Plant Physiol. (1977) 59, 738-740

Alpha-Ketoglutarate Supply for Amino Acid Synthesis in Higher Plant Chloroplasts

INTRACHLOROPLASTIC LOCALIZATION OF NADP-SPECIFIC ISOCITRATE DEHYDROGENASE[1]

Received for publication October 27, 1976 and in revised form December 14, 1976

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ABSTRACT

Isocitrate dehydrogenase was found in *Pisum sativum* chloroplasts purified on sucrose density gradients. A chloroplast-enriched pellet obtained by differential centrifugation formed two chlorophyll-containing bands. The lower one containing intact chloroplasts had NADP-specific isocitrate dehydrogenase and triose-phosphate isomerase activities. Mitochondria and peroxisomes were observed to band well away from the intact chloroplast region, as indicated by peak activities of fumarase and catalase, respectively. The presence of isocitrate dehydrogenase in chloroplasts suggests that chloroplasts may generate at least some of the α-ketoglutarate required for glutamate synthesis.

At least three possibilities exist for the supply of appropriate keto acid carbon skeletons for the synthesis of key amino acids by chloroplasts. The first involves a partial tricarboxylic acid cycle as is found in some blue-green algae (16). Evidence that the chloroplast was originally derived from free-living procaryotes (2, 12, 19, 22) would suggest that this may be a definite possibility. An alternative possibility involves the synthesis of keto acids in the cytosol or in other organelles, such as the mitochondria or peroxisomes, and the transport of these acids into the chloroplasts (8, 14, 24). Some workers have favored this second hypothesis, but until recently there has been little direct evidence to support it. A third possibility is the chloroplastic synthesis of keto acids by pathways not directly related to the tricarboxylic acid cycle (21). The source of chloroplastic keto acids is reviewed elsewhere (6).

The existence of a chloroplastic isocitrate dehydrogenase was claimed by Leech (10) working on *Vicia faba*. This finding is of considerable interest, since a chloroplastic IDH[2] could mediate the formation of α-ketoglutarate from isocitrate. Leech's finding, however, was not confirmed by other workers (25), who studied spinach chloroplasts purified on isopycnic sucrose gradients. Controversy over the presence of IDH in chloroplasts has probably been due to technical difficulties in chloroplast isolation and purification. It is essential to rule out cross-contamination and to ensure the retention of the chloroplast envelopes to avoid loss of soluble chloroplastic enzymes. There is some doubt about the ability of isopycnic density gradient methods to purify intact chloroplasts free of mitochondrial contamination (20). It was necessary to purify chloroplasts by a different method in order to assess critically the presence or absence of chloroplastic IDH.

In the present study, we adopted the sucrose gradient method developed by Miflin and Beevers (13) and used it to demonstrate localization of NADP-specific isocitrate dehydrogenase in intact pea chloroplasts. The significance of this finding is discussed in relation to amino acid synthesis.

MATERIALS AND METHODS

Chloroplast Isolation. Chloroplasts were isolated from 2-week-old *Pisum sativum* shoots (var. Laxton Superb) using a modification of Walker's technique (23). The grinding medium consisted of 0.33 M D(−) sorbitol, 0.1% BSA, and 50 mM HEPES (pH 6.5). The chloroplast pellet obtained (washed twice with same medium or unwashed) was resuspended in the same medium. The suspension contained a mixture of intact and broken (type C) chloroplasts (7). Five ml of the suspension were layered slowly on to a linear density gradient composed of 30 to 60% (w/w) sucrose solution made up in 50 mM HEPES (pH 7.4). A plateau of 42% sucrose was included in the gradient to increase the separation between the broken and the intact plastids (13). The gradients were spun in a Beckman L2-65B preparative ultracentrifuge at 4,000*g* for the first 5 min and at 10,000*g* for next 10 min. The brief centrifugation time employed in this technique does not give sufficient time for peroxisomes and mitochondria to reach the equilibrium density position in the gradient, but both the broken and intact plastids may approach nearer to equilibrium (13). The gradient was eluted into 24 fractions and each was assayed for the appropriate enzymes. Chlorophyll was determined by the method of Arnon (1) and sucrose concentrations by refractometry.

