

Purification of the Chloroplast Pyruvate Dehydrogenase Complex from Spinach and Maize Mesophyll

Hans-Jürgen Treede and Klaus-Peter Heise

Institut für Biochemie der Pflanze der Universität Göttingen, Untere Karspüle 2,
D-3400 Göttingen, Bundesrepublik Deutschland

Z. Naturforsch. **41c**, 1011–1017 (1986); received June 30, 1986

Pyruvate Dehydrogenase Complex, Chloroplasts

This paper deals with the partial purification of the pyruvate dehydrogenase complex (PDC) from chloroplasts of spinach and maize mesophyll which hitherto has been isolated only from pea chloroplasts. Starting with membrane free suspensions of lyophilized chloroplasts, and following a high-speed ($140\,000 \times g$) centrifugation of this "stromal extract", the initial specific PDC-activities were concentrated by a factor 10 in the sediment. While most of the purification procedures described earlier resulted in almost complete loss of enzyme activities, a rate zonal sedimentation on linear glycerol gradients allowed for an additional up to 100-fold enrichment of the labile multienzyme complex, albeit with low yields. In contrast to chloroplast PDC from maize mesophyll, inactivation of the spinach complex during glycerol fractionation was due to the dissociation of its loosely bound dihydrolipoyl dehydrogenase component which collected in a lower density fraction of the gradient. Its recombination with PDC constituents of the bottom layer nearly restored initial activities.

The chloroplast complex has been identified as true PDC by its substrate specificity for pyruvate, NAD^+ , and coenzyme A and the 1:1:1 stoichiometry of its reaction products NADH, CO_2 , and acetyl-CoA.

The chloroplast PDC of both plant species showed the well known higher pH- and Mg-requirements than the mitochondrial complex. The observed species-specific differences in the stability of this multienzyme system suggest a connection with the aggregation state of its components. Apparently, the individual subcomplexes are able to function either together in acetyl-CoA formation or independently from each other, e.g. in the synthesis of acetolactate *via* hydroxyethyl-thiamine pyrophosphate or dihydrolipoyl dehydrogenase activities.

Introduction

In photosynthetically active chloroplasts from spinach, peas and maize mesophyll two acetyl-CoA synthesizing enzyme systems have been demonstrated [1–6]. The acetyl-CoA synthetase catalyses the reaction $\text{acetate} + \text{CoASH} + \text{ATP} \xrightarrow{\text{ACS}} \text{acetyl-CoA} + \text{AMP} + \text{pyrophosphate}$ while the pyruvate dehydrogenase complex is involved in the overall reaction $\text{pyruvate} + \text{NAD}^+ + \text{CoASH} \xrightarrow{\text{PDC}} \text{acetyl-CoA} + \text{CO}_2 + \text{NADH} + \text{H}^+$. Both pathways appear to be controlled by the stromal pools of acetate and pyruvate which in turn seem to depend on the cellular concentrations of these substrates [5, 6] and on the mechanism of their uptake [7, 8]. As

further regulation mechanisms, the feedback control of the PDC by their end products (acetyl-CoA and NADH) and differences in the stromal cofactor levels which, like MgATP, influence both enzyme systems either in a stimulatory or inhibitory manner, have been discussed [5]. That the PDC is composed of the three enzymes pyruvate decarboxylase, dihydrolipoyl transacetylase and dihydrolipoyl dehydrogenase has been supported after its purification from pea chloroplasts [4]. In order to generalize this notion, appropriate enrichment studies have been extended to the PDC from spinach and maize mesophyll chloroplasts.

Materials and Methods

The cultivation of spinach [9] and maize [8] has been described earlier.

Chloroplasts were prepared from spinach as described in [9] and from maize mesophyll as described in [10]. Starting with 600–800 g leaf material of each species 150 g batches were homogenized in 250 ml of the corresponding extraction medium. In order to

Abbreviations: ACS, acetyl-CoA synthetase; CoA, coenzyme A; DTT, dithiothreitol; PDC, pyruvate dehydrogenase complex; PEG, polyethylene glycol; RuBPCase, ribulose 1,5-bisphosphate carboxylase; TPP, thiamine pyrophosphate.

