Studies of protein–protein interaction using countercurrent distribution in aqueous two-phase systems

Partition behaviour of six Calvin-cycle enzymes from a crude spinach (*Spinacia oleracea*) chloroplast extract

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The partition behaviour of six enzymes of the Calvin cycle in extracts of chloroplasts from spinach (*Spinacia oleracea*) between two aqueous phases has been studied by countercurrent distribution. The enzymes showed distribution patterns which indicate heterogeneity and the presence of two or three fractions of most of the enzymes. When two of the enzymes, phosphoglycerate kinase and fructose-bisphosphate aldolase, were partitioned in both purified and partially purified form, they behaved like homogeneous substances. These results indicate that countercurrent distribution of crude extracts in aqueous two-phase systems is a useful method to study protein–protein interaction.

INTRODUCTION

The interplay among the easily extractable ('soluble') enzyme activities in the biological cell is the missing link between our present knowledge of the individual enzymes and a better understanding of cellular ultrastructure. Extensive work has shown that these enzyme activities are often located in restricted (membrane-surrounded) sections of the cell. Less is known about the organization within such a compartment, with the exception of the enzymes which are attached to the membrane. Is it possible that another level of organization exists (micro-compartments)? Such conceivable, but not unambiguously demonstrated, spatially organized enzyme systems have long been discussed (see, e.g., [1]). The main difficulty has been in studying the enzymes under conditions that can be regarded as similar to the situation that obtains *in vivo*.

In the present study the surface properties of six enzymes of the Calvin cycle (reductive pentose phosphate pathway), in extracts from spinach (*Spinacia oleracea*) chloroplasts, have been compared by studying their partition between two liquid phases with high water concentrations. The liquid phases are obtained by dissolving the polymers dextran and poly(ethylene glycol) (PEG) in water. The water concentration in these phases is 88–94% [2], which approaches the stromal concentration of around 70% [3]. In order to obtain accurate partition coefficients for the enzymes and to establish their possible heterogeneity, a countercurrent distribution (CCD) process was applied to the two-phase systems.

MATERIALS AND METHODS

All manipulations were carried out at 4 ± 1 °C unless otherwise indicated.

MATERIALS

Dextran ($\bar{M}_r = 2000000$; dextran T 2000), was purchased from Pharmacia (Uppsala, Sweden). Poly(ethylene glycol) (PEG) ($\bar{M}_r = 6500$ – 8000) was obtained from Union Carbide (New York, NY, U.S.A.). $\text{NaH}^{14}\text{CO}_3$ was from The Radiochemical Centre (Amersham, Bucks., U.K.). Biochemicals and auxiliary enzymes were provided by Sigma Chemical Co. (St. Louis, MO, U.S.A.). The water was singly distilled and then passed through an ion-exchanger. All other chemicals were, with a few exceptions, of analytical grade.

Chloroplast preparation

Spinach (*Spinacia oleracea* L., cv. Viking II; Weibulls, Sweden) was grown in a nutrient solution as described by Siegenthaler & Depéry [4], except that the amount of FeSO_4 was doubled. The temperature in the growth chamber was 17 °C in the dark and 23 °C during the 12 h daily light period. The light was provided by fluorescent tubes (Power Groove, 'cool white'; General Electric) and dysprosium bulbs (HQI-E 400W/DH; Osram), which together produced a light intensity of about $400 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$.

The chloroplasts were prepared as described by Jensen & Bassham [5], with some modifications in the following manner: batches of 50 g freshly harvested leaves (5–6 weeks old) were de-ribbed, cut into pieces and homogenized for 2×5 s with 150 ml of 'semifrozen' [6] solution A (50 mM-potassium phosphate/0.33 M-sorbitol/10 mM-NaCl/2 mM-EDTA, 5 mM-L-ascorbic acid/4 mM-L-(+)-cysteine at pH 6.1 and 4 °C; the last two ingredients were added as solids just before use) in a household homogenizer with rotating knives (model no. A520, Kenwood Manufacturing Co., U.K.) equipped with a specially designed lid which restricted its volume to 500 ml. The resulting slurry was filtered through four layers of nylon

