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CONFORMATIONAL CHANGES ASSOCIATED WITH THE REVERSIBLE COLD INACTIVATION OF RIBULOSE-1,5-BISPHOSPHATE CARBOXYLASE-OXYGENASE *

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Summary

Crystalline ribulose-1,5-bisphosphate carboxylase-oxygenase (3-phospho-D-glycerate carboxy-lyase (dimerising), EC 4.1.1.39) isolated from tobacco (*Nicotiana tabacum* L.) leaf homogenates is partially inactivated by cold treatment and fully reactivated by simple heating in the absence of sulfhydryl reagents and effectors. Since the reversible cold inactivation of this bifunctional enzyme does not involve a gross change in the association state of subunits, a subtle conformational change induced by low temperatures was implicated (Chollet, R. and Anderson, L.L. (1976) Arch. Biochem. Biophys. 176, 344—351). Chemical modification of the cold-inactivated and heat-reactivated enzymes by 5,5′-dithiobis-(2-nitrobenzoate) and *p*-mercuribenzoate at 25°C revealed no difference in the number of free -SH groups per mol protein. However, the reactivity of the sulfhydryl residues on the inactivated protein was considerably greater than that of the reactivated enzyme. Pretreatment of the two proteins with sodium dodecyl sulfate completely abolished the difference in -SH reactivity, indicating its dependence on protein conformation. Both the cold-inactivated and heat-reactivated enzymes enhanced the fluorescence intensity of 8-anilino-1-naphthalenesulfonate (ANS) and caused a blue shift of the emission maximum from 510 to 472 nm. When the inactivated enzyme was reactivated by heating, the increase in catalytic activity was closely paralleled by a concomitant decrease in the fluorescence intensity of the ANS · protein complex at 25°C. Fluorescence titration experiments revealed that the decrease in fluorescence intensity accompanying heat reactivation of the inactivated enzyme was due to a reduction in the number of hydrophobic

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Abbreviations used: Ru-P_2, ribulose 1,5-bisphosphate; 6-*P*-GlcA, 6-phosphogluconate; DTNB, 5,5′-dithiobis-(2-nitrobenzoate); SDS, sodium dodecyl sulfate; ANS, 8-anilino-1-naphthalenesulfonate; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

sites available for ANS binding rather than to a change in the dissociation constant of the ANS · protein complex.

These results indicate that the reversible cold inactivation of ribulose-1,5-bisphosphate carboxylase-oxygenase is associated with a reversible change in the conformation of the protein. This cold-induced conformational change results in a greater exposure of sulfhydryl groups and hydrophobic regions to the external environment and is closely paralleled by changes in the catalytic activity of the protein. By analogy to other oligomeric enzymes also subject to reversible cold inactivation, perhaps low temperatures induce a partial dissociation of the octameric structure of the hydrophobic catalytic subunits, but complete dissociation is arrested in some unknown manner by the small hydrophilic subunits.

Introduction

Ribulose-1,5-bisphosphate carboxylase (3-phospho-D-glycerate carboxylyase (dimerising), EC 4.1.1.39) catalyzes the initial reaction in the photosynthetic carbon reduction cycle, the carboxylation of Ru-P_2 to yield two molecules of 3-*P*-glycerate. Recent work with purified preparations of the carboxylase isolated from prokaryotic and eukaryotic organisms has demonstrated that the enzyme also functions as an oxygenase, catalyzing the oxygenation of Ru-P_2 to yield 3-*P*-glycerate and 2-*P*-glycolate, the presumed precursor of the photorespiratory substrate, glycolic acid. The enzyme has been obtained in a highly purified crystalline state from tobacco and is a huge protein ($s^0_{20,w}$ = 18.3 S, $M_r \approx 550\,000$) composed of eight large, catalytic subunits and eight small, non-catalytic subunits [1,2], the former being much more hydrophobic than the latter [3—5].

The carboxylase and oxygenase activities of the dissolved crystalline tobacco enzyme are partially inactivated (50—70%) by cold treatment and fully reactivated by simple heating in the absence of sulfhydryl reagents and effectors [5—7]. Unlike other oligometric enzymes also subject to reversible cold inactivation such as chicken liver pyruvate carboxylase [8] and phosphofructokinase [9], yeast aspartic β-semialdehyde dehydrogenase [10], and glycogen phosphorylase *b* of muscle [11], Ru-P_2 carboxylase-oxygenase shows no indication of dissociation-reassociation of subunits or reversible aggregation during cold inactivation and heat reactivation [5—7]. In addition, cold inactivation of the tobacco leaf enzyme is completely reversed by heating even after prolonged incubation in the cold or repeated cycles of cooling and rewarming [5], suggesting that there is a true equilibrium between active and reversibly inactive forms of the enzyme [6], with no concurrent slower irreversible inactivation component as in the case of pyruvate carboxylase [8].

