Control of Isocitrate Lyase Synthesis in *Chlorella fusca* var. *vacuolata*

THE BASAL ACTIVITY OF THE ENZYME AND THE KINETICS OF INDUCTION

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1. Isocitrate lyase activity was measured in non-induced *Chlorella fusca* var. *vacuolata* cells. 2. During exponential autotrophic growth about 1–2 molecules of the enzyme per cell were present. 3. In light-limited cultures the amount of the enzyme increased to 10–20 molecules/cell. 4. When autotrophic cultures were placed in the dark, the basal activity of isocitrate lyase increased after a 2 h lag so that after 8 h in the dark there was a 500-fold increase in activity. 5. When isocitrate lyase was induced (by addition of acetate and removal of illumination) in autotrophic cultures which had been growing exponentially, the full induced rate of enzyme synthesis was obtained after 70–80 min. 6. When light-limited autotrophic cultures were induced, the rate of isocitrate lyase synthesis was maximal after only 40–50 min. 7. These data are consistent with a catabolite-repression control co-ordinated with photosynthetic activity, which may be independent of the specific inducing effect of acetate.

In *Chlorella fusca* var. *vacuolata*, induced synthesis of isocitrate lyase (*threo*-D₃-isocitrate glyoxylate-lyase, EC 4.1.3.1) occurs only when acetate is the sole carbon source for growth (Syrett *et al.*, 1963; Syrett, 1966). Nevertheless, when autotrophic cultures of the alga become light-limited, mRNA for isocitrate lyase accumulates without being translated (Scragg *et al.*, 1975), and we have suggested that control of synthesis of this enzyme is exerted at two levels: induction of synthesis by acetate is a post-transcriptional control and synthesis of isocitrate lyase mRNA is repressed by high rates of photosynthesis or when glucose is supplied in the medium (Scragg *et al.*, 1975), although repression may also be exerted at the translational level (McCullough & John, 1972a).

In the present paper the amount of isocitrate lyase in non-induced cells (the basal activity of the enzyme) has been studied in an attempt to correlate non-induced enzyme activity with photosynthetically mediated catabolite repression.

Experimental

Culture of organism

Chlorella fusca var. *vacuolata* Cambridge Culture Collection strain 211/8p was grown autotrophically as described by John *et al.* (1970). Cultures (2 litres) grew exponentially to a cell density of 10^7 cells/ml, after which growth was light-limited, cell density increasing linearly to about 5×10^7 cells/ml. Cell density was determined with a Coulter counter Z_{B1} (Coulter Electronics Ltd., Dunstable, Beds., U.K.).

Frozen-cell assay of isocitrate lyase

Isocitrate lyase activity was measured in intact cells whose semipermeability had been destroyed by freezing and thawing, as described by Syrett (1966), except that the glyoxylate formed was determined by the method of Kramer *et al.* (1959). Enzyme activity measured in this way was expressed as μmol of glyoxylate formed/min per 10^{10} cells.

Preparation of cell-free extracts

This procedure was performed at 0–4°C. Cells were harvested by centrifugation, washed and resuspended in TMCE buffer (10 mM-triethanolamine hydrochloride/NaOH/5 mM-MgCl₂/1 mM-dithiothreitol/0.5 mM-EDTA, pH 7.6). After two passages through a French pressure cell at 1.35×10^8 Pa, the broken cell suspension was centrifuged at 120 000g for 1 h. The supernatant was applied to a Sephadex G-25 column (27 cm $\times$ 2.5 cm, void volume 61 ml) equilibrated with TMCE buffer, which was also used for elution. Fractions containing protein were pooled and concentrated by pressure dialysis in a collodion bag (SM 13200; Sartorius-Membranfilter, Göttingen, Germany). Cell density, measured with a haemocytometer, before and after treatment with the French pressure cell, was used to calculate the number of cells broken. Protein concentration was measured in the supernatant before Sephadex G-25 treatment and in the final extract by the method of Lowry *et al.* (1951), with dry crystalline bovine plasma albumin as standard; 1.00 mg of extract protein was obtained from 10^8 cells.