Abbreviations used: CCD, countercurrent distribution; PEG, poly(ethylene glycol); DTT, DL-dithiothreitol; RuBPC, ribulose-bisphosphate carboxylase (EC 4.1.1.39); PGK, phosphoglycerate kinase (EC 2.7.2.3); GAPDH, glyceraldehyde-phosphate dehydrogenase (NADP^+) (phosphorylating) (EC 1.2.1.13); TPI, triose-phosphate isomerase (EC 5.3.1.1); FBPase, fructose bisphosphatase or hexose diphosphatase (EC 3.1.3.11); PEP carboxylase, phosphoenolpyruvate carboxylase (EC 4.1.1.31).

net (20 μ m), prewetted with solution A, and centrifuged (Sorvall SS-34) in four tubes at 1320 *g* (3800 rev./min, r_{av} 8.19 cm) for 20 s. After a further 10 s the rotor was stopped by hand braking. The supernatants were discarded and the pellets drained by placing the tubes upside down on a paper towel for 15–30 s. The pellets were suspended in a small volume (1–2 ml per pellet) of solution B (10 mM-potassium phosphate/0.33 M-sorbitol/10 mM-NaCl/2 mM-EDTA at pH 6.7 and 4 °C) by gentle swirling and then diluted to a total volume of 40 ml. This suspension was centrifuged in two new tubes at 206 *g* (1500 rev./min; r_{av} 8.19 cm) for 60 s (without hand braking). The supernatants were discarded and the pellets were drained, suspended in the same way and diluted to a volume of 20 ml with solution B in one new centrifuge tube. After centrifugation as described above the final pellets (one per batch of 50 g of leaves) were suspended in 250–500 μ l of 10 mM-potassium phosphate/0.33 M-sorbitol/10 mM-NaCl/2 mM-EDTA at pH 7.6 and 4 °C, pooled, and stored on ice.

The proportion of intact chloroplasts varied between 60 and 90 % as judged by the ferricyanide test [7], and the cytoplasmic contamination was less than 0.5 % when monitored by the levels of PEP carboxylase [8].

Preparation of chloroplast extracts

Chloroplast extracts were prepared immediately before use by treating chloroplast suspensions in a Potter–Elvehjem glass/Teflon homogenizer (A 15864; Thomas, Philadelphia, PA, U.S.A.) on ice for 5 min (20 strokes by hand), followed by centrifugation (Sorvall SE-12) at 10440 *g* (10000 rev./min, r_{av} 9.33 cm) for 5 min. The resulting clear supernatant had a slight yellowish-green colour and was used without further manipulations.

Preparation of purified and partially purified enzymes

Spinach chloroplast aldolase was purified as described by Persson [9] and spinach chloroplast PGK as described by Persson & Olde [10].

To obtain partially purified enzymes, finely ground PEG was added to the chloroplast extract to a final PEG concentration of 15 % (w/w), in the case of PGK, or 40 % (w/w), in the case of aldolase. The resulting PEG solutions were centrifuged (Sorvall SS-34) at 47900 *g* (20000 rev./min, r_{av} 10.70 cm) for 60–90 min and the supernatants, containing the enzyme activities, were used as the PEG stock solutions for the preparation of the phase system that was added to chamber 0 (see below).

Aqueous two-phase systems

The phase systems contained 5.4 % (w/w) dextran T 2000, 3.6 % (w/w) PEG, potassium phosphate (10 mmol/kg of system), NaHCO₃ (10 mmol/kg), MgCl₂ (3 mmol/kg), DTT (1 mmol/kg) and EDTA (0.1 mmol/kg). They were prepared by mixing 48.6 g of 20.0 % (w/w) dextran T 2000, 16.2 g of 40.0 % (w/w) PEG, 18 ml of 0.1 M-potassium phosphate buffer, pH 8, 3.6 ml of 500 mM-NaHCO₃, 1.1 ml of 500 mM-MgCl₂, 27.8 mg of DTT, 0.2 ml of 100 mM-EDTA. The pH was adjusted to 7.9 at 4 °C with 0.5 M-phosphoric acid under vigorous stirring, and the weight was made up to 180 g with water. The mixtures were shaken and allowed to separate in a funnel. The phases were separated (interface discarded) and stored separately.