Reprint requests to Dr. K.-P. Heise.

Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0341–0382/86/1100–1011 \$ 01.30/0



Dieses Werk wurde im Jahr 2013 vom Verlag Zeitschrift für Naturforschung in Zusammenarbeit mit der Max-Planck-Gesellschaft zur Förderung der Wissenschaften e.V. digitalisiert und unter folgender Lizenz veröffentlicht: Creative Commons Namensnennung-Keine Bearbeitung 3.0 Deutschland Lizenz.

Zum 01.01.2015 ist eine Anpassung der Lizenzbedingungen (Entfall der Creative Commons Lizenzbedingung „Keine Bearbeitung“) beabsichtigt, um eine Nachnutzung auch im Rahmen zukünftiger wissenschaftlicher Nutzungsformen zu ermöglichen.

This work has been digitalized and published in 2013 by Verlag Zeitschrift für Naturforschung in cooperation with the Max Planck Society for the Advancement of Science under a Creative Commons Attribution-NoDerivs 3.0 Germany License.

On 01.01.2015 it is planned to change the License Conditions (the removal of the Creative Commons License condition "no derivative works"). This is to allow reuse in the area of future scientific usage.

eliminate mitochondrial contaminations, the chloroplast suspensions of both spinach [11] and maize mesophyll [10] were finally purified on Percoll gradients, pelleted and washed twice in grinding buffer. The final pellet was resuspended in 50 mM Glycylglycine (pH 8.0), 2 mM MgCl_2 and 2 mM DTT (buffer A), frozen in liquid N_2 , lyophilized and stored at -20°C until further processing.

The isolation of mitochondria was slightly modified according to Ebbighausen *et al.* [12] and storage was at -84°C until used.

Preparation of chloroplast PDC

Lyophilized chloroplasts from both species were resuspended in 20 ml ice-cold buffer medium containing 50 mM Glycylglycine (pH 8.0), 5 mM MgCl_2 , 0.2 mM TPP, 2 mM DTT and 1 mM NAD^+ (final protein concentration: $20\text{ mg}\cdot\text{ml}^{-1}$). The resulting suspension ("chloroplast extract") was stirred for 20 min at 4°C , followed by centrifugation at $25000\times g$ for 15 min to remove membranes. The light greenish to yellowish supernatant ("stromal extract" in Table II) was then centrifuged at $140000\times g$ for 2.5 h to give a gelatinous pellet of the same colour and enriched in PDC activities ("ultracentrifugation 1" in Table II). At this stage of purification the PDC was resuspended in a minimum volume of buffer A, stored at -20°C and was then stable for 1–2 weeks, but very sensitive to freezing and thawing.

Glycerol gradients

0.3 ml aliquots from the first ultracentrifugation step were layered onto 13 ml linear 10 to 50% (v/v) glycerol gradients containing 50 mM Glycylglycine (pH 8.0), 2 mM MgCl_2 , 1 mM NAD^+ , 0.1 mM TPP, 2 mM DTT and 1 mM pyruvate. Gradients were centrifuged for 12 h at 25000 rpm in a Kontron TGA-65 ultracentrifuge using a SW28 rotor. 0.5 ml fractions of the gradients were collected for enzyme- and protein assays. The PDC-containing fractions were generally concentrated by a second ultracentrifugation. Glycerol concentrations were monitored by refractometry.

