REACTIVATION BY A BACTERIAL ACETATE: ENZYME LIGASE OF PLANT GLYOXYSOMAL ISOCITRATE LYASE

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1. Introduction

Isocitrate lyase, when found under heterotrophic growth conditions in higher plants, fungi or eucaryotic algae, is housed in microbodies (glyoxysomes) [1,2]. Fatty acid degradation and the glyoxylate cycle take place in these organelles. Little is known about regulation mechanisms, especially about the onset of the glyoxylate cycle some days after germination. Besides the acetyl-CoA utilizing processes, the isocitrate lyase reaction is a likely candidate acting as target of control mechanisms, as glyoxysomes contain two enzymes for isocitrate consuming reactions, isocitrate lyase and isocitrate dehydrogenase.

Recently, it has become obvious that another carbon–carbon lyase, namely citrate lyase of some bacteria, can be reactivated, after splitting off a citryl or acetyl group, by an enzyme-acylating system, an acetate:enzyme ligase [3,4]. As both enzyme reactions, mediated by citrate lyase and isocitrate lyase, are likely to follow the same mechanism, also the type of control mechanism by which the enzyme activity is switched on and off may be the same.

In this paper we report on experiments with a heterologous system, a bacterial enzyme-acylating ligase and a plant protein, the isocitrate lyase. That may become a valuable model system for further search on homologous activating systems controlling the isocitrate lyase activity in glyoxysomes.

2. Materials and methods

2.1. *Purification of the acetate: enzyme ligase from* Klebsiella pneumoniae

K. pneumoniae No. 94973 was cultured anaerobically in a medium described elsewhere [5] with sodium citrate (15 g/l) as the sole carbon source. Cells from 1 l of liquid culture were collected, washed with physiological saline and sonically disrupted. The purification of the ligase followed essentially the same steps as used by Schmellenkamp and Eggerer [3] for the characterization of the *K. aerogenes* enzyme. After streptomycin sulphate precipitation and fractionation with ammonium sulphate the enzyme preparation was dialyzed, concentrated and further purified by gel filtration on Sephadex G-100. Fractions with highest acetate: enzyme ligase activity had almost no isocitrate lyase activity (<0.5 mU/mg protein).

2.2. *Purification of the glyoxysomal isocitrate lyase*

Glyoxysomes were prepared from homogenates of cotyledons of 5-day old cucumber seedlings (7 mU isocitrate lyase/mg protein) by isopycnic density gradient centrifugation [6,7]. The matrix enzymes, liberated by osmotic shock, were subjected to isoelectric focussing using the range pH 5–8 [8]. Fractions with pH 5.8–6.1 containing the isocitrate lyase were pooled. Finally, after dialysis and concentration, the enzyme was purified by sedimentation velocity centrifugation on a sucrose gradient

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(25–40 %, w/w) utilizing a SW-27 rotor at 25 000 rev/min during 68 h. The isocitrate lyase sedimented only slightly slower than the glyoxysomal catalase whose mol. wt was determined to be 225 000 [9]. Thus, a more than 150 times purification yielded an isocitrate lyase with a specific activity of 1070 mU/mg protein. Details about purification and characterization of the enzyme will be published elsewhere [10].

2.3. *Assay procedures*

For the measurement of isocitrate lyase activity, the photometrical assay described by Dixon and Kornberg [11] was modified as follows. A stabile MES/HEPES buffer was prepared by heating 20 mmol MES, 10 mmol HEPES and 12 mmol $MgCO_3$ in 800 ml distilled water. After cooling 10 mmol DTT and 50 mmol phenylhydrazine were added, the mixture adjusted to pH 7.4 with 2 N NaOH and diluted to 1 l. This buffer is kept frozen till use.

1.5 ml MES/HEPES buffer were preincubated with 100 µl enzyme for 10 min, then the reaction was initiated by addition of 100 µl 100 mM DL-isocitrate (free of *allo*). A Gilford spectrophotometer 2400 S with reference compensation was used and the extinction difference between sample and blank (without isocitrate) was recorded during 15 min.