Enzyme Assays. Assays were run at specific temperatures in a Perkin Elmer 156 spectrophotometer. The spectrophotometer was usually operated in the dual wavelength mode for assays involving measurements of oxidation or reduction of pyridine nucleotides at 340 nm (9). Isocitrate dehydrogenase was determined by the method of Cox (3), triose-phosphate isomerase by the method of Gibbs and Turner (5), fumarase by the method of Racker (18), and catalase by the method of Lück (11).

RESULTS

Figures 1, a and b, and 2, a and b show data from separate centrifugation runs. Triose-phosphate isomerase can be seen to peak together with the lower band of Chl (Fig. 1a), thus confirming this band to be that of intact plastids (13). In gradients using unwashed chloroplasts, triose-phosphate isomerase activity was normally high at the top of the gradient (in the supernatant). To define mitochondrial and peroxisomal bands, unwashed chloroplasts were used on the gradient. Washing the chloroplast pellet twice usually removed considerable amounts of mitochondria and peroxisomes so that peak activities of their marker enzyme have become very low and difficult to detect.

[1] This work was supported by the Malaysian Rubber Research and Development Board.

[2] Abbreviation: IDH: isocitrate dehydrogenase.

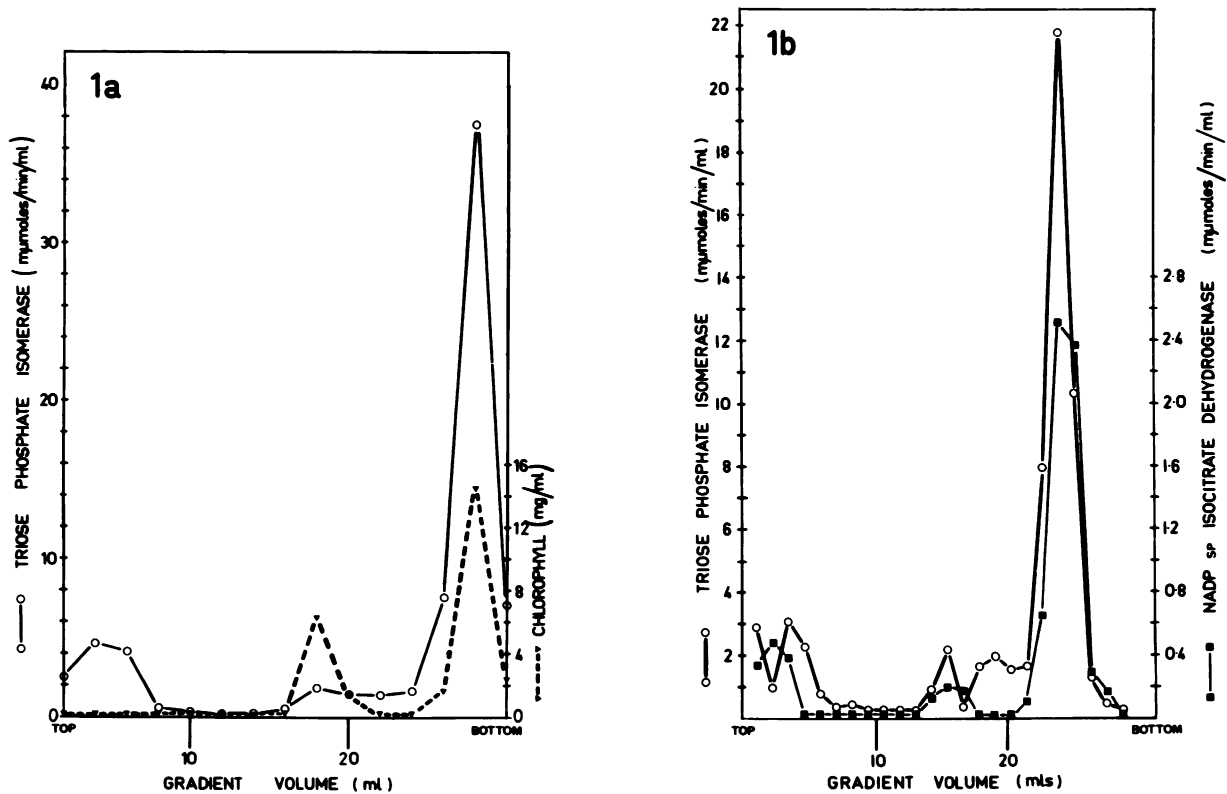

FIG. 1. Density gradient elution profiles of washed chloroplasts. a: Co-banding of triose-phosphate isomerase activity with the lower band of Chl; b: coincidental peak activities of triose-phosphate isomerase and NADP-specific IDH. Note: a and b are from separate centrifugation runs.