Assay procedures

1. Chloroplast PDC

a) Pyruvate-driven, CoA-dependent NAD-reduction was measured with the modified method of Ran-

dall *et al.* [13] using a double beam spectrophotometer (Sigma ZFP 22). The standard assay contained: 50 mM Tricine/KOH (pH 8.0), 0.2 mM TPP, 5 mM MgCl_2 , 1.5 mM NAD^+ , 0.5 mM CoA, 2 mM DTT and 1.2 mM pyruvate. For reconstitution experiments 15 μg of dihydrolipoyl dehydrogenase from spinach (specific activity: $37\text{ nmol}\cdot\text{mg}^{-1}\text{protein}\cdot\text{min}^{-1}$) was added to the standard assay.

b) Pyruvate decarboxylation was assayed radiochemically with $[1\text{-}^{14}\text{C}]$ pyruvate ($2\text{ }\mu\text{Ci}\cdot\mu\text{mol}^{-1}$) according to Roughan *et al.* [14] using the same reaction mixture as for the spectrophotometric test.

c) Acetyl-CoA production was determined after trapping with dithioerythritol as described by Liedvogel [3]. The assay mixture was the same as in the foregoing tests, but the reaction was started by adding $[2\text{-}^{14}\text{C}]$ pyruvate ($2\text{ }\mu\text{Ci}\cdot\mu\text{mol}^{-1}$).

d) NAD-reduction by dihydrolipoyl dehydrogenase (EC 1.6.4.3) was measured spectrophotometrically according to Reid *et al.* [15].

2. Mitochondrial PDC was measured in reaction mixtures containing 0.1% Triton X-100, 50 mM Tricine/KOH (pH 8.0), 0.2 mM TPP, 1 mM MgCl_2 , 1.5 mM NAD^+ , 0.5 mM CoA, 2 mM DTT and 1 mM pyruvate (modified after [13]).

All determinations were performed at 20°C and started with CoASH [5]. Protein was assayed by the method of Bradford [16] using BSA as the standard. NAD^+ -isocitrate dehydrogenase [17], citrate synthetase [18] and hydroxypyruvate reductase [19] were measured by standard procedures.

Results

The elimination of mitochondrial and peroxisomal contaminants in the chloroplast preparations used was assessed by measuring NAD^+ -dependent isocitrate dehydrogenase (EC 1.1.1.41) and citrate synthetase (EC 4.1.3.7) activities as mitochondrial or hydroxypyruvate reductase (EC 1.1.1.29) as peroxisomal marker enzymes (Table I). Only few of the procedures described for PDC-purifications from other sources [4, 20] were simultaneously suitable for that from spinach and maize mesophyll chloroplasts. This selection of methods is summarized in a typical purification scheme in Table II. The purification was started with resuspended lyophilized chloroplasts ("chloroplast extracts") containing specific PDC-activities of $1\text{ nmol}\cdot\text{mg}^{-1}\text{protein}\cdot\text{min}^{-1}$ for both preparations from spinach and maize mesophyll chloro-

plasts. This amount is only 13% of the initial activities recently used for PDC-enrichments from pea chloroplasts [4]. The $25000 \times g$ supernatants ("stromal extracts") of these suspensions showing only slightly higher PDC-activities (by a factor 1.4) were then pelleted by ultracentrifugation ($140000 \times g$) with a 10 to 12-fold purification but with lower recoveries of the enzyme from maize mesophyll chloroplasts (18%) than that of spinach (86%). Application of ultracentrifugation followed by rate zonal sedimentation on linear glycerol gradients proved as the most stabilizing method for further enrichment of the labile multienzyme complex, although low yields had to be tolerated (Table II). Thus, the remarkable loss of PDC-activities from spinach chloroplasts during fractionation on glycerol was due to a dissociation of the loosely

bound dihydrolipoyl dehydrogenase subcomplex collecting separately in a lower density fraction of the gradient (Fig. 1, peak A). Reconstitution of this fraction with the PDC-containing bottom layer (Fig. 1, peak B), however, restored 56% of the initial PDC-activities (bracket values in Table II). In order to reach the initial activity of the enzyme complex before dissociation (see Table II, ultracentrifugation 1) the addition of $15 \mu\text{g}$ dihydrolipoyl dehydrogenase ($37 \text{ nmol} \cdot \text{mg}^{-1} \cdot \text{protein} \cdot \text{min}^{-1}$) to the standard assay (see Materials and Methods, 1a) was required. Subsequent high-speed sedimentation ($140000 \times g$) of the recombined fractions finally resulted in a 113-fold enrichment of the specific PDC-activities with 49% recovery which resembles that recently found for the pea chloroplast complex [4]. Although showing a similarly high loss in recovery on

Table I. Marker enzyme activities ($\mu\text{mol} \cdot \text{mg}^{-1} \cdot \text{protein} \cdot \text{h}^{-1}$) in Percoll purified chloroplast preparations from maize (mesophyll) and spinach, and in mitochondrial fractions from etiolated peas.