Preparation of [6-¹⁴C]isocitrate

The aqueous incubation mixture had a final volume of 4.5 ml in a 10 ml glass tube sealed with a vaccine stopper. It contained 100 mg of NADPH, 80 mg of potassium 2-oxoglutarate and 10 mg of isocitrate dehydrogenase, adjusted to pH 8.0 with Tris. NaH¹⁴CO₃ (5 mCi) was added and the mixture incubated at 30°C for 26 h, after which 4.5 ml of acetic acid/ethanol (1:19, v/v) was added and the supernatant after centrifugation was concentrated to a syrup under a stream of compressed air (in a fume hood). The syrup was made up to 2.5 ml with water and applied to a Sephadex G-10 column (90 cm × 1.5 cm, void volume 50 ml) and the fractions containing significant radioactivity were pooled and freeze-dried, resuspended in 0.3 ml of water and applied again to the column. After a third passage through the column under the same conditions and fractionation on a Bio-Gel P-2 column of the same dimensions, eluted in the same way, a radioactive fraction containing 13.8 μCi was obtained. This was freeze-dried and resuspended in unlabelled 25 mM-*threo*-D₅(+)-isocitrate to give a specific radioactivity of 0.38 μCi/μmol. Assay for nicotinamide nucleotides by A₂₆₀ and for 2-oxoglutarate by formation of its phenylhydrazone showed that over 99% of the starting concentration of these compounds had been removed. To confirm the chemical concentration of isocitrate in this solution, a continuous spectrophotometric assay of isocitrate lyase (Kornberg, 1965) was used, with enzyme supplied as an induced *C. fusca* extract; 5 μl of the solution gave 0.125 μmol of glyoxylate when the reaction was allowed to go to completion, and compared with glyoxylate standards. With a smaller amount of enzyme (cell-free extract), the initial rate of glyoxylate formation was the same for the [¹⁴C]isocitrate solution and the unlabelled isocitrate used as carrier, indicating that there were no inhibitors of isocitrate lyase in the radioactive substrate preparation. Radiochemical purity was assessed by radioautography of a two-dimensional t.l.c. separation as described below. The principal radioactive spot co-chromatographed with isocitrate detected chemically as described below. Two other radioactive spots were detected, containing together less than 1% of the radioactivity applied to the plate.

Radiochemical assay of isocitrate lyase

The reaction was started by addition of 0.1 ml of [6-¹⁴C]isocitrate solution (2.5 μmol, 0.95 μCi) to 0.9 ml of cell-free extract and TMCE buffer at 30°C. Immediately and after 60 and 120 min, 0.2 ml amounts were withdrawn and mixed with 0.4 ml of acetic acid/ethanol (1:19, v/v), boiled for 30 s, centrifuged and the supernatant was retained. A further 0.4 ml of acetic acid/ethanol was used to wash the pellet and

was pooled with the supernatant. The mixture was blown dry and resuspended in 40 μl of succinic acid in methanol (6 mg/ml), followed by 40 μl of methanol, all of which was spotted on a t.l.c. plate. The succinic acid spot, separated and located as described below, was scraped off the plate and extracted from the cellulose powder by two successive 0.5 ml portions of water, which were pooled and counted for radioactivity as described previously (Thurston *et al.*, 1973). Sample counts were converted into d.p.m. by using an external-standard channels-ratio method. Enzyme activity was expressed as pmol of succinate formed/min per mg of extract protein.

Thin-layer-chromatographic separation of isocitrate and succinate

A two-dimensional separation on 20 cm × 20 cm cellulose plates (Art. 5716; Merck, Darmstadt, Germany) used diethyl ether/formic acid/water (6:2:1, by vol.) as first solvent and diethyl ether/formic acid/methanol (8:2:1, by vol.) as the second. *R_F* values in the two solvents were respectively isocitrate 0.53, 0.27, and succinate 0.77, 0.63. When thoroughly dry the plates were sprayed with 0.1% 2,6-dichlorophenol-indophenol in ethanol, made alkaline by addition of NaOH. Succinic acid and isocitric acid gave red spots on a blue background.

Chemicals

NaH¹⁴CO₃ (59.1 Ci/mol) and sodium [2,3-¹⁴C]-succinate (23 Ci/mol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Crystalline bovine plasma albumin was from Armour Pharmaceutical Co., Eastbourne, Sussex, U.K. Sigma (London) Chemical Co., Kingston-upon-Thames, U.K., supplied the following: *threo*-D₅(+)-isocitric acid, monopotassium salt; isocitrate dehydrogenase [*threo*-D₅-isocitrate-NADP⁺ oxidoreductase (decarboxylating), EC 1.1.1.42], type IV from pig heart, 3.6 μmol/min per mg; NADPH, tetrasodium salt; 2-oxoglutarate, monopotassium salt; sodium glyoxylate.