Since the reversible cold inactivation of tobacco leaf Ru-P_2 carboxylase-oxygenase does not involve a gross change in the state of association of subunits, a subtle change in the conformation of the protein induced by cold temperatures has been implicated [5—7]. To characterize this presumed conformational change, we have investigated the effects of cold inactivation and heat reactivation on the sulfhydryl group reactivity and the binding of the

fluorescent probe 8-anilino-1-naphthalenesulfonate to the crystalline tobacco enzyme.

Materials and Methods

Reagents. All biochemicals were purchased from Sigma Chemical Co. unless noted otherwise. The sodium salts of Ru-P_2, 6-*P*-GlcA, and NADPH were dissolved in 10 mM Tris · HCl (pH 8.2) and used without further purification. Solutions of DTNB, SDS, and the sodium salt of *p*-mercuribenzoate were prepared in 100 mM Tris · HCl (readjusted to pH 7.4) just prior to use. The ammonium salt of ANS (Eastman Organic Chemicals) was recrystallized four times from hot water and dissolved in 100 mM Tris · HCl (pH 7.4) just prior to use. $NaH^{14}CO_3$ was obtained from New England Nuclear.

Enzyme preparation. Crystalline Ru-P_2 carboxylase-oxygenase was obtained from tobacco (*Nicotiana tabacum* L. cv. Xanthi) leaf homogenates and recrystallized twice as described previously [7]. The protein crystals were dissolved and subsequently diluted in 25 mM Tris, 100 mM NaCl, pH 7.4.

Assays. All assays were performed at 25°C following a temperature pretreatment step of cold inactivation and/or heat reactivation of the protein. Cold inactivation consisted of storing a 10 mg/ml solution of the protein for 24—48 h at 4°C. The minimum state of Ru-P_2 carboxylase activity is reached after 20 h of cold storage and does not change thereafter [6]. The inactivated protein was diluted and stored at 4°C throughout the course of the experiment. Unless noted otherwise, heat reactivation consisted of heating aliquots of the cold-inactivated protein (10 mg/ml) in glass tubes for 20 min at 50°C. This treatment fully reactivates the inactivated protein (see Fig. 8 and ref. 6). The reactivated enzyme was diluted and stored at 25°C.

Ru-P_2 carboxylase activity was determined by $^{14}CO_2$ incorporation at 25°C in the presence of $NaH^{14}CO_3$ and Ru-P_2. Unless noted otherwise, the reaction vessels contained 100 mM HEPES, 10 mM $MgCl_2$, 0.1 mM Na_2EDTA (entire solution adjusted with NaOH to pH 7.9), 0.6 mM Ru-P_2, 20 mM $NaH^{14}CO_3$ (0.25 Ci/mol), and approx. 20 μg of protein in a final volume of 1.0 ml. Vessels containing the buffer/salt solution and enzyme were sealed and repeatedly evacuated and refilled with He, followed by preincubation for 10 min at 25°C in the presence of $NaH^{14}CO_3$. The reactions were initiated by injecting Ru-P_2 and terminated after 3—5 min by injecting 0.1 ml of 6 M acetic acid. Contents of the flasks were thoroughly mixed, 0.5-ml aliquots were dried at 90°C, and dpm were determined by liquid scintillation spectroscopy. Blanks were treated in an identical manner except that Ru-P_2 was added following acidification.

The reaction of cold-inactivated and heat-reactivated Ru-P_2 carboxylase with *p*-mercuribenzoate was monitored spectrophotometrically at 255 nm and 25°C [12,13]. For the determination of the number of free sulfhydryl groups, the assay tubes contained 63 mM Tris, 50 mM NaCl, approx. 5 mg of protein (9 nmol), and 0.3, 0.6, 1.25, or 1.63 mM *p*-mercuribenzoate in a final volume of 1.0 ml at pH 7.4. After 30 and 60 min at 25°C, 0.1-ml aliquots were diluted with 3 ml of 100 mM Tris · HCl (pH 7.4) and the increase in absorbance at 255 nm determined. In the time course experiments, the assay tubes contained 50 mM Tris, 67 mM NaCl, approx. 0.76 mg of protein (1.4 nmol), and 16 or

63 μM *p*-mercuribenzoate in a final volume of 3.0 ml at pH 7.4. Following 5 min preincubation at 25°C, the reaction was initiated with *p*-mercuribenzoate and the ΔA_{255nm} was monitored over a 40 min period. In all experiments the A_{255nm} values were corrected for absorbance due to protein (minus *p*-mercuribenzoate) and *p*-mercuribenzoate (minus protein). For calculating the number of sulfhydryl groups in the protein samples, standard calibration curves for -SH residues were constructed under identical experimental conditions using freshly prepared solutions of crystalline glutathione.

