HETEROGENEITY OF LARGE SUBUNITS OF RIBULOSE-1,5-BISPHOSPHATE CARBOXYLASE FROM *HYDROGENOMONAS EUTROPHA*

by

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SUMMARY: Homogeneous ribulose-1,5-bisphosphate carboxylase purified from autotrophically grown *Hydrogenomonas eutropha* can be dissociated with sodium dodecylsulfate into small 15,000-dalton subunits and large 56,000- and 52,000-dalton subunits (the latter in a mole ratio of 5:3). The overall mole ratio of small to large subunits is 1.08. Considering the molecular weight of the native enzyme (516,000), the simplest quarternary structure of this enzyme consists of 8 large (mixed) and 8 small subunits. Isolation of the enzyme from cells under conditions that should minimize proteolysis has no effect upon the observed heterogeneity of the large subunits.

We have postulated that the structural gene for the large 55,000-dalton subunits of ribulose-1,5-bisphosphate (RuBP) carboxylase [3-phospho-D-glycerate carboxy-lyase (dimerizing), EC 4.1.1.39], was established prior to that encoding the small 15,000-dalton subunits (1). RuBP carboxylases lacking small subunits have been isolated in a stable dimeric state from *Rhodospirillum rubrum* (2), a stable hexameric state from *Chlorobium limicola* f. *thiosulfatophilum* (3), and a stable octomeric state from *Thiobacillus intermedius* (4) and *Agmenellum quadruplicatum* (5). In contrast, the dominant form of RuBP carboxylase consists of large and small subunits and occurs in eukaryota including higher plants (for reviews see 1 and 6; see also 7) and a few prokaryota (1,6,8,9). We have suggested that the ancient structural gene specifying the 55,000-dalton polypeptide mutated to a descendent gene which specified a gene product of higher aggregation state, *i.e.*, the expression of this gene as reflected in *R. rubrum* was more ancient than that reflected in *C. limicola* (1,6). One simple mechanism by which this may have occurred is through gene duplication followed by mutation (10).

Such a mechanism, however, required the existence of two kinds of products of these genes in organisms in which both structural genes persisted. Indeed, Kung et al. (11) reported heterogeneity of large subunits derived from RuBP carboxylase of Nicotinia tabacum in which chloroplast DNA encodes these large subunits (12). However proteolysis after cell breakage was not excluded by these authors as an explanation of the observed heterogeneity. We now report heterogeneity of large subunits of RuBP carboxylase isolated from the bacterium Hydrogenomonas eutropha under conditions which should minimize proteolysis.

MATERIALS AND METHODS

H. eutropha RuBP carboxylase was purified by centrifugation of the ribosome-free cell-extract in a 0.2-0.8 M discontinuous sucrose gradient (13,14), precipitation of the enzyme from the pooled and dialysed fractions at 35% saturation with respect to $(NH_4)_2SO_4$, and, finally, by centrifugation in another sucrose gradient identical to that just mentioned. The homogeneity of the enzymatically active fractions was analyzed by electrophoresis on gels polymerized from 5% acrylamide. The fractions showing a single Coomassie blue-positive band and corresponding RuBP carboxylase activity were pooled, dialysed, and concentrated with Aquacide. Homogeneity of the pooled fractions was assessed by electrophoresis on gels polymerized from 4-7.5% acrylamide. The concentrated and dialysed enzyme with a specific activity of 1.76 μmoles CO_2 fixed/min/mg protein, was used in all experiments (15). The buffer used was TEMBD (16) and the sucrose solutions for gradients were prepared in the same buffer.

During purification in the presence of proteinase inhibitors (17), dithiothreitol (DTT) was omitted from the buffer and the concentration of Mg^{+2} and EDTA was also changed. In addition to the usual purification, the enzyme was purified in the presence of: (i) 1 mM 1-chloro-3-tosylamido-7-amino-L-2-heptanone·HCl(TLCK), (ii) 1 mM diisopropyl fluorophosphate (DFP), (iii) 1 mM Mg^{+2} and 10 mM EDTA without DTT or (iv) in the absence of DTT. RuBP carboxylase was assayed as described by McFadden et al. (18). Sodium dodecyl sulfate (SDS) was recrystallized from 95% ethanol (19). Electrophoresis was done by the method of Laemmli (20) with gels polymerized from Eastman electrophoresis-grade acrylamide containing SDS (0.1%) and also with gels without SDS.

The native enzyme was dissociated with 1% SDS and 10 mM β-mercaptoethanol in boiling water for 2-3 min. The subunits were separated by gel filtration on a Sephadex G-100 column (particle size: 140-400 mesh), preequilibrated with 1% SDS and 10 mM β-mercapto-ethanol in water. The fractions corresponding to the subunits, as measured by absorbance at 280 nm and also protein determination (21), were pooled and lyophilized. The SDS was extracted two times with 30 ml of chilled 80% acetone and the precipitated protein corresponding to the large and small subunits was solubilized by adding 10 and 3 mg of SDS respectively.