Enzyme	Chloroplast extract ^a from maize	Chloroplast extract ^a from spinach	Mitochondrial extract ^b from etiolated peas
Isocitrate dehydrogenase (NAD-dependent)	0	0	1.1
Citrate synthetase	0.04	0.02	8.0
Hydroxypyruvate reductase	0.04	0.09	0.7

^a Chloroplasts purified with Percoll were resuspended in 100 mM Glycylglycine, 2 mM MgCl_2 , and 2 mM DTT and centrifuged at $25000 \times g$ for 20 min. The supernatant was used as chloroplast extract.

^b Mitochondria were incubated in 0.1% Triton X-100 and then centrifuged at $25000 \times g$ for 20 min. The resulting supernatant was taken as mitochondrial extract.

Table II. Purification scheme of the chloroplast pyruvate dehydrogenase complex from spinach (A) and maize mesophyll (B).

	Total activity [m units] ^a		Total protein [mg]		Specific activity [m units $\cdot \text{mg}^{-1} \cdot \text{protein}$]		Purification [-fold]		Recovery [%]	
	A	B	A	B	A	B	A	B	A	B
Chloroplast extract	180	140	185	145	1	1	1	1	100	100
Stromal extract	160	65	117	45	1.4	1.4	1.4	1.4	89	46
Ultracentrifugation 1	155	24.7	14.3	2	10.8	12.4	11.1	12.4	86	17.6
Glycerol gradient	(101) ^b	3.2	(1.2) ^b	0.03	(84.2) ^b	107.0	(86.8) ^b	107.0	(56) ^b	2.3
Ultracentrifugation 2	(88) ^b	n.d.	(0.8) ^b	n.d.	(110.0) ^b	n.d.	(113.4) ^b	n.d.	(48.9) ^b	n.d.

^a One unit of activity is defined as CoASH-dependent formation of 1 μmol NADH per min.

^b The values in brackets reflect the conditions after reconstitution of residual PDC activities (Fig. 1, peak B) with the dissociated dihydrolipoyl dehydrogenase activities (Fig. 1, peak A).

n.d. = not determined.