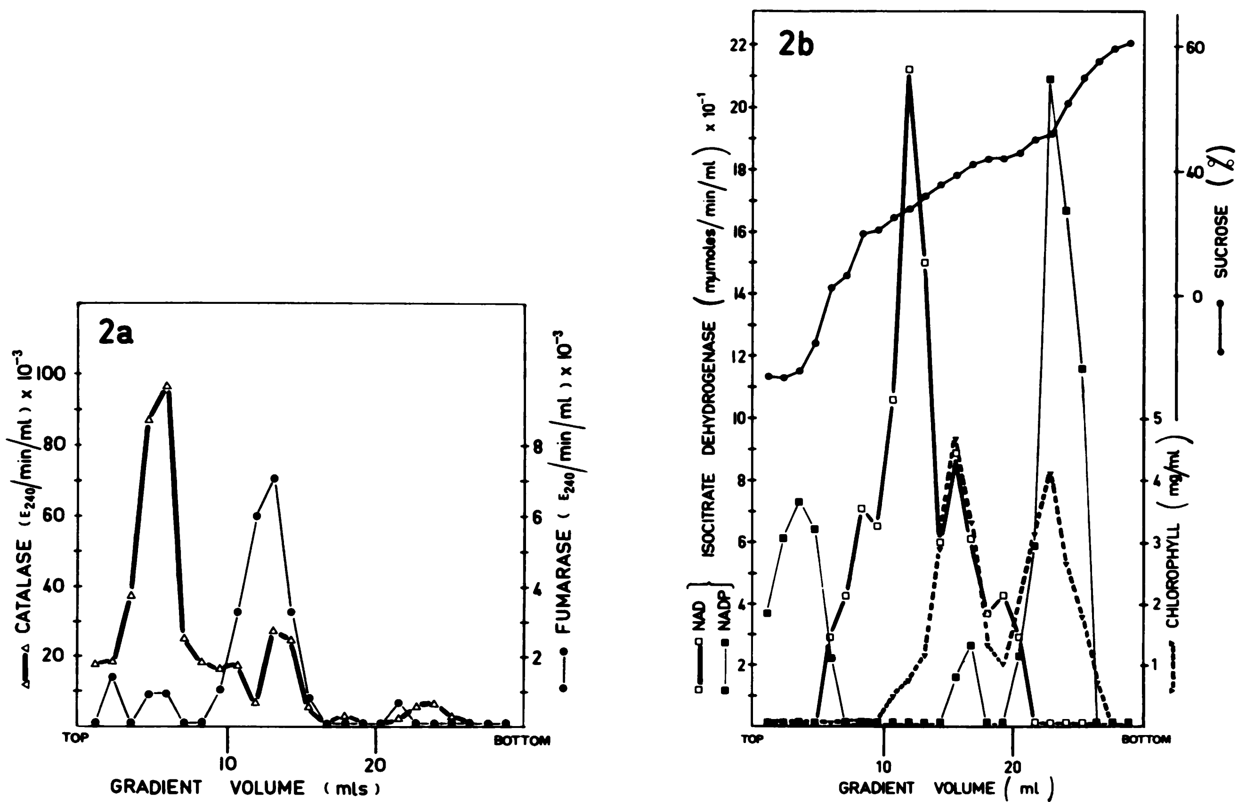

FIG. 2. Density gradient elution profiles of unwashed chloroplasts. a: Positions of mitochondria and peroxisomes on the gradient as marked out by peak activities of fumarase and catalase, respectively; b: peak activities of the NAD-specific IDH in the mitochondrial region and the NADP-specific IDH in the intact chloroplast region. A plot of sucrose concentrations is also shown in b. Note: a and b are from separate centrifugation runs.

Mitochondria and peroxisomes can be seen to band well away from the intact chloroplast region, as indicated by fumarase and catalase activities, respectively (Fig. 2a). Percentage mitochondrial contamination of the intact plastid region as measured by fumarase activity was about 2.8% and that of catalase was about 1.5%. This was in an unwashed chloroplast preparation. We believe that washing the chloroplast pellet would have further diminished the possibility of mitochondrial and peroxisomal contamination in the intact chloroplast region. Figure 1b contains data for washed chloroplasts. NADP-specific IDH peaked coincidentally with triose-phosphate isomerase. As with triose-phosphate isomerase, the amount of the soluble IDH was considerably reduced by washing. Data from the unwashed chloroplast (Fig. 2b) show peak activity of NADP-specific IDH in intact chloroplasts to be about 2.5 μmol/mg Chl·hr.

In addition to the apparent reduction in contamination obtained by washing, the inclusion of BSA in the grinding medium minimizes the possibility of nonspecific binding of soluble enzymes to chloroplastic membranes (4). NAD-specific IDH is mainly localized in the mitochondria (Fig. 2b) as observed by others (*e.g.* 3). Considerable activity of the NAD-specific enzyme is also found in the fractions containing broken chloroplasts; this could be due to some contamination from the mitochondria, since the broken plastids and the mitochondria band close to each other in this procedure. The broken chloroplast band also showed trace activities of triose-phosphate isomerase and NADP-specific IDH, but these were extremely low in comparison to the intact chloroplasts. NADP-specific IDH is therefore probably a soluble enzyme of the chloroplast stroma and is not firmly bound to the lamellae. The localization of the enzyme on the envelope, however, is not ruled out.

