Incorporation of Large Subunits into Ribulose Bisphosphate Carboxylase in Chloroplast Extracts

INFLUENCE OF ADDED SMALL SUBUNITS AND OF CONDITIONS DURING SYNTHESIS

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ABSTRACT

The incorporation of newly synthesized large subunits into ribulose bisphosphate carboxylase/oxygenase (RuBisCO) in pea chloroplast extracts occurs at the expense of intermediate forms of the large subunit which are complexed with a binding protein. Most subunits of this binding protein are found in dodecameric complexes in chloroplast extracts. Addition of small subunits to these extracts results in approximately 40 to 60% increased incorporation of newly made large subunits into RuBisCO at low or zero concentrations of ATP, but is without significant effect at high concentrations of ATP, a condition in which the dodecameric binding protein complex is dissociated into subunits. Overall, these data support the assumption that the incorporation of large subunits into RuBisCO in chloroplast extracts reflects de novo assembly rather than 'mere' exchange of subunits. The in vitro assembly of large subunits into RuBisCO is a function of the conditions under which the large subunits are synthesized in organello. When the large subunits are made in chloroplasts suspended in 188 millimolar sorbitol, they are approximately 2- to 3-fold better able to assemble into RuBisCO when subsequently incubated in vitro than when they are synthesized in chloroplasts suspended in 375 millimolar sorbitol. This observation indicates that mere synthesis of large subunits is not sufficient to confer maximal assembly competence on large subunits.

RuBisCO\(^2\) (EC 4.1.1.39) catalyzes the CO\(_2\) fixation step in the Calvin cycle of photosynthesis. It also catalyzes the competing O\(_2\) fixation step in photorespiration (12). The enzyme consists of large subunits encoded in chloroplast DNA (13) and small subunits encoded in nuclear DNA. The small subunits are synthesized as precursors, taken up and processed by chloroplasts, and assembled with large subunits into RuBisCO in the chloroplast stroma (7). The mechanism of these final assembly steps appears to involve a large subunit binding protein, which is not part of the final structure of the enzyme (3, 5, 6, 9, 14–16, 18).

The large subunits which are incorporated into RuBisCO in pea chloroplast extracts are derived from 7S complexes (15). The bulk of the large subunits, however, occur in association with a 29S binding protein complex (3, 5, 18). ATP brings about the dissociation of this complex into 7S complexes and monomeric (about 60 kD) binding protein subunits (5, 11, 15). Recently our laboratory has shown that antibody directed against this binding protein inhibits the in vitro incorporation of 7S large subunits into RuBisCO (6). It was also found that very low concentrations of ATP appear to support a reaction which limits the incorporation of large subunits into RuBisCO (6). The incorporation of large subunits into RuBisCO could be due to de novo assembly or it could reflect some kind of exchange reaction. If the incorporation is due to de novo assembly, then it should be dependent on small subunits; data presented here indicate that added small subunits can stimulate the incorporation of large subunits into RuBisCO in chloroplast extracts.

We observed an upper limit to the ability of added small subunits to stimulate RuBisCO assembly. Searching for limiting factors, we investigated the effect of pH and sorbitol concentration during in organello protein synthesis on subsequent in vitro assembly of large subunits into RuBisCO. Significant effects of sorbitol concentration were observed, indicating that optimal in vitro assembly was obtained after large subunits were synthesized in chloroplasts under hypotonic conditions.

MATERIALS AND METHODS

Growth of Plants. Pea seedlings (Pisum sativum, var Progress No. 9, Agway, Rochester, NY) were grown on a 12 h light/dark cycle as described previously (6). Light intensity was 700 to 1000 \(\mu\)E/m\(^2\)/s.

Isolation of Intact Chloroplasts for Protein Synthesis. Chloroplasts were isolated from P. sativum plants using Percoll gradients and resuspended in 330 mM sorbitol, 35 mM HEPES-KOH (pH 8.3), 0.96 mM DTT, 200 \(\mu\)M MgCl\(_2\), 50 \(\mu\)M Na\(_2\)EDTA, 200 \(\mu\)M isoleucine, 200 \(\mu\)M threonine (17), and illuminated in the presence of \(^35\)S-methionine as indicated in figure captions. Alternatively, various components were altered as described in captions.

