivity was determined [8].

dialysis, revealed that the low temperature form has probably larger hydrophobic regions. The higher temperature form can be characterized by its higher apparent V_{max} as the more efficient conformation for CO_2 fixation.

The study was financially supported by Deutsche Forschungsgemeinschaft.

[1] N. Kawashima and T. Ayabe, Plant and Cell Physiol. **13,** 523–530 [1972].
[2] N. Kawashima, S. Singh, and S. G. Wildman, Biochem. Biophys. Res. Commun. **42,** 664–668 [1971].
[3] S. Singh and S. G. Wildman, Plant and Cell Physiol. **15,** 373–379 [1974].
[4] K. E. Moon and E. O. P. Tompson, Austr. J. Biol. Sci. **22,** 463–470 [1969].
[5] B. A. McFadden, J. M. Lord, A. Rowe, and D. Dilks, Eur. J. Biochem. **54,** 195–206 [1975].
[6] G. F. Wildner, Z. Naturforsch. **31 c,** 267–271 [1976].
[7] G. F. Wildner and G. Hauska, Arch. Biochem. Biophys. **164,** 127–135 [1974].
[8] W. R. Andersen, G. F. Wildner, and R. S. Criddle, Arch. Biochem. Biophys. **137,** 84–90 [1970].
[9] B. J. Davis, Ann. N. Y. Acad. Sci. **121,** 404–427 [1964].
[10] K. Weber and M. Osborne, J. Biol. Chem. **244,** 4406–4412 [1969].
[11] S. Y. Kwok and S. G. Wildman, Arch. Biochem. Biophys. **161,** 354–359 [1974].
[12] D. K. Chu and J. A. Bassham, Plant Physiol. **52,** 373–379 [1973].
[13] D. K. Chu and J. A. Bassham, Plant Physiol. **55,** 720–726 [1975].