CCD with 58 transfers was carried out in a standard

automatic thin-layer CCD apparatus [2,11] equipped with a plate containing 60 chambers numbered 0–59. The capacity of each bottom chamber was 1.05 ml. Chambers 1–59 were each loaded with 0.95 ml of the upper phase and 0.95 ml of the lower phase. Under these conditions the lower phase plus 0.10 ml of the upper phase were stationary. Chamber 0 was loaded with 1.90 ml of the two thoroughly mixed phases containing the sample. The shaking time after each transfer was 20 s, and the time allowed for separation between successive transfers was 5 min. After the 58 transfers, 0.70 ml of 10 mM-potassium phosphate, 66 mM-MgCl₂, 10 mM-NaHCO₃, 1 mM-DTT, 0.1 mM-EDTA at pH 7.9, were added to each chamber and the plate was shaken for 3 $\times$ 5 s to obtain homogeneous solutions. The content of each chamber was collected and analysed for enzyme activity and protein content.

Enzyme assays

Enzyme activities were determined photometrically at 340 nm using a Hitachi 100-60 double-beam spectrophotometer connected to a LKB 2066 potentiometric recorder. The cuvette holder was thermostatically controlled either at 25 $\pm$ 0.2 °C or at 30 $\pm$ 0.2 °C (RuBPC). The assays used have been described for GAPDH [12], except that in the present experiment pH was 8.5 [13] and NADPH was used instead of NADH, for TPI [14], with dihydroxyacetone phosphate as substrate, for aldolase (fructose-bisphosphate aldolase, EC 4.1.2.13) [15], and for FBPase [16].

The PGK activity was measured by the method of Scopes [17], except that bovine serum albumin was omitted and the initial velocity was determined. The RuBPC activity was measured either photometrically, as described by Bränden *et al.* [18], with 0.3 mM-ribulose 1,5-bisphosphate, or by measuring the fixation of NaH¹⁴CO₃, as described by Lorimer *et al.* [19]. Before assay, the enzyme was activated by incubating the chloroplast fractions at 50 °C for 30 min [20]. In order to avoid decreased activity of the other chloroplast enzymes due to heat inactivation, RuBPC was measured as the last enzyme.

Protein determination

Protein was assayed as described by Bradford [21], calibration being obtained with bovine serum albumin.

Calculations

The partition of a substance in a two-phase system may be described by its partition coefficient, *K*, defined as its concentration (at equilibrium) in the upper phase divided by its concentration in the lower phase. When two-phase systems are used in CCD processes, the partition is described by the partition ratio, *G*. This factor is defined as the ratio between the percentages of the substance (at equilibrium) in the mobile part of the system (here the main volume of the upper phase, $V_{u,m}$) and the percentage in the stationary part (here the volume of the lower phase, V_L , and a small volume of the upper phase, $V_{u,s}$). *K* and *G* are thus related via eqn. (1):

$$G = \frac{KV_{u,m}}{KV_{u,s} + V_L} \quad (1)$$

The fraction of material in tube number *i* (*i* = 0–*n*)

Fig. 1. CCD of crude spinach chloroplast extracts

Two-phase systems were composed of 5.4% (w/w) dextran T 2000, 3.6% (w/w) PEG, potassium phosphate (10 mmol/kg), NaHCO_3 (10 mmol/kg), MgCl_2 (3 mmol/kg), DTT (1 mmol/kg) and EDTA (0.1 mmol/kg), pH (4 °C) 7.9. Sample systems containing 0.2–0.3 mg of protein/ml were added to chamber 0. The number of transfers was 58 and the temperature was 4 °C. Each chamber contained 0.95 ml of upper and 0.95 ml of lower phase. ■, Experimental values; ····, theoretical curves; —, the sum of the theoretical curves. (a) RuBPC; (b) PGK; (c) GAPDH; (d) TPI; (e) aldolase; (f) FBPase; and (g) total protein.

after n transfers, $T_{n,i}$, can be calculated from eqn. (2) [22–23]:

$$T_{n,i} = \frac{n!}{i!(n-i)!} \cdot \frac{G^i}{(1+G)^n} \quad (2)$$

Theoretical CCD curves were calculated from eqn. (2) and their sums (if more than one was necessary) were fitted to the experimental CCD patterns by varying the assumed G values.