Isolation of P. sativum RuBisCO Small Subunits. RuBisCO purified by ammonium sulfate fractionation and sucrose gradient centrifugation (2). It was kept frozen as an (NH\(_4\))\(_2\)SO\(_4\) suspension at \(-80^\circ\)C until needed, when frozen beads were thawed and centrifuged. The pellets were dissolved in 10 mM sodium phosphate (pH 7.6), 1 mM Na\(_2\)EDTA, 500 mM NaCl, and dialyzed against the same buffer overnight. The protein concentration was adjusted to 1.0 mg/ml and the pH lowered to 5.0 by the addition of 0.25 N acetic acid (1). The sample was left on ice for 60 min, centrifuged to remove insoluble material, and the pH of the supernatant was returned to 7.6 by the addition of 1 mM Tris base. The small subunit preparation contained no

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2 Abbreviations: RuBisCO, ribulose 1,5 bisphosphate carboxylase/oxygenase; PMSF, phenyl methyl sulfon fluoride; in organello refers to events occurring in isolated intact chloroplasts; in vitro refers to events occurring in solution outside of organelles or cells.
detectable large subunits.

Polyacrylamide Gel Electrophoresis. Samples were subjected to electrophoresis at room temperature in the presence of SDS by the method of Laemmli (10), and at 4°C using the same procedure but omitting SDS from all solutions (14). Gels were stained with Coomassie blue or treated with En3Hance (New England Nuclear Corp., Waltham, MA) and fluorographed as previously described (15). Occasionally gel slices were counted as described by Roy et al. (19).

RESULTS

Stimulation of Large Subunit Incorporation into RuBisCO by Small Subunits. Small subunits were added to pea chloroplast extracts containing radioactive large subunits which had been synthesized in organello (Fig. 1a). It has been demonstrated repeatedly that almost all the soluble radioactive protein produced under these conditions is RuBisCO large subunits (3-6, 14-16, 18). Lane 1 shows the radioactivity found in RuBisCO following incubation at 0°C. Modest additional incorporation of radioactivity into RuBisCO occurred at room temperature (compare lane 2 with lane 1). The addition of small subunits caused a marked increase in RuBisCO radioactivity, as judged by visual inspection of the fluorogram (compare lane 3 with lane 2). Hexokinase and glucose were added to samples 4 and 5 before incubation, in order to restrict the dissociation of the 29S form of the binding protein complex. Under these conditions, the incorporation of radioactive large subunits into RuBisCO occurs at the expense of 7S large subunits which are not associated with the 29S form of the binding protein (6). These can be resolved by sucrose gradient centrifugation and SDS-PAGE (6, 18), but cannot be resolved on nondenaturing gels. Accordingly, the increase in RuBisCO radioactivity in lane 4 as compared with lanes 1 and 2 was not accompanied by a decrease in any resolved band in the gel lanes. The addition of small subunits in the presence of hexokinase and glucose led to a perceptible increase in incorporation of radioactive large subunits into RuBisCO (compare lane 5 with lane 4). However, because the lane widths varied somewhat, it was necessary to obtain a quantitative measurement to verify this perception (see below). ATP was added before incubation of samples 6 and 7, in order to dissociate the 29S form of the binding protein (5, 15). As expected, this led to loss of radioactivity in the 29S binding protein complex and an increase in RuBisCO radioactivity (compare lanes 6 and 7 with lane 2). The amount of radioactivity lost from the 29S complex exceeded that appearing in RuBisCO; previous work has shown that most of the large subunits released by ATP treatment, while not resolved on these nondenaturing gels, can be accounted for: they sediment at 7S in sucrose gradients (15).) Interestingly, the addition of small subunits in the presence of 5 mM ATP did not lead to greater incorporation than ATP alone (compare lane 7 with lane 6). The concentration of small subunits chosen in this experiment was very high—comparable with protein in vivo values (see “Discussion”). In other experiments, lower concentrations of small subunits showed similar effects, even when the small subunits were derived from spinach RuBisCO (Fig. 1b, compare lane 3 with lane 2).

To check the visual impressions gained from the foregoing, repeat experiments were assessed by liquid scintillation counting of gel slices containing the RuBisCO band (Fig. 2). Small subunits stimulated the incorporation of large subunits into RuBisCO in the absence of added ATP by 60% or more (compare C with B). Small subunits stimulated large subunit incorporation into RuBisCO in the absence of endogenous ATP by about 40% (compare D with E). Small subunits did not have a significant effect on incorporation of large subunits into RuBisCO in the presence of added ATP (compare H with F). The same result was obtained when the added ATP was subsequently removed by addition of hexokinase and glucose (compare G and F). The data in Figure 2 suggest that there is an upper limit to the amount of incorporation of large subunits into RuBisCO under these conditions. This corresponded to about 20% of the total large subunits in the extract in this experiment.