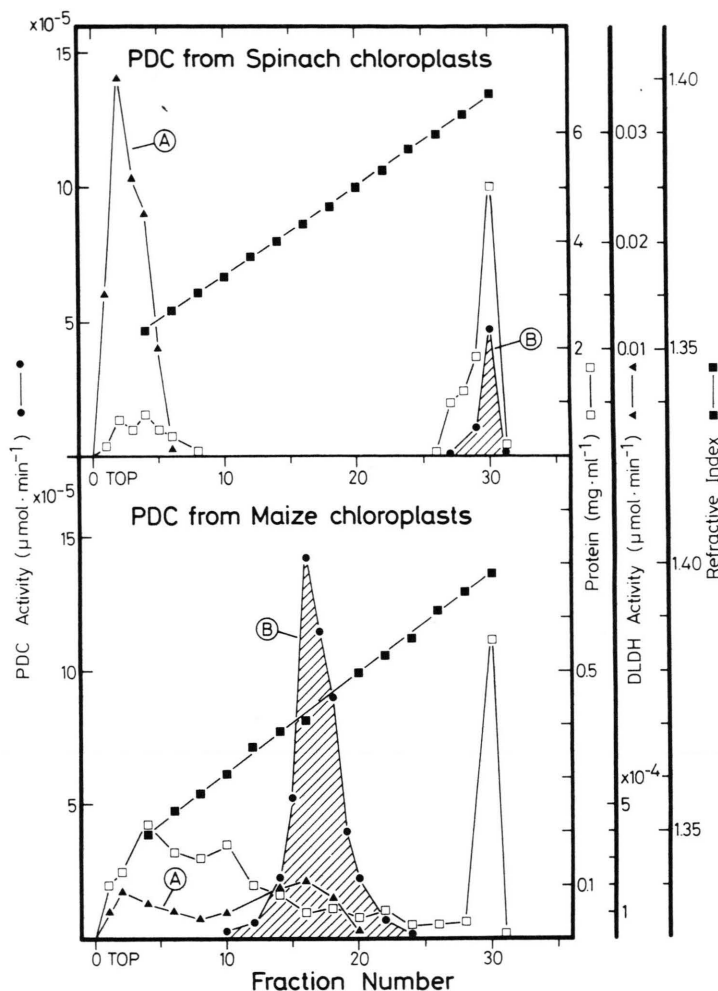


Fig. 1. Distribution of chloroplast PDC from spinach and maize (mesophyll) after rate-zonal sedimentation of high-speed stroma pellets on linear (10–50%) glycerol gradients. Dissociation of the spinach chloroplast complex during this procedure is indicated by a separation of the component dihydrolipoyl dehydrogenase (peak A) from the residual complex activities (peak B). Reconstitution of both activities (from peak A and B) nearly restored initial PDC activities.

glycerol gradients, the small PDC-activities of maize mesophyll chloroplasts banded as total complex in a fraction of intermediate density with a 107-fold purification, but only 2.3% recovery (Fig. 1, Table II). The above observed lability of the chloroplast PDC points to a remarkable sensibility against shearing forces. This is especially expressed by the strong reduction in recovery after rate zonal sedimentation on glycerol (Table II) and is thus similar to gel permeation on Sepharose 2B and affinity chromatography methods on ethyl-agarose (data not shown).

Substrate specificity and cofactor requirements

The chloroplast PDC from both spinach and maize mesophyll chloroplasts showed an absolute require-

ment for pyruvate, CoASH and NAD^+ (Table III). Both partially purified complexes (after first ultracentrifugation) utilized hydroxypyruvate at 18% (spinach) and 10% (maize mesophyll) of the rate of pyruvate but showed no activity with glyoxylate or 2-ketoglutarate. In the absence of TPP only 64% (spinach) and 60% (maize mesophyll) of the control activity has been observed. Substitution of NAD^+ by NADP^+ resulted in only 7% (spinach) or 6% (maize mesophyll) of the rate of NAD^+ .

It has been recently shown, that the chloroplast PDC from peas [4] and spinach [5] can be distinguished from the plant mitochondrial complex by a higher magnesium requirement and an optimal reaction rate at a more alkaline pH. In accordance with these findings the optimal Mg^{2+} -concentrations of

Table III. Substrate and cofactor requirements of chloroplast PDC from spinach and maize mesophyll. For spectrophotometric determinations the partially enriched enzyme of the pellet after the first ultracentrifugation (Table I, ultracentrifugation 1) was taken. The assay procedure was as in Materials and Methods.

Assay mixture	Relative activity [%]	
	spinach	maize
Complete	100 ^a	100 ^b
– TPP	64	60
– CoA	0	0
– NAD	0	0
+ 1.6 mM NADP	7	6
– Pyruvate	0	0
+ 1.5 mM hydroxypyruvate	18	10
+ 1.5 mM glyoxylate	0	0
+ 1.5 mM 2-ketoglutarate	0	0

PDC-activities determined with the complete assay:

^a spinach chloroplasts : 197 nmol · mg⁻¹protein · h⁻¹.

^b maize mesophyll chloroplasts: 864 nmol · mg⁻¹protein · h⁻¹.

the chloroplast complex from spinach (app. $K_m = 0.6$ mM) and maize mesophyll (app. $K_m = 1$ mM) were 10-fold larger than that required for the mitochondrial complex from peas (app. $K_m = 65$ μ M) (Fig. 2).