The incubation mixture of the acyl-ligase test (reactivation of isocitrate lyase) contained in a total volume of 1.7 ml: 200 µl (100 µl) deactivated isocitrate lyase, 20 µl 10 mM magnesium succinate, 10 µl 50 mM ATP, 50 µl 100 mM isocitrate and 1.4 ml MES/HEPES buffer (described above). The reaction is started with 20 µl (20 µg) *Klebsiella* enzyme and the formation of glyoxylate phenylhydrazone monitored at 324 nm.

3. Results

3.1. *Inactivation of isocitrate lyase*

Both bacterial citrate lyase [12,13] and plant glyoxysomal isocitrate lyase are very sensitive towards hydroxylamine. While hydrazines, e.g. 1–10 mM phenylhydrazine, have no effects on these enzymes, 1 mM NH_2OH in the incubation mixture led to 98 % inhibition of the plant enzyme.

In another experiment, isocitrate lyase was inactivated and only then used as substrate for the reactivation test. A 15-min treatment of 110 mU isocitrate lyase with 5 µmol NH_2OH (in a total volume of 4.5 ml) was followed by dialysis or gel filtration on Sephadex G-100 in order to separate the protein from low mol. wt compounds, resulting in an 80% deactivation of the enzyme.

Inactivation of isocitrate lyase which resembles the one of watermelon seedlings [14] also occurred upon chromatography on Sepharose 6B. If no attention is paid to preserve the enzyme by adding magnesium succinate and sucrose to the buffer, most of the isocitrate lyase activity is lost during the gel filtration. Fractions with the remaining isocitrate lyase activity corresponding to a mol. wt of 210 000 were used as 'inactivated enzymes' for the following tests.

3.2. *Reactivation of inactivated isocitrate lyase*

Reactivation experiments using the de-acylated plant isocitrate lyase and a purified, already described [3] acetate:enzyme ligase from the bacterium are summarized in table 1. When freshly prepared ligase is employed, always full reactivation is achieved with 20 µg of the purified enzyme. Diluting the preparation from *Klebsiella* by more than 1:10 is necessary to show that the glyoxylate formation is dependent on ligase concentration. Included in the figure are also controls in which ATP or succinate was omitted. Acetyl-CoA or acetate could not substitute for succinate.

4. Discussion

While citrate lyase from *K. aerogenes* [3] and *Rhodopseudomonas gelatinosa* [4] can clearly be separated from their acetylating enzymes (acetate:-HS-citrate lyase ligase), the lyase from *Streptococcus diacetilactis* [4] was found to be associated with its acetylating ligase. There are no findings that the plant isocitrate lyase is coupled with an acylating enzyme. Towards activation point the results of Foo et al. [15] who reported a 50 % increase of activity of isocitrate lyase from the green alga *Gloemonas* upon incubation with 1 mM succinyl CoA. In contrast, the isocitrate lyase from *Pseudomonas indigofera* seems to differ considerably from the algal and plant enzyme, as Dimroth et al. [16] found neither inhibition by NH_2OH nor succinylation.

Table 1
Reactivation of various inactivated isocitrate lyase preparations

Isocitrate lyase preparation	Reactivation with Ligase	Succinate	ATP	Isocitrate lyase activity mU	%
Isocitrate lyase,	–	–	–	110	100
active	+	+	+	119	108
Preparation a,	–	–	–	50	45
NH_2OH-inactivated	+	+	+	113	103
Preparation b,	–	–	–	22	20
NH_2OH-inactivated	+	+	+	112	102
	boiled	+	+	20	18
	+	–	+	27	25
	+	+	–	44	40
Preparation c	–	–	–	0.90	100
	+	+	+	1.53	170

Isocitrate lyase (110 mU, 100 μg) was inactivated with 5 μmol NH_2OH for 15 min and then dialyzed (preparation a). Another 110 mU isocitrate lyase was inactivated in the same manner and then, after additional dialyzation against 10 mM NH_2OH, filtered through a bed of Sephadex G-100 (preparation b). Preparation c: 56 mU isocitrate lyase gave a protein with 0.90 mU after gel filtration on Sepharose 6B.

For many reasons the understanding how isocitrate lyase activity is controlled in vivo or in vitro has important implications. Using the now reported acylating system as a model, work is in progress to demonstrate a lyase-activating mechanism which is intrinsic to glyoxysomes.

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