Results and Discussion

Basal activity of isocitrate lyase during autotrophic growth

Attempts to measure isocitrate lyase activity in non-induced *C. fusca* cultures by using colorimetric assays of glyoxylate derivatives were unsuccessful. Syrett (1966) showed that assays based on formation of glyoxylate 2,4-dinitrophenylhydrazones were unsuitable, as 2-oxoglutarate present in extracts or frozen-thawed cells gave an absorbing derivative preventing the precise measurement of small amounts

Table 1. Basal activity of isocitrate lyase in *Chlorella fusca* var. *vacuolata*, measured with a [¹⁴C]isocitrate assay
The assay procedure and synthesis of [6-¹⁴C]isocitrate are described in the Experimental section. Rate of [¹⁴C]succinate formation was obtained by subtracting zero-time radioactivity from the value measured in a sample taken after 1 h incubation. No. of molecules of isocitrate lyase per cell was calculated by using 9.88×10^{-3} amol/min as the activity of one molecule of isocitrate lyase at pH7.6 and 30°C, which was derived from the data of John & Syrett (1967) for purified enzyme from the same strain of *Chlorella*.

Source of <i>C. fusca</i> extracts		Assay data				
Conditions of growth of the alga	No. of cells/ml when harvested	Protein concn. (mg/ml)	Rate of succinate formation (d.p.m./h per 0.2ml sample)	Isocitrate lyase activity		No. of molecules of isocitrate lyase per cell
				(pmol/min per mg of protein)	(amol/min per cell)	
Exponential, autotrophic	4.0 × 10 ⁶	16.90	305	1.80	0.018	1.8
Light-limited, autotrophic	1.3 × 10 ⁷	7.92	914	11.5	0.115	11.7
	2.9 × 10 ⁷	6.96	967	14.4	0.145	14.6
Incubated in the dark for 2 h	(4 × 10 ⁷)	1.37	7540	542	5.44	550

of glyoxylate. The formation of glyoxylate formazan (Kramer *et al.*, 1959) and of glyoxylate phenylhydrazone (Kornberg, 1965) were subject to the same restriction (results not shown). The radiochemical assay described here took advantage of the specific radiolabelling in C-6 of isocitrate formed by the back reaction of isocitrate dehydrogenase with H¹⁴CO₃⁻ and unlabelled 2-oxoglutarate as substrates. When [6-¹⁴C]isocitrate is a substrate for isocitrate dehydrogenase, the radioactive carbon goes to bicarbonate, whereas isocitrate lyase action gives labelled succinate as a reaction product. By measuring the rate of formation of [¹⁴C]succinate from [6-¹⁴C]isocitrate, isocitrate lyase activity was detected in extracts from autotrophic *C. fusca* cells growing exponentially (Table 1). The amount of enzyme activity is extremely small, representing 1–2 molecules of isocitrate lyase per cell. When growth of autotrophic cultures was no longer exponential because of light-limitation, extracts contained increased enzyme activity equivalent to 10–20 molecules of enzyme per cell (Table 1). Radioactive succinate formation was inhibited by 35% when 10mm-phosphoenolpyruvate was included in an assay (Fig. 1), which was consistent with the inhibition of pure isocitrate lyase by this metabolite (John & Syrett, 1968).

Isocitrate lyase assays performed as described here are probably underestimates of activity for two reasons. First, when 0.136 μCi of [¹⁴C]succinate was added to a dummy assay, 0.123 μCi (90%) was recovered. Secondly, even after removal of free NAD(P)⁺ from *C. fusca* extracts by Sephadex G-25 treatment, there was residual isocitrate dehydrogenase activity present. After 1 h incubation, up to 30% of [¹⁴C]isocitrate was lost by decarboxylation. As the K_m for isocitrate of *C. fusca* isocitrate lyase is 0.023

Fig. 1. Assay of isocitrate lyase with [6-¹⁴C]isocitrate
Assays were done without (●) and with (○) 10mm-phosphoenolpyruvate. The method of assay and separation of succinate are described in the Experimental section: 0.95 μCi of [6-¹⁴C]isocitrate and 6.96mg of extract protein from light-limited organisms were included in each 1 ml incubation. Radioactivity in succinate from 0.2ml samples of assay mixture was corrected by subtraction of radioactivity in zero-time samples. After 120 min, 25% of [6-¹⁴C]isocitrate had been lost, owing to isocitrate dehydrogenase activity in the cell-free extract.

mm (John & Syrett, 1967) lowering the isocitrate concentration from 0.25 to 0.175mm would produce a relatively small decrease in reaction rate, although further loss of isocitrate during the second hour of incubation could have contributed to the obvious lowering of reaction rate (Fig. 1).