As further documented in Fig. 3 for the mitochondrial PDC from peas, a pH-optimum lower than 7.5 has been measured, while that of the chloroplast

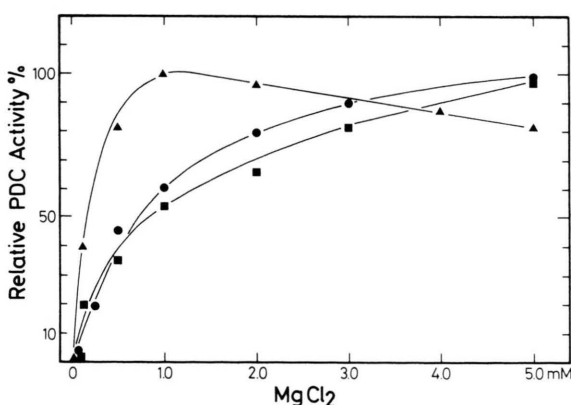


Fig. 2. Comparison of the Mg-dependence of chloroplast PDCs from spinach (—●—●—) and maize mesophyll (—■—■—) with that of mitochondrial PDC from peas (—▲—▲—). For spectrophotometric determinations, the high-speed pellets of either chloroplast or mitochondrial extracts were suspended in the standard assay media with varying Mg-concentrations.

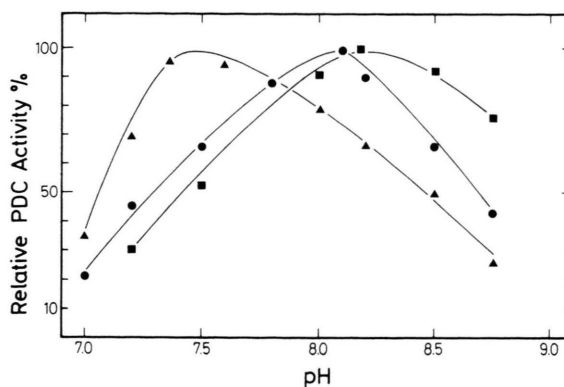


Fig. 3. Difference between the pH-dependence of chloroplast PDCs from spinach (—●—●—) and maize mesophyll (—■—■—) on one hand and of mitochondrial PDC from etiolated peas (—▲—▲—) on the other. For spectrophotometric determinations the high-speed pellets of either chloroplast or mitochondrial extracts were suspended in the standard assay media which were adjusted with 50 mM Hepes-Tricine to the pH-values indicated.

PDCs investigated was shifted into a more alkaline region (pH 7.8–8.1).

Products of plastid PDC

The PDC reaction equation predicts a 1:1:1 stoichiometry of the resultant products, CO₂, acetyl-CoA and NADH. In order to determine, whether the reaction products were indeed formed in the postulated proportion, they have been assayed by three techniques (see Materials and Methods). Each experiment was repeated several times with at least three replications per point. Simultaneous measurements of the three component activities of the PDC, expressed in nmol · mg⁻¹protein · h⁻¹, showed mean values of 56 ± 6 for the chloroplast complex from spinach, 158 ± 18 for that from maize and 266 ± 49 for the mitochondrial complex from etiolated peas (Table IV).

Discussion

Techniques like PEG-fractionation and ethyl-agarose affinity chromatography, which were recently applied for the purification of chloroplast PDC from peas [4], have not been as successful in the enrichment of PDC from spinach and maize mesophyll. This observation points to species-specific differences in the stability of the chloroplast complex. A direct high-speed sedimentation (140 000 × g) prior

Table IV. Stoichiometries of the pyruvate dehydrogenase complex (PDC) from spinach and maize mesophyll chloroplasts and from mitochondria of etiolated peas.