For amino acid analysis, aliquots of SDS-solubilized subunits were hydrolysed in 6N HCl under N_2 for 72 h at 110-113°C in sealed vials. The hydrolysates were analysed with a Beckman 121 Automatic amino acid analyzer. After performic acid oxidation at 0°C for 3 h followed by acid hydrolysis, methionine was determined as methionine sulfone and cysteine and cystine as cysteic acid (22).

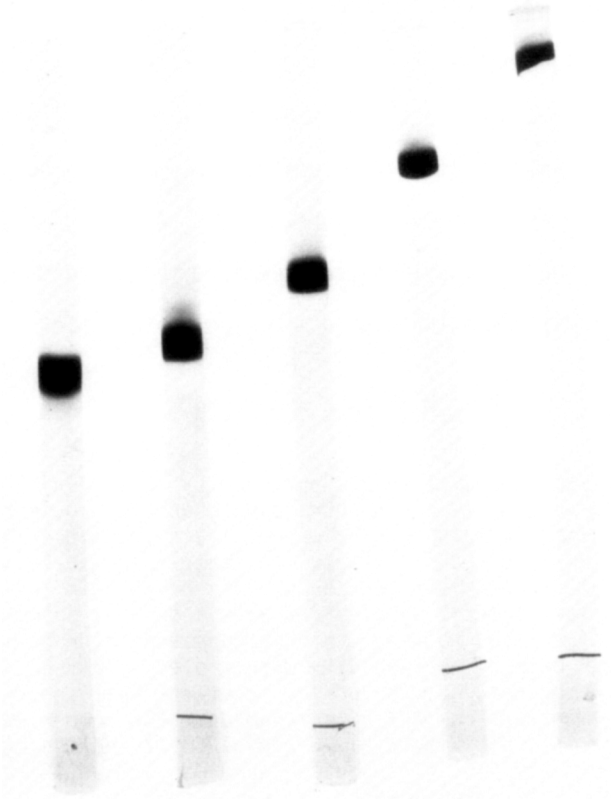

Fig. 1 Polyacrylamide gel electrophoretogram of 9.5 μg H. eutropha RuBP carboxylase. The electrophoresis was done on gels polymerized from (left to right) 4, 5, 5.5, 6.5 and 7.5% acrylamide.

RESULTS

In Figure 1, a single Coomassie blue-positive band is shown after electrophoresis of RuBP carboxylase in each of a series of gels polymerized from 4% to 7.5% acrylamide. This approach greatly raises the probability of detecting contaminating proteins of closely similar charge:mass ratio (23).

In Figure 2, the dissociation of this homogeneous enzyme is depicted after isolation of the enzyme under a variety of conditions. In all cases when a higher protein concentration was examined after dissociation in the presence of β-mercapto-ethanol and SDS and electrophoresis in polyacrylamide containing SDS

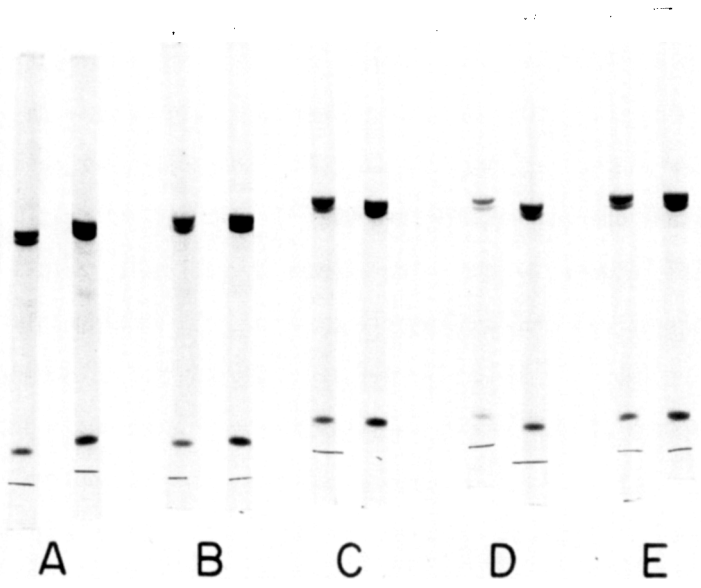

Fig. 2 SDS (0.1%) polyacrylamide (10%) gel electrophoretogram of dissociated H. eutropha RuBP carboxylase purified, (A) in the absence of DTT, or in the presence of: (B) TLCK, (C) DFP, (D) 1 mM Mg^{+2} and 10 mM EDTA, (E) TEMBD buffer (the control). Each gel pair represents two protein concentrations; the gels on the left in each pair were subjected to electrophoresis with 2.5 to 6 μg protein per gel with twice as much protein applied to each gel on the immediate right.