RESULTS

The partition behaviour of the six enzyme activities, present in the extracts of spinach chloroplasts, between the two aqueous phases of the dextran/PEG/water system, was studied by CCD.

CCD of spinach chloroplast extracts

The typical CCD pattern of the six measured enzyme activities of the Calvin cycle (RuBPC, PGK, GAPDH, TPI, aldolase and FBPase), as well as of total protein after CCD of crude chloroplast extract (mainly stroma material) are shown in Fig. 1. Three experiments, performed on different batches of extract, yielded similar patterns for all the seven measured variables. The theoretical curves for homogeneous components were calculated according to eqn. (2) and adjusted so that their

Table 1. Partition ratios (G) and relative amount of material in the estimated subfractions of the CCD experiments on crude chloroplast extracts (exemplified in Fig. 1)

Results are from three experiments performed on different batches of extract. Values represents means $\pm$ S.D. ($n = 2$ or 3).

Enzyme activity or total protein	G value of subfraction	Relative amount of material (%)
RuBPC	0.50 ± 0.05	32 ± 13
	0.88 ± 0.08	68 ± 13
PGK	0.31 ± 0.05	18 ± 3
	0.53 ± 0.07	54 ± 9
	0.92 ± 0.10	28 ± 9
GAPDH	0.33 ± 0.04	27 ± 17
	0.60 ± 0.07	73 ± 17
TPI	0.49 ± 0.05	55 ± 0
	0.84 ± 0.13	45 ± 0
Aldolase	0.65 ± 0.06	45 ± 4
	1.05 ± 0.15	55 ± 4
FBPase	0.60 ± 0	57 ± 2
	1.00 ± 0.08	44 ± 2
Total protein	0.55 ± 0	43 ± 8
	0.88 ± 0.04	58 ± 8

Fig. 2. CCD of purified and partially purified PGK from spinach chloroplasts

The two-phase systems were composed and CCDs were performed as given for Fig. 1. ■, Experimental values; ···, theoretical curves; —, sum of the theoretical curves. When the data can be fitted to a single theoretical curve, a continuous line only has been used. (a) Partially purified PGK; 1.4 mg of protein/ml in chamber 0. (b) Purified PGK; 0.2 mg of protein/ml in chamber 0. - - -, CCD pattern of PGK in the crude extract (from Fig. 1b).

Fig. 3. CCD of purified and partially purified aldolase from spinach chloroplasts

The two-phase systems were composed and the CCDs were performed as given for Fig. 1. ■, Experimental values; —, theoretical curves. (a) Partially purified aldolase; 0.3 mg of protein/ml in chamber 0. (b) Purified aldolase; 0.3 mg of protein/ml in chamber 0. ---, CCD pattern of aldolase in crude extract (from Fig. 1e).

sums fit the distribution profiles of the measured activities. Table 1 summarizes the G values and the relative amounts of material for the theoretical curves from all three experiments. The distribution of total protein could be fitted by two theoretical curves with G values 0.55 and 0.88. The distribution of the six enzyme activities demanded at least two, and, in the case of PGK, three, theoretical curves in order to attain an acceptable fit (Figs. 1a–1f). In these cases the G values ranged from 0.31 to 1.05. For each activity one theoretical curve was in the interval 0.49–0.65 and another, with the exception of GAPDH, in the interval 0.84–1.05.

CCD of purified and partially purified enzymes

Fig. 2(a) shows the CCD pattern of a partially purified PGK preparation containing 100% of the total activity and 48% of the total protein (in the chloroplast extract). In contrast with the case when the crude chloroplast extract was used (Fig. 1b), only two theoretical curves are necessary in order to attain an unacceptable fit. One dominating component (82% of the recovered enzyme activity) with a G value of 0.53 and a minor one with a G value of 0.90 were found. When a purified PGK preparation was used (Fig. 2b) a single homogeneous peak with a G value of 0.53 was attained. For comparison purposes, the CCD pattern of PGK from the crude extract is included in Fig. 2b (from Fig. 1b).