Modification of enzyme sulfhydryl groups with DTNB was determined spectrophotometrically at 25°C by following the increase in absorbance at 412 nm of 2-nitro-5-thiobenzoate liberated ($\epsilon_{412} = 13\,600\ M^{-1} \cdot cm^{-1}$) [14]. The assay tubes contained 50 mM Tris, 67 mM NaCl, protein, and DTNB in a final volume of 3.0 ml at pH 7.4 as specified in the figure legends. Following 5 min preincubation at 25°C, the reaction was initiated with DTNB and the ΔA_{412nm} was monitored over a 45 min period. When SDS was included in the reaction mixture, it was added 2 min prior to DTNB addition to give a final concentration of 1—2%. All A_{412nm} values were corrected for absorbance due to DTNB (minus protein, ±SDS); the crystalline tobacco enzyme has negligible absorbance at 412 nm [15].

Fluorescence measurements were performed at 25°C in an Hitachi MPF-2A spectrofluorimeter using excitation and emission wavelengths of 382 and 472 nm, respectively. Unless noted otherwise, the cuvettes contained 25 mM Tris, 100 mM NaCl, approx. 1.5 mg of protein (2.7 nmol), and 20—26 μM ANS in a final volume of 3.05 ml at pH 7.4. Following 5 min preincubation at 25°C, the assays were initiated with 50 μl of ANS and the fluorescence recorded after 4 min. When SDS was included, it was added 2 min prior to ANS addition to give a final concentration of 1%. In all experiments the observed fluorescence was corrected for emission by dye (minus protein, ±SDS) and protein (minus ANS); all such corrections were negligible except for the ANS · SDS complex. The emission spectra were uncorrected for photomultiplier response. The concentration of ANS was determined spectrophotometrically from the molar absorbance of $4950\ M^{-1} \cdot cm^{-1}$ at 350 nm [16].

Protein concentration, as mg per ml, was calculated by the factor $A_{280} \times 0.7$ [15]. Protein content estimated by this method was about 1.6 times that determined by the Lowry method [17] using crystallized bovine albumin as the standard. The molar concentration of tobacco Ru-P_2 carboxylase-oxygenase was calculated using a molecular weight of 550 000 [4].

Results and Discussion

Number and reactivity of sulfhydryl groups

Recent work by Takabe and Akazawa [18] has demonstrated that -SH groups play an important role in the Ru-P_2 carboxylase and oxygenase reactions. It was thus of considerable interest to determine the number and reactivity of -SH residues in the cold-inactivated and heat-reactivated proteins. To determine the number of free -SH groups, titration with *p*-mercuribenzoate was employed. As shown in Fig. 1, 65 -SH groups were titrated per mol protein at saturating levels of *p*-mercuribenzoate with both the cold-inactivated and heat-