(0.1%), two Coomassie blue-positive bands were obtained. Analogous experiments in which electrophoresis was conducted in gels polymerized from 10% acrylamide instead of from 5% acrylamide resulted also in only two stained bands (not shown). When, however, the concentration of dissociated protein was lowered for electrophoretic analysis, the slower band was resolved into two components of closely similar mobility (Fig. 2, gels to the left in each pair). The same band pattern was observed after dissociation of the enzyme isolated in the absence of DTT or in the presence of TLCK, DFP, or a high concentration of EDTA. Isolation of RuBP carboxylase under these four conditions should greatly restrict proteolysis (17).

The molecular weights of the three polypeptides oberved after dissociation

were 56,000, 52,000 and 15,000, respectively (24). Electrophoresis of the dissociation products and standards employing a running buffer of pH 9.3 instead of 8.3 yielded the same molecular weights. Six densimetric scans of various stained gels typified in Fig. 2 revealed a major peak with a well defined shoulder. By extrapolation to the baseline, the area under each peak could be estimated; this approach established that the average mole ratio of 56,000: 52,000 dalton subunits is 0.6. The average overall mole ratio of small to large subunits ascertained from densimetric scans of gels showing only 2 bands was 1.08. In the face of a molecular weight of 516,000 (25), the most likely quaternary structure of this enzyme consists of eight large and eight small subunits. It is possible that this RuBP carboxylase exists in distinct molecular species which contain varying proportions of the two kinds of large subunits.

TABLE 1. Relative Amino Acid Composition of Mixed Large and of Small Subunits of RuBP Carboxylase from Hydrogenomonas eutropha.

Amino Acid	Large (mixed) subunits[a]	Small subunits
Aspartic	2.61	2.00
Threonine	1.35	1.22
Serine	1.05	0.98
Glutamic	1.92	2.22
Proline	1.02	1.19
Glycine	2.39	1.39
Alanine	2.56	1.36
1/2 Cystine	0.33	1.90
Valine	1.56	0.30
Methionine	0.56	0.45
Isoleucine	0.89	1.05
Leucine	1.98	1.25
Tyrosine	0.69	0.74
Phenylalanine	(1.00)	(1.00)
Lysine	1.12	0.15
Histidine	0.53	0.31
Arginine	1.59	1.48
Tryptophan	-	-

[a]An average of duplicate analyses.

The mole content of amino acids normalized with respect to phenylalanine is given in Table 1 for mixed large and for small subunits.

DISCUSSION

The present data establish that one prokaryotic RuBP carboxylase consists of small subunits and of two kinds of large subunits that differ subtly in molecular weight. The latter were detectable only after electrophoresis of the dissociation products which had been applied at lower concentrations to gels. In contrast, examination of the enzyme from T. intermedius under comparable conditions reveals dissociation into a single 54,500-dalton electrophoretic species (Purohit and McFadden, unpublished observation). In light of this variability of large subunit structure, we urge the reexamination of the quaternary structure of other RuBP carboxylases. This should be done after isolation of gel-electrophoretically homogeneous enzymes under conditions that minimize proteolysis. In this connection, heterogeneity of both large and small subunits of the tobacco enzyme has geen detected by isoelectric focusing (26). If the chloroplast DNA-encoded large subunits are indeed heterogeneous in this enzyme from tobacco, heterogeneity of analogous subunits from prokaryota would be compatible with the endosymbiont theory of chloroplast origin (27). Of special interest in this connection would be a reinvestigation of the quaternary structure of RuBP carboxylases from certain blue-green (28,29) and unicellular green algae (30-32).

The amino acid composition of large (mixed) subunits of RuBP carboxylases from H. eutropha is closely similar to that of large subunits from several sources. In 1971 Marchalonis and Weltman (33), introduced a parameter, SΔQ, which is the sum of squares of individual differences in mole percent content of each amino acid in two proteins. Over 5000 pairs of proteins, many of known primary structure, were compared and for 98% of unrelated proteins SΔQ was >100. Values of less than 50 were taken to indicate relatedness or homology of primary structure. The following SΔQ values reflecting large subunit comparisons have been calculated from our present data: H. eutropha-R. rubrum, 16 (2); H. eutropha-

T. intermedius, 16 (4); H. eutropha-C. ellipsoidea, 17 (30); and H. eutropha-spinach, 18 (30,34). Once again, the data suggest that large subunits, which contain catalytic activity or potential (1), have been conserved in evolution. Moreover, the close similarity between mixed large and homogeneous large subunits from the H. eutropha and T. intermedius enzymes, respectively, predicts that the mixed large subunits will prove to be homologous. The composition, tryptic fingerprints, and immunological relationship of the two large subunits of the hydrogenemonad enzyme will be of considerable interest.

The SΔQ values for small subunits, which are still of unknown function, suggest considerable divergence. For enzymes from the following sources the values are: H. eutropha-Chlorella ellipsoidea, 77 (30); H. eutropha-spinach, 111 (30,34).

In closing, we emphasize that the exact function of the two kinds of large subunits observed in the present research remains to be established.

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