Dependence of in Vitro RuBisCO Assembly on Conditions of in Organello Synthesis of Large Subunits. The chloroplast protein synthesizing system used here has been optimized with respect to the rate and duration of amino acid incorporation into protein (8, 17). The extracts appeared to be unable to support incorporation of all newly synthesized large subunits into RuBisCO, even when excess small subunits were added. Therefore we began to optimize the system with respect to subsequent in vitro assembly of the large subunits. As a preliminary to this analysis we determined optimal sorbitol concentrations for synthesis of total and soluble proteins by isolated chloroplasts. There were two optima, at 188 and 330 mM (Fig. 3).

We resuspended intact chloroplasts at different pH or sorbitol concentrations, and carried out in organello protein synthesis in the presence of 35S-methionine. We then added small subunits to extracts of these chloroplasts in the absence of ATP, and monitored the in vitro assembly of the radioactive large subunits into RuBisCO. We carried out the assembly steps in the absence of ATP and the presence of small subunits in order to eliminate two known limiting factors. A series of controls lacking these components was run in parallel.

The optimum sorbitol concentration for in organello synthesis of large subunits which subsequently could assemble into RuBisCO in vitro occurred between 165 and 220 mM (Fig. 4). It is important to note that the assembly steps in this experiment were carried out in vitro, in the absence of sorbitol. The sorbitol effect therefore persists from the prior step of in organello protein synthesis, through lysis of the chloroplasts and removal of membranes. It is important to note that there was no peak of assembly in the region between 200 and 377 mM sorbitol.

Previous experiments had been done using chloroplasts suspended at pH 8.0 to 8.3 and 330 mM sorbitol, which had been found to be optimal for in organello protein synthesis (8). At 188 mM sorbitol, the optimum pH for in organello synthesis of large subunits which subsequently could assemble into RuBisCO in vitro was also between 8.0 and 8.3 (Fig. 5). As before, the pH effects persist from the in organello synthesis stage. The observed pH optimum is not significantly different from the optimum pH for in organello protein synthesis at this sorbitol concentration (data not shown).

DISCUSSION

Both large subunits and small subunits are incorporated into RuBisCO in pea chloroplast extracts. For this reason, most investigators have tacitly assumed that de novo assembly of RuBisCO occurs in the extracts (5, 20). The availability of soluble small subunits (1) made it possible to test this assumption. The data indicate that the incorporation of large subunits into RuBisCO can be stimulated by addition of small subunits. Large subunit incorporation into RuBisCO at 0°C is minimal (Fig. 1a, lane 1). The slightly higher baseline radioactivity seen in Figure 2A is due to an elevated background in the gel lane in that experiment. Thus, the percentage stimulation of assembly of RuBisCO by added small subunits is about 60% in the absence of other additions, and about 40% in the presence of AMP. This strengthens the idea that de novo assembly is occurring, and it also indicates that small subunits are one limiting factor in these extracts. In the presence of high concentrations of added ATP, however, addition of small subunits does not lead to additional assembly. This phenomenon is not fully understood, but it could be related to the ATP-dependent assembly limitation discovered.
earlier by our laboratory (6).

The pH optima for formation of assembly competent large subunits and for in organello protein synthesis were comparable to each other and occurred between pH 8.0 and 8.5 (Fig. 5; Ref 8).

In contrast to this, we observed a different sorbitol optimum for the production of assembly competent large subunits as compared to that for in organello protein synthesis. Maximum in vitro assembly was observed when the large subunits were made by chloroplasts which had been suspended in 165 to 220 mM sorbitol. But the maximum in organello protein synthesis showed two optima, one at 188 and the other at 330 mM sorbitol (Fig. 3). The latter observation at first appeared different from that of Fish and Jagendorf, who reported one optimum at 340 to 350 mM (8). However, it appears that those investigators did not examine the effect of sorbitol below 220 mM (L Fish, personal communication). In our hands, large subunits were synthesized at comparable rates at both 188 and 377 mM sorbitol. However, the assembly of those made at the lower sorbitol concentration was 2- to 3-fold more extensive than the assembly of those made at the higher sorbitol concentration.

Barraclough and Ellis (3) reported that in organello assembly of RuBisCO is dependent on the use of sorbitol rather than KCl as an osmotic. However, it is not clear why the osmotic conditions of synthesis in organello should affect the subsequent in vitro assembly of the large subunits into RuBisCO.