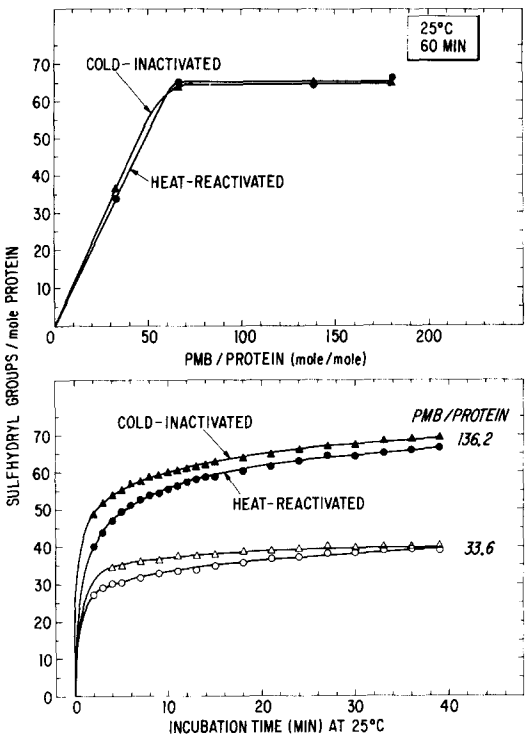


Fig. 1. Reaction of cold-inactivated (▲,△) and heat-reactivated (●,○) Ru-P_2 carboxylase with *p*-mercuribenzoate (PMB) at 25°C. Upper panel: the assay tubes contained 63 mM Tris, 50 mM NaCl, 4.95 mg of protein (9.0 nmol), and 0.3, 0.6, 1.25, or 1.63 mM *p*-mercuribenzoate in a final volume of 1.0 ml at pH 7.4. After 60 min at 25°C, 0.1-ml aliquots were diluted and the ΔA_{255nm} determined. Lower panel: the cuvettes contained 50 mM Tris, 67 mM NaCl, 763 μg of protein (1.39 nmol), and 15.7 or 63 μM *p*-mercuribenzoate in a final volume of 3.0 ml at pH 7.4. The reactions were initiated with *p*-mercuribenzoate and the ΔA_{255nm} determined over a 40 min period.

reactivated enzymes. This value is in good agreement with that reported for the wheat [13] and spinach [18] leaf enzymes (96 -SH groups/mol) when normalized with respect to Lowry protein (see Materials and Methods). When the time course of *p*-mercuribenzoate reaction with Ru-P_2 carboxylase was followed at several molar ratios of modifier: protein, the initial reactivity of -SH groups in the inactivated enzyme was always greater than in the reactivated sample (Fig. 1). Since this differing -SH reactivity may reflect a difference in conformation between the two proteins, subsequent sulfhydryl group studies were performed using a more bulky modifier, DTNB.

The rate of DTNB reaction with protein -SH residues is thought to be influenced primarily by steric factors, as the rather bulky DTNB molecule requires a certain amount of space in the proximity of the -SH group in order to react [19]. Indeed, Guidotti [20] has attributed the difference in reactivity of the sulfhydryls in oxyhemoglobin and carbon monoxyhemoglobin with DTNB to a subtle difference in the conformation of the two proteins. Similarly, the rate of DTNB reaction with -SH groups in rabbit muscle phosphorylase *b* [19] and several β-lactoglobulins [21] has been used to detect conformational changes. The reactivity of the sulfhydryl residues on the cold-

inactivated carboxylase-oxygenase with DTNB was considerably greater than that of the reactivated enzyme (Figs. 2 and 3). This difference in -SH reactivity was observed at all concentrations of DTNB examined ([DTNB] : [protein] ratios varied from 48 : 1 to 1012 : 1), indicating that both fast-reacting and slow-reacting sulfhydryls on the two proteins differed in reactivity. Pretreatment of the inactivated and reactivated enzymes with SDS completely abolished the difference in -SH reactivity between the two proteins (Fig. 3), indicating its dependence on gross protein conformation. The greater reactivity of sulfhydryls on the inactivated enzyme is thus attributed to a reversible conformational change in the carboxylase protein induced by cold temperature, making its -SH groups more readily available for reaction with *p*-mercuribenzoate and DTNB.

Némethy and Scheraga [22] have suggested that of the types of bonds postulated to maintain protein structure only hydrophobic bonds have properties consistent with a decrease in stability at low temperature. The reversible cold inactivation of Ru-P_2 carboxylase-oxygenase activities and the unusual heat stability of the protein (50°C for 20 min) [5—7,15] suggest that hydrophobic interactions are involved in maintaining the active conformation of this enzyme. It is thus possible, due to the hydrophobic nature of the large catalytic subunits, that the interaction between the large subunits is reduced at low temperatures and the conformation of the carboxylase protein changes from