Compartment	Species	Component enzyme activities (nmol · mg ⁻¹ protein · h ⁻¹) of the PDC*		
		Pyruvate decarboxylase	Dihydrolipoyl transacetylase	Dihydrolipoyl dehydrogenase
chloroplast	spinach	51 ± 5	59 ± 11	58 ± 2
	maize (mesophyll)	159 ± 16	157 ± 24	158 ± 15
mitochondrion	peas (etiol.)	291 ± 54	245 ± 53	263 ± 40

* The component enzyme activities of the pyruvate dehydrogenase complex have been determined in the fraction after the first ultracentrifugation step (see Table II). The data are mean values from at least 3 experiments ± S.D.

to a complete clarification of the greenish "stromal extracts" resulted, however, in pellets which were contaminated with co-sedimenting membrane fragments and other major stromal proteins like RuBPCase in spinach [4] and pyruvate P_i dikinase [21] in maize mesophyll preparations. This contamination by foreign proteins did not allow for a critical electrophoretic analysis parallel with purification, although a decrease of lower molecular weight proteins together with an accumulation of bands typical for PDC components (approximate mol wts: 95000, 65000 and 58000) has been observed (data not shown). We have been satisfied, therefore, with demonstrating the purification by increasing the specific PDC-activities alone (Table II). From this point of view, the enrichment found with linear glycerol gradients cannot deceive about the remarkable loss in activities during this procedure. Nevertheless, the relative sharp peak obtained with the chloroplast PDC from maize mesophyll after fractionation on glycerol (Fig. 1) suggests that this complex, like that from pea chloroplasts, is comparable in size to the *E. coli* complex [4]. This observation as well as apparent similarities in regulatory properties [4] give rise to speculations on the homology between the prokaryotic and the chloroplast PDC. Recent findings that antibodies to the *E. coli* complex did not cross-react with pea chloroplast PDC [4] do not contradict these ideas as long as it has not been shown, that antibodies made to specific components of the *E. coli* complex are as ineffective. Possible variations in the aggregation state of both chloroplast PDCs investigated are further documented by their different stability during the purification procedures applied. Thus, the complex from maize mesophyll appeared to be highly labile in all centrifugation steps (Table II), while the most pronounced loss in enzyme activities of the spinach preparation seemed to be

due to the dissociation of its dihydrolipoyl dehydrogenase component (Fig. 1, peak A) during glycerol fractionation. In accordance with binding studies using the PDC from other sources [22], the hydrolyzed subcomplex could be reconstituted with residual PDC-activities in the bottom layer (Fig. 1, peak B) of the gradient. This procedure nearly restored initial activities of the complex before separation (Table II).

In order to ensure that the observed pyruvate decarboxylation is not attributable to other keto-acid dehydrogenase complexes or to acetolactate synthetase, as recently proposed by other authors [1], the substrate specificities and the stoichiometry of the reaction products have been investigated in detail.

Both PDCs showed an absolute requirement for pyruvate, which could not be substituted by other keto acids. Also, the reaction was obligatorily linked to NAD⁺ and CoA (Table III). In spite of the large NADP⁺-pool in chloroplasts, this substrate could replace NAD⁺ only to a small extent (6–7%). In accordance with the findings of other authors [4, 15], both PDCs investigated were only stimulated 40% by exogenous TPP. This observation points to a tighter association of TPP with the chloroplast than with the mitochondrial complex, the very rapid dissociation of which has been attributed to a possible regulation of mitochondrial PDC [23].

The quantitative examination of the three PDC reaction products CO₂, acetyl-CoA and NADH confirms the predicted 1:1:1 stoichiometry (Table IV) and, thus, verifies the chloroplast complex as a true PDC similar to that of mitochondria.

Like other stromal enzymes, the PDC of green [4–6] as well as colorless plastids [15] evidently requires high Mg²⁺-concentrations (≥ 5 mM) and an alkaline pH (pH 8) for optimal conditions. This requirement distinguishes them from the mitochon-

drial complex (Fig. 2 and 3). Since light-dependent variable parameters in the stroma space (pH, Mg^{2+} , ATP and reducing equivalents) are known to be involved in the regulation of stromal enzymes [24], increasing pH and Mg^{2+} -concentrations in illuminated chloroplasts have been suggested to control acetyl-CoA formation from pyruvate [4]. From this point of view, the stromal acetyl-CoA-synthesis from acetate with its additional requirement for MgATP [9, 25, 26] appears better adapted to illuminated chloroplasts than the PDC which, in contrast, seems to be rather inhibited by ATP (e.g. by Mg-complexation) [5, 27]. If one further assumes, that a light-dependent control of fatty acid synthesis from acetate does