The effect cannot be due to differential small subunit pool sizes, since it occurs in the presence of excess added small
into subunits: the small subunits added to the extracts are at a concentration 0.8 to 2.4 μM. The in vivo concentration of free small subunits is believed to be in the μM range, as judged by analysis of radioactivity in unassembled large and small subunit pools, and Western blots of unassembled large subunits associated with the 29S binding protein (18; H Roy, M Gilson, unpublished data). Since the extracts are considerably more dilute than the chloroplast stroma, the added small subunits are in vast molar excess over large subunits under in vitro conditions.

The effect cannot be attributed entirely to increased rates of large subunit synthesis, since the optima for synthesis of protein and assembly of RuBisCO are not the same. However, we can speculate that the low sorbitol concentration leads to a less compact stroma than is normally the case during in organello protein synthesis, which is usually carried out at concentrations greater than 300 mM. This less compact stroma might be 'better' for the formation of assembly-competent large subunits, either by permitting favorable protein-protein interactions during or after synthesis, or by inhibiting unfavorable ones. One possibility is that the lower concentration of binding protein subunits in hypotonic chloroplasts leads to less sequestration of large subunits in the 29S complex, thus increasing the relative concentration of 7S large subunits. Since these are the assembly-competent form of the large subunits (6, 15), greater assembly of these into RuBisCO would be expected. Experiments are under way to test this hypothesis. More indirect effects could also be imagined, since the volume changes caused by the lower sorbitol concentration would lead to decreases in ion concentrations in the stroma. The data available do not allow a firm conclusion to be drawn on this point. The data do indicate that mere synthesis of large subunits is not enough—the conditions of their synthesis in organello appear to affect their ability to subsequently assemble into RuBisCO in vitro.

![Graph of CPM x 10^-3 vs. Sorbitol Conc. (mM)](image)

**Fig. 2.** Stimulation of RuBisCO assembly by *P. sativum* small subunits. Chloroplasts were prepared and illuminated in the presence of 35S-methionine, and an extract was prepared as described in Figure 1. The extract was divided into 45 μl aliquots, and additions were made as follows: A, No addition; B, 0.9 μg pea small subunits; C, 5 mM glucose and 15 units hexokinase; D, 5 mM glucose plus 15 units hexokinase plus 0.9 μg pea small subunits; E, 5 mM ATP; F, 5 mM ATP plus 0.9 μg small subunits; G, 5 mM ATP plus 0.9 μg small subunits, followed 20 min later by 5 mM glucose plus 15 units hexokinase. All the samples were incubated on ice for 30 min and then at 24°C for 60 min. At the end of this period, 70 μl of each sample was analyzed by nondenaturing PAGE, followed by staining with Coomassie blue. Slices of the gel containing stained RuBisCO were dissolved in 1.0 ml 30% (V/V) H2O2 at 55°C overnight, mixed with 10 ml Liquiscint scintillation fluid, and counted in an Intertechnique scintillation spectrometer. Error bars indicate the standard deviation of multiple samples (n = 3).

![Graph of Radioactivity (CPM x 10^-5) vs. Sorbitol Conc. (mM)](image)

**Fig. 3.** Direct effect of various concentrations of sorbitol on in organello total and soluble protein synthesis in pea chloroplasts. Chloroplasts were isolated as described in "Materials and Methods" and resuspended in various concentrations of sorbitol (range 137.4-440 mM), 35 mM HEPES-KOH (pH 8.0), 0.96 mM DTT, 200 μM MgCl2, 50 mM Na2EDTA, 200 μM isoleucine, 200 μM threonine. The Chl concentration was adjusted to 400 μg/ml. The samples (0.5 ml) were illuminated for 24 min at 22°C in the presence of 500 μCi/ml of 35S-methionine. At the end of this period duplicate 10 μl aliquots were removed to Whatman 3MM filter paper discs and the remaining chloroplasts were lysed (Fig. 2). Aliquots (10 μl) of the cleared lysates were removed to Whatman 3MM filter paper discs. All the filter paper discs were exposed to 5% TCA at 100°C for 15 min, washed several times with cold 5% TCA, ethanol, ether, dried, and counted by liquid scintillation. Open circles (top), total chloroplast samples; filled circles (bottom), supernatants of chloroplast lysates. Mean and range of replicate samples of each reaction are shown. (One of five replicate experiments.)

The fact that assembly occurs best after synthesis under hypotonic conditions suggests that under normal circumstances the assembly of RuBisCO may not be occurring with optimal efficiency. It is conceivable, though not proven, that the assembly of RuBisCO may be a limiting factor in the biogenesis of the photosynthetic apparatus.

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LITERATURE CITED


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