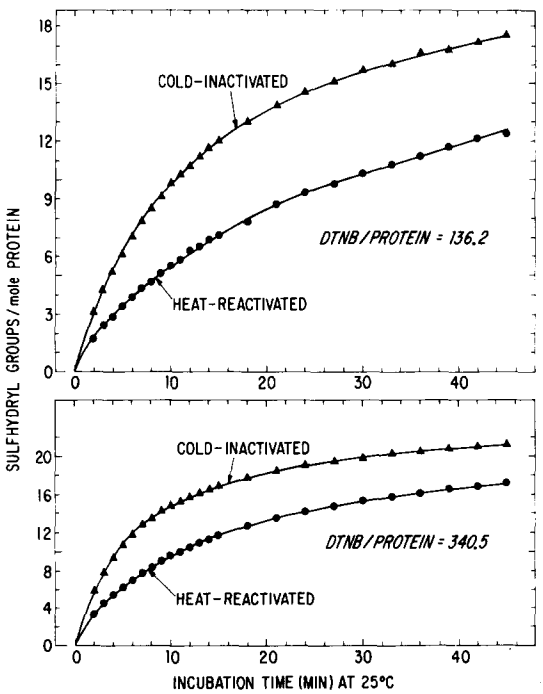


Fig. 2. Reaction of cold-inactivated and heat-reactivated Ru-P_2 carboxylase with DTNB at 25°C. The cuvettes contained 50 mM Tris, 67 mM NaCl, 1.05 mg of protein (1.91 nmol), and 87 (upper panel) or 217 (lower panel) μM DTNB in a final volume of 3.0 ml at pH 7.4. The reactions were initiated with DTNB and the ΔA_{412nm} determined over a 45 min period.

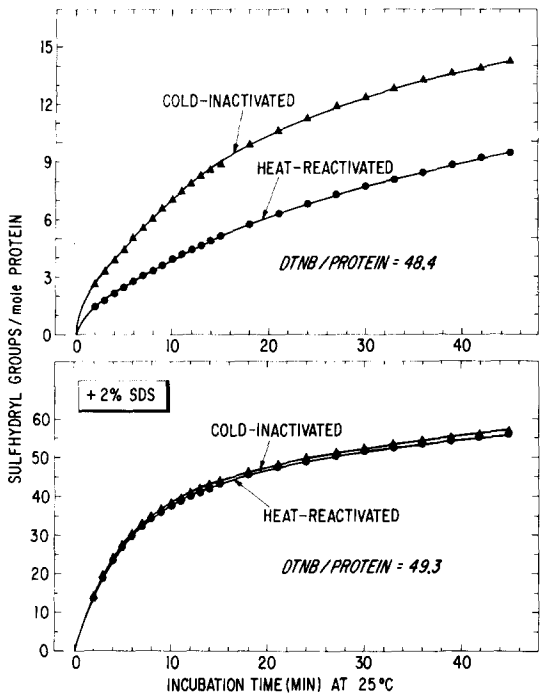


Fig. 3. The effect of 2% (w/v) SDS on the reaction of cold-inactivated and heat-reactivated Ru-P_2 carboxylase with DTNB at 25°C. The experimental protocol was identical to that described for Fig. 2 except that the protein content was 1.54 mg (2.8 nmol) and the concentration of DTNB was 46 μM. When SDS was included in the reaction mixture, it was added 2 min prior to DTNB addition.

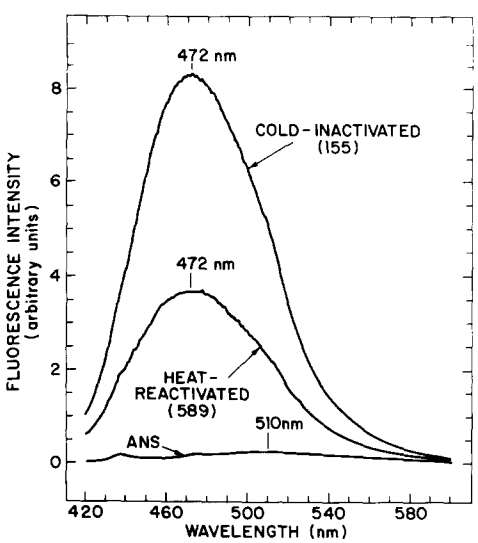
that of a more compact macromolecule to a slightly less compact form, as first postulated by Wildman's laboratory [5]. This change in conformation would be consistent with the -SH reactivity studies described above in that 75% of the total sulfhydryl groups in the higher plant carboxylase are located on the large hydrophobic subunits [18].

Interaction of ANS with cold-inactivated and heat-reactivated Ru-P_2 carboxylase-oxygenase

8-Anilino-1-naphthalenesulfonate has been widely used as a fluorescent probe for conformational changes [16,23—26] and hydrophobic regions [27—29] in proteins, including Ru-P_2 carboxylase [30]. Weber and Laurence [27] and Stryer [28] were the first to report that ANS is essentially non-fluorescent in aqueous solution but exhibits intense blue fluorescence as solvent polarity is reduced or on binding to non-polar sites on proteins. If, as discussed above, low temperatures cause a loosening of the subunits of the tobacco carboxylase protein, this partial dissociation would possibly result in the appearance of hydrophobic regions which had previously formed contact sites between subunits. Such sites would be expected to interact strongly with a hydrophobic probe such as ANS.