not precede malonyl-CoA formation [9], a regulatory significance of Mg- and pH-effects on the chloroplast PDC appears questionable. As a possible control mechanism which determines the relative involvement of both acetyl-CoA synthesizing systems within the different types of chloroplasts, the stromal availability of acetate and pyruvate and feedback control of the PDC by acetyl-CoA or NADH have been discussed [5, 6].

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft.

- [1] D. J. Murphy and P. K. Stumpf, *Arch. Biochem. Biophys.* **212**, 730–739 (1981).
- [2] B. Liedvogel, *Z. Naturforsch.* **40c**, 182–188 (1985).
- [3] B. Liedvogel, *Anal. Biochem.* **148**, 182–189 (1985).
- [4] P. J. Camp and D. D. Randall, *Plant Physiol.* **77**, 571–577 (1985).
- [5] H. J. Treede and K.-P. Heise, *Z. Naturforsch.* **40c**, 496–502 (1985).
- [6] H. J. Treede, B. Riens, and K.-P. Heise, *Z. Naturforsch.* **41c**, 733–740 (1986).
- [7] M. O. Proudlove and D. A. Thurman, *New Phytol.* **88**, 255–265 (1981).
- [8] U. I. Flügge, M. Stitt, and H. W. Heldt, *FEBS Lett.* **183**, 335–339 (1985).
- [9] A. Sauer and K.-P. Heise, *Z. Naturforsch.* **39**, 268–275 (1984).
- [10] C. L. D. Jenkins and V. J. Russ, *Plant Sci. Lett.* **35**, 19–24 (1984).
- [11] G. Mouriaux and R. Douce, *Plant Physiol.* **67**, 470–473 (1981).
- [12] H. Ebbighausen, H. Chen, and H. W. Heldt, *Biochim. Biophys. Acta* **810**, 184–199 (1985).
- [13] D. D. Randall, P. M. Rubin, and M. Fenko, *Biochim. Biophys. Acta* **485**, 336–349 (1977).
- [14] P. G. Roughan, R. Holland, and C. R. Slack, *Biochem. J.* **184**, 193–202 (1979).
- [15] E. E. Reid, P. Thompson, C. R. Lyttle, and D. T. Dennis, *Plant Physiol.* **59**, 842–848 (1977).
- [16] M. M. Bradford, *Anal. Biochem.* **72**, 248–254 (1976).
- [17] G. F. Cox, *Meth. Enzymol.* **13**, 47–51 (1969).
- [18] J. A. Miernyk and R. N. Trelease, *Plant Physiol.* **67**, 875–881 (1981).
- [19] M. R. Schmitt and G. E. Edwards, *Plant Physiol.* **70**, 1213–1217 (1982).
- [20] F. H. Pettit and L. J. Reed, *Meth. Enzymol.* **89**, 376–386 (1982).
- [21] J. N. Burnell and H. D. Hatch, *Arch. Biochem. Biophys.* **237**, 490–503 (1985).
- [22] S. Furata and T. Hashimoto, *Meth. Enzymol.* **89**, 414–420 (1982).
- [23] P. M. Rubin, W. L. Zahler, and D. D. Randall, *Arch. Biochem. Biophys.* **188**, 70–77 (1978).
- [24] B. B. Buchanan, *Ann. Rev. Plant Physiol.* **31**, 341–374 (1980).
- [25] D. N. Kuhn, M. Knauf, and P. K. Stumpf, *Arch. Biochem. Biophys.* **209**, 441–450 (1981).
- [26] B. J. Nikolau and J. C. Hawke, *Arch. Biochem. Biophys.* **228**, 86–96 (1984).
- [27] P. Thompson, E. E. Reid, C. R. Little, and D. T. Dennis, *Plant Physiol.* **59**, 854–858 (1977).