DISCUSSION

Since NAD-specific IDH occurs in the mitochondria, the possibility exists that α-ketoglutarate generated in the mitochondria can be transferred into the chloroplast for amino acid synthesis. Thus far there is no evidence on the extent to which such a transfer takes place *in vivo*.

Despite the widespread assumption that α-ketoglutarate required for chloroplastic amino acid synthesis comes from the mitochondria, data on this point are few. It has been shown recently (24) that alanine, succinate, oxaloacetate, glutamine, glycine, glutamate, and α-ketoglutarate permeate 1- to 2-hr greened etiochloroplasts more readily than they permeate envelopes of mature chloroplasts. The NADP-specific IDH present in the chloroplast may play a role in the supply of α-ketoglutarate for glutamate and glutamine synthesis. However, the source of chloroplastic isocitrate is at present unclear.

The presence of a chloroplastic IDH might suggest a partial tricarboxylic acid cycle in the chloroplast comparable to that found in blue-green algae. Thus far, however, we have detected no citrate synthase or aconitase in the chloroplast (although these enzymes are readily detectable in the mitochondria). A complete tricarboxylic acid cycle is almost certainly absent, as several workers have found no fumarase or succinic dehydrogenase (10, 15, 17). On present evidence, it is most probable that the primitive chloroplasts, when first derived from a blue-green algal organism, may have had a partial tricarboxylic acid cycle, but that it is no longer present. The chloroplasts may rely extensively on compounds imported from the rest of the cell to provide the necessary carbon precursors for glutamic acid synthesis.

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