Addition of Ru-P_2 carboxylase-oxygenase to a solution of ANS results in a large enhancement of the fluorescence intensity of the dye and a blue shift of

the emission maximum from 510 to 472 nm. Fig. 4 contrasts the fluorescence emission spectra of 26 μM ANS in the presence and absence of 0.91 μM (0.50 mg/ml) cold-inactivated and heat-reactivated enzyme. Although the excitation and emission maxima were identical for both ANS · protein complexes (382 and 472 nm, respectively), the enhancement of ANS fluorescence on binding to the inactivated protein was considerably greater than that with the reactivated sample (48- versus 21-fold, respectively). The fluorescence enhancement occurred immediately upon addition of either protein to the dye and the same intensity was maintained for at least 10 min. Pretreatment of the inactivated and reactivated enzymes with 1% SDS completely abolished the enhancement of ANS fluorescence, indicating its dependence on gross protein conformation. When the cold-inactivated Ru-P_2 carboxylase-oxygenase was reactivated by heating, the fluorescence intensity of the ANS · protein complex decreased dramatically as the catalytic activity of the protein increased (Figs. 4 and 5). As shown in the lower panel of Fig. 5, the percentage of total change in fluorescence intensity closely paralleled the reactivation of carboxylase activity



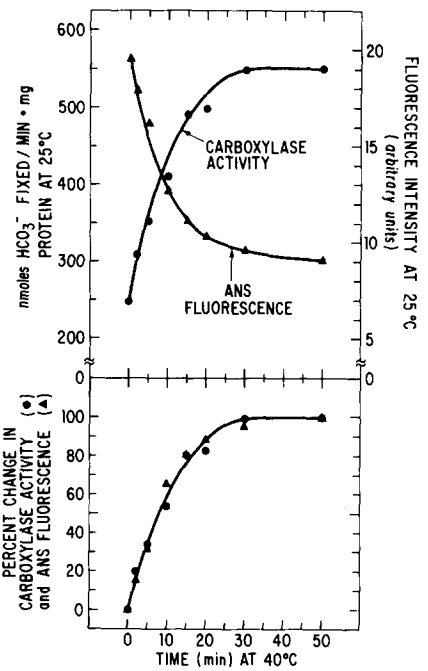


Fig. 4. Fluorescence emission spectra of 26 μM ANS in the presence and absence of 1.52 mg (2.76 nmol) of cold-inactivated and heat-reactivated Ru-P_2 carboxylase. Final volume = 3.05 ml, temperature = 25°C, λ_{ex} = 382 nm. The numbers in parentheses refer to the carboxylase activity (nmol HCO_3^- fixed/min per mg protein) of the cold-inactivated and heat-reactivated proteins determined at 25°C as described in Materials and Methods.

Fig. 5. The increase in Ru-P_2 carboxylase activity (•) and the decrease in ANS fluorescence (▲) during reactivation of the cold-inactivated enzyme at 40°C. Aliquots of the inactivated protein were heated at 40°C for the specified time and immediately assayed for enzyme activity and ANS fluorescence at 25°C. The carboxylase assays were performed exactly as described in Materials and Methods. The conditions for the fluorescence measurements were as follows: 26 μM ANS, 1.49 mg of protein (2.71 nmol), λ_{ex} = 382 nm, λ_{em} = 472 nm, final volume = 3.05 ml.

when the cold-inactivated protein was heated at 40°C. Apparently the fluorescence of the inactivated protein · ANS complex changes in response to variations in protein conformation accompanying the reactivation of enzyme activity.

In order to further characterize the interaction of ANS with the cold-inactivated and heat-reactivated proteins, fluorescence titration experiments were performed. In the experiment shown in Fig. 6 ANS concentration was held constant at 20 μM while enzyme concentration was varied between 0.13 and 2.7 μM (0.07—1.5 mg/ml). The results are presented in a double-reciprocal plot from which estimates of F_0 (the fluorescence intensity due to complete binding of the dye to the protein) and the enhancement of ANS fluorescence at infinite protein concentration can be obtained from the extrapolated intercept on the ordinate [25]. The maximum enhancement of ANS fluorescence (270-fold) and F_0 were identical for both proteins. In the experiment depicted in Fig. 7 enzyme concentration was held constant at 0.85 μM (0.47 mg/ml) while ANS concentration was varied between 6.7 and 124 μM. The data are again presented in a double-reciprocal plot from which F_{max} (the maximal fluorescence intensity at infinite dye concentration) and K_D (the dissociation constant of the ANS · protein complex) can be estimated from the extrapolated intercepts on the ordinate and abscissa, respectively [25]. Whereas the dissociation constant of the ANS · protein complex was essentially identical