Fig. 3(a) shows the CCD pattern of a partially purified aldolase preparation containing 94% of the total activity and 22% of the total protein (in the chloroplast extract),

Table 2. Partition ratios (G) of the CCD curves in Figs. 2 and 3

Enzyme	G value of subfractions	Relative amount of material (%)
Partially purified PGK	0.53	82
	0.90	18
Purified PGK	0.53	100
Partially purified aldolase	0.67	100
Purified aldolase	0.67	100

whereas Fig. 3(b) shows the pattern when a purified aldolase preparation was used. Both preparations yielded a single homogeneous peak with a G value of 0.67. To allow for comparison, the CCD pattern of aldolase from the crude extract is included in Fig. 3(b) (from Fig. 1e).

Table 2 summarizes the G values and the relative amounts of material for the theoretical curves in Figs. 2 and 3.

DISCUSSION

Single proteins partition between the aqueous phases of a dextran/PEG system with partition coefficients in the range of 0.05–1.5 when the polymer content is close to the one used in the present work [24]. The partition of a protein depends on several factors, such as net charge

and the presence of exposed hydrophobic groups, as well as other solvation-determining groups [25]. When these two-phase systems are used in a CCD process, a single, homogeneous protein will move like a band in column chromatography and, when the distribution is completed, the concentration of the protein along the row of CCD tubes will show a distribution peak (as in Fig. 3). A mixture of several proteins, not interacting with each other and differing in their partition coefficients, will give rise to more- or less-separated peaks. With the above range of partition coefficients the expected positions of the peaks (maximal concentration of protein) after the distribution should be within the range of tube numbers 3–39.

The six measured Calvin-cycle activities in crude spinach chloroplast extracts all showed heterogeneous activity peaks after CCD (Fig. 1), whereas pure PGK and aldolase yielded single, homogeneous, peaks (Figs. 2*b* and 3*b*). This heterogeneity points to the presence of at least two fractions of each enzyme and indicates that the enzymes in each fraction are closely related in partition behaviour. A possible explanation for this distribution is that one of the fractions consists of a protein complex or conglomerate exhibiting different partition behaviour as compared with non-associated enzymes. One of the two intervals, mentioned above, should then represent such a complex.

The heterogeneity is not due to the presence of multiple molecular forms of the enzymes [26–27]. The chloroplast extracts contained less than 0.5% of cytoplasmic contamination, as judged from the levels of PEP carboxylase activity, which excludes the presence of isoenzymes from the cytoplasm. Furthermore, the results from CCD of partially or extensively purified aldolase, as well as of purified PGK (Figs. 3*a*, 3*b* and 2*b*), do not indicate the presence of more than one molecular form of the measured activities in the chloroplast. CCD of the partially purified PGK yielded, however, two peaks (82:18; Fig. 2*a* and Table 2), but the extract yielded three peaks and showed a more even distribution of the activity (14:53:33; Figs. 1*b* and Table 1). In the case of partially purified aldolase, containing 94% of the total activity (but only 22% of the total protein) present in the chloroplast extract, the CCD yielded one homogeneous peak (Fig. 3*a*), whereas the stroma extract yielded two peaks containing 50% of the total activity each (Fig. 1*e* and Table 1). These results clearly show that interactions between the enzymes studied and other components of the stroma take place. In the partially purified aldolase preparation these associating components have been removed, which results in a homogeneous CCD pattern of the aldolase activity. In the partially purified PGK preparation, which contain a larger fraction of the total protein in the extract (48 as against 22%), the remaining minor heterogeneity in the CCD pattern of the PGK activity is caused by some of these components that are still present.