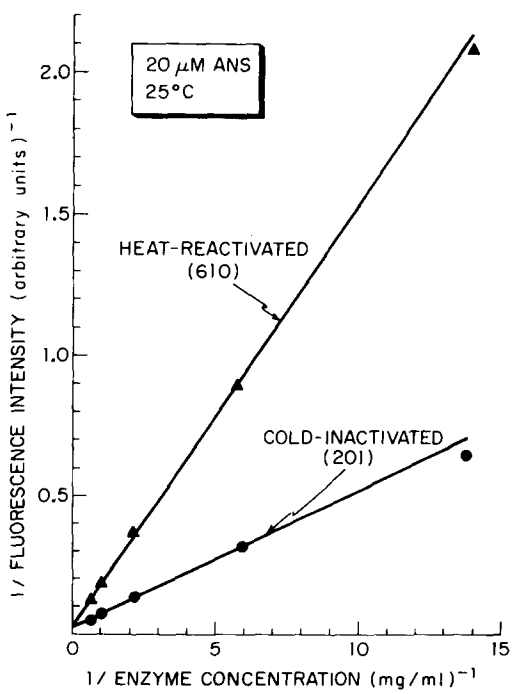


Fig. 6. Fluorescence titration of 20 μM ANS with cold-inactivated and heat-reactivated Ru-P_2 carboxylase. Protein content = 0.22—4.57 mg (0.40—8.31 nmol), λ_{ex} = 382 nm, λ_{em} = 472 nm, final volume = 3.05 ml. The numbers in parentheses refer to the carboxylase activity (nmol HCO_3^- fixed/min per mg protein) of the cold-inactivated and heat-reactivated proteins determined at 25°C as described in Materials and Methods.

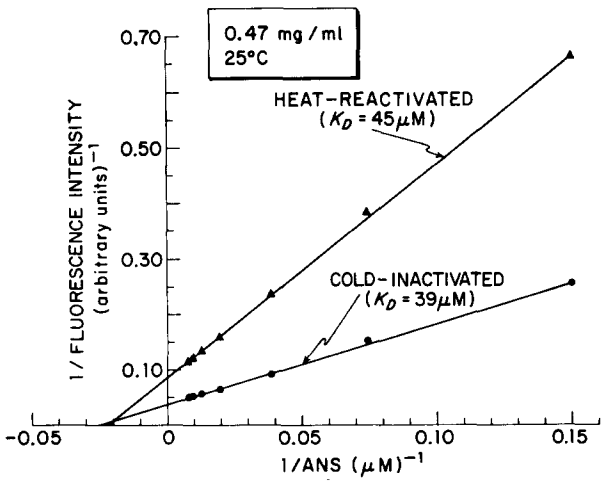


Fig. 7. Fluorescence titration of 1.43 mg (2.60 nmol) cold-inactivated and heat-reactivated Ru-P_2 carboxylase with ANS. Final volume = 3.05 ml, ANS concentration = 6.7—124 μM, λ_{ex} = 384 nm, λ_{em} = 474 nm.

for the two proteins (K_D = 40—45 μM), estimates of F_{max} for the inactivated protein · ANS complex were 2.4-fold greater than with the reactivated enzyme. Identical results were obtained when similar ANS titration experiments were performed at a much lower protein concentration of 0.05 mg/ml (data not shown). Since the number of ANS binding sites per mol protein is proportional to the ratio F_{max} : F_o [25], the results from the fluorescence titration experiments indicate that the cold-inactivated enzyme has more than twice the number of hydrophobic sites available for ANS binding as does the heat-reactivated protein.

In conclusion, the results from the ANS experiments summarized in Figs. 5—7 indicate that (a) cold storage of the tobacco enzyme induces a reversible change in the conformation of the protein which results in a greater exposure of hydrophobic regions to the external environment, and (b) that this reversible conformational change is closely paralleled by changes in the catalytic activity of the protein.

Solubility studies

Previous studies with the crystalline tobacco enzyme have indicated that the solubility of this protein is markedly dependent on ionic strength [31,32]. For example, the solubility of the enzyme is greater than 100 mg/ml in 100 mM NaCl at pH 7.4 and 20°C, whereas less than 1 mg/ml is soluble in 10 mM NaCl [31]. If, as indicated above, cold inactivation of Ru-P_2 carboxylase-oxygenase is associated with a reversible change in conformation of the protein exposing hydrophobic regions to the external aqueous environment, this cold-induced conformational change may also lead to a decreased solubility of the protein under conditions of low ionic strength. When solutions of heat-activated enzyme were stored for 23 h at 4 or 25°C in the presence of various NaCl concentrations, the solubility of the protein kept at 4°C and low salt was less than half of that of the sample maintained at 25°C (Table I). As the solubility

TABLE I

SOLUBILITY OF HEAT-ACTIVATED Ru-P_2 CARBOXYLASE-OXYGENASE AS A FUNCTION OF TEMPERATURE AND NaCl CONCENTRATION

A 1.45 mg/ml solution of heat-activated (50°C, 20 min) enzyme was stored undisturbed at 4 or 25°C in the presence of various NaCl concentrations, 25 mM Tris, pH 7.4, for 23 h, which is sufficient time to reach equilibrium between protein in solution and precipitated protein [3,32]. The solutions were centrifuged at 20 000 × *g* to remove any recrystallized protein, and the protein content of the supernatant was determined spectrophotometrically at 280 nm.

NaCl (mM)	Solubility (mg/ml)	
	4°C	25°C
10.0	0.31	0.81
12.5	0.43	1.02
15.0	0.55	1.20
100	>1.45	>1.45

of a protein molecule is generally a reflection of the nature of the surface groups exposed to the external environment, these data are consistent with the concept that cold storage of the tobacco enzyme induces a reversible conformational change of the protein which results in an increase in hydrophobicity.

Concluding remarks

The results from this study indicate that the reversible cold inactivation of Ru-P_2 carboxylase-oxygenase is associated with a reversible change in the conformation of the protein. This cold-induced conformational change results in a greater exposure of sulfhydryl groups and hydrophobic regions to the external environment and is closely paralleled by changes in the catalytic activity of the protein. By analogy to other oligomeric enzymes also subject to reversible cold inactivation [8—10], perhaps low temperatures induce a partial dissociation of the octameric structure of the hydrophobic catalytic subunits, but complete dissociation is arrested in some unknown manner by the small hydrophilic subunits. It is interesting to note that this cold-induced change in conformation of the protein which has such profound effects on its catalytic activities does not influence the modulation of enzyme activity by either positive (NADPH, 6-*P*-GlcA) (Fig. 8) or negative (0.5 mM ribose-5-*P*) (data not shown) effector molecules [7].

The physiological significance of the reversible cold inactivation of Ru-P_2 carboxylase-oxygenase is uncertain. Singh and Wildman [6] have suggested that the reversible inactivation of the enzyme observed in vitro offers a molecular basis for explaining the reversible inhibition of photosynthetic CO_2 fixation in plants acclimatized to low temperatures [33]. However, until this suggestion is supported by additional CO_2 uptake experiments with intact plants, it seems possible that this in vitro property may simply be a chance consequence of the particular primary, tertiary, and quaternary structures of this bifunctional enzyme.

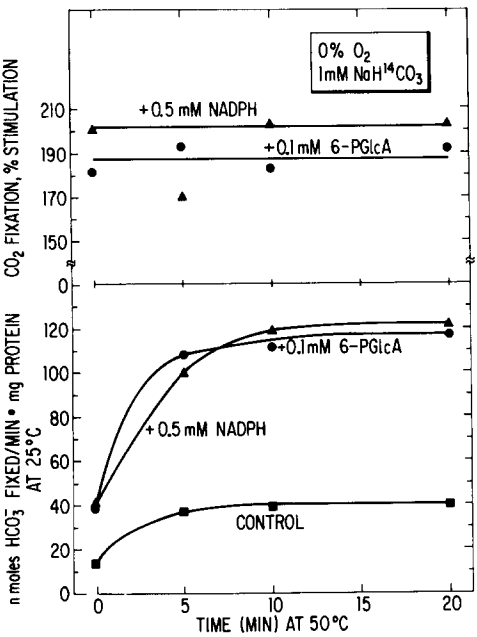


Fig. 8. The increase in Ru-P_2 carboxylase activity during reactivation of the cold-inactivated enzyme at 50°C. The inactivated protein was heated at 50°C and at the specified times aliquots were withdrawn and assayed for enzyme activity at 25°C. The carboxylase assays were started by adding 0.6 mM Ru-P_2 to the enzyme (approx. 20 μg), which had been preincubated for 10 min in a 50 mM Tris-buffered reaction medium (pH 7.7) containing 10 mM Mg^{2+}, 1 mM $NaH^{14}CO_3$ (2.7 Ci/mol), ±0.5 mM NADPH (▲) or 0.1 mM 6-*P*-GlcA (●). The reactions were run for 5 min at 0% O_2.

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