The homogeneity of a number of chloroplast enzymes in higher plants has been reported by several other workers [28–34]. DEAE-cellulose chromatography of crude supernatant (40000 *g*, 30 min) from spinach-leaf extract showed only one peak of RuBPC activity [28]. Two-dimensional gel electrophoresis of extracts from normal and white plastid mutant leaves and chloroplasts from barley (*Hordeum vulgare*) resulted in the identification of a single isoenzymic form of chloroplast PGK

[29–30]. Non-denaturing gel electrophoresis of extract from spinach chloroplasts revealed one enzymically active form of GAPDH [31]. DEAE-Sepharose chromatography of chloroplast extract from rye (*Secale cereale*) showed one activity peak of TPI [32]. DEAE-cellulose chromatography of spinach chloroplast extract showed one activity peak of aldolase [33], and the same result was obtained for FBPase when DEAE-Sephadex [34] was used, although the activity peak was severely distorted, and multiple forms of this enzyme in chloroplasts cannot therefore be ruled out.

The observed heterogeneity is not caused by entrapment of the enzymes by membrane vesicles or their adhesion to membrane fragments. The presence of a membrane form of RuBPC in spinach chloroplasts is well established [35–40], and activities associated with membranes have also been reported for PGK, GAPDH and aldolase from pea (*Pisum sativum*) chloroplasts [41–43] and for FBPase from soybean (*Glycine max*) chloroplasts [44]. However, the chloroplast extracts used in our experiments (Fig. 1) contained only minor amounts of membrane fragments, since they appeared transparent, with only a slight yellowish-green colour. Furthermore, enzyme-membrane aggregates would, as do particulate materials in general, exhibit an extreme partition behaviour in the phase system described here and would thus result in activity peaks at the beginning or the end of the CCD profiles.

The addition of polymers, in concentrations less than those causing non-specific precipitation, has been shown to give rise to specific interaction between metabolically related enzymes [45]. This may explain why CCD in aqueous two-phase systems has been a successful method of detecting weak enzyme-enzyme interactions. As far as interaction studies are concerned, this method has only been applied to purified proteins [46–49]. This approach has the obvious advantage of a well-defined experimental system. However, factors essential for the interaction may be missing and the purification procedure may imply such alterations in the protein structure that recognition sites are altered and interaction is reduced. CCD of crude extracts is an interesting alternative, since it at least partly overcomes these disadvantages. Thus, a combination of CCD on crude extracts and on purified enzymes may prove to be a powerful tool in studies of weak protein-protein interactions.

Several multienzyme complexes (enzyme clusters) catalysing two or more sequential steps in a metabolic pathway, e.g. α -oxo-acid dehydrogenase complexes, type I fatty acid synthetases and the *arom* complex, are well known and well characterized [50–53]. Owing to several major advantages of multienzyme complexes for the metabolic flux in the cell [52,54], the existence of complexes involving weakly bound enzymes has been considered, and numerous reports of interaction between many 'soluble' sequential enzymes have been published (for recent reviews, see, e.g. [55–56]). In the case of the Calvin cycle, the carbon-reducing pathway of photosynthesis, a possible complex between RuBPC and a CO₂-generating enzyme has been suggested [57–59]. In recent years several papers have been published supporting the existence of an interaction between enzymes belonging to the Calvin cycle. These enzyme combinations include GAPDH and phosphoribulokinase in the green alga *Scenedesmus obliquus* [60–61] and RuBPC, ribose-phosphate isomerase and phosphoribulokinase in

pea chloroplasts [62]. Data supporting channelling of ribulose 5-phosphate from ribulose-phosphate isomerase to phosphoribulokinase have also been presented [63]. During the preparation of the present paper, a description of the isolation of a functional multienzyme complex from spinach chloroplasts containing RuBPC, PGK, GAPDH, ribose-phosphate isomerase and phosphoribulokinase was published by Gontero *et al.* [64] and supports our findings.

Weak enzyme complexes existing in the living cell may well dissociate during the breakage of cells or cell organelles and dilution of their content. These possible complexes represent a challenging problem concerning extraction techniques and detection of weak protein-protein interactions. The present work indicates that CCD of crude extracts in aqueous two-phase systems is a useful method for detecting and isolating of such fragile enzyme superstructures.

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