Fig. 2. Effect of incubation in the dark on the basal activity of isocitrate lyase in a *C. fusca* culture

Isocitrate lyase activity was measured in samples (5×10^7 cells) taken from an autotrophic *C. fusca* culture (1.25×10^7 cells/ml) which had been put in the dark. The frozen-cell assay method (Syrett, 1966) was used and the points are average values from duplicate measurements.

Change in the basal activity of isocitrate lyase when *C. fusca* cells were incubated in the dark

As decreased photosynthetic activity owing to light-limitation was accompanied by an increase in the basal activity of isocitrate lyase, the effect of inhibition of photosynthesis by removing light completely was examined. After 2 h in the dark a substantial increase in isocitrate lyase activity had occurred (Table 1); however, between 2 and 8 h after the culture had been kept in darkness, a much larger increase in enzyme activity took place, as shown in Fig. 2, where isocitrate lyase became measurable in the frozen-cell assay method (Syrett, 1966). The activity of isocitrate lyase after 8 h in the dark was $0.696 \mu\text{mol/min per } 10^{10}$ cells, which was approximately a 500-fold increase over the value in light-limited cells, but was still less than 1% of the fully induced activity of the enzyme, $233 \mu\text{mol/min per } 10^{10}$ cells as measured with the frozen-cell assay (Syrett *et al.*, 1963). Longer periods of incubation in the dark did not increase the basal activity of isocitrate lyase.

Kinetics of induced isocitrate lyase synthesis

When acetate was supplied to *C. fusca* cultures, isocitrate lyase synthesis was induced after a short lag (Syrett, 1966; Fig. 3 of the present paper). The time taken to obtain maximum rate of enzyme synthesis was about 75 min when exponentially growing autotrophic cultures were induced and about 45 min for cultures that had been growing under light-limitation (Fig. 3). The maximum rate of induced synthesis was not obtained more quickly if cultures

Fig. 3. Onset of induced isocitrate lyase synthesis. Isocitrate lyase synthesis was induced by addition of sodium acetate (final concn. 12 mM) and transfer of cultures to the dark. Enzyme activity was assayed by the frozen-cell method (Syrett, 1966). Culture conditions before induction were: autotrophic cultures growing exponentially (●), average enzyme activities from three experiments where cell densities were 3.8×10^6 , 4.8×10^6 and 9.5×10^6 cells/ml; light-limited cultures (○), average enzyme activities from three experiments where cell densities were 3.3×10^7 , 3.6×10^7 and 3.8×10^7 cells/ml; cultures incubated in the dark for 4 h (▲), and 8 h (△), before the addition of sodium acetate.

of the alga were incubated in the dark for 4 or 8 h before the addition of sodium acetate. Preincubation in the dark led, however, to an increase in the maximum rate of enzyme synthesis (Fig. 3), for which two possible explanations are suggested. First, in the dark, where exogenous carbon and energy supply to the organisms is cut off, protein synthesis may become limited by mRNA supply, so that when mRNA for isocitrate lyase can be translated more ribosomes are available to do this. Secondly, in the dark the organisms may accumulate in that part of the cell cycle where induced isocitrate lyase synthesis is allowed. In asynchronous cultures such as we have used here, a proportion of the cells would be (at any one time) in that part of the cell cycle where isocitrate lyase cannot be induced (McCullough & John, 1972b).

Photosynthetically mediated repression and the inducing effect of acetate may be separate factors bearing on the control of isocitrate lyase synthesis

The repression of isocitrate lyase synthesis exerted by photosynthesis is clearly a complex phenomenon. Our previous results (Scragg *et al.*, 1975) showed that repression was in part relieved when growth of *C. fusca* became light-limited, and this was confirmed by the change in the basal activity of the enzyme accompanying the onset of light-limitation shown in Table 1.

It is also evident from Syrett's (1966) elegant experiments that light-driven carbon fixation, rather than photophosphorylation alone, was required for repression of isocitrate lyase synthesis. In addition, the change in basal activity of isocitrate lyase when organisms were aerated in the dark (Fig. 2) shows that partial de-repression of enzyme synthesis occurred for up to 8 h in the dark, which suggests a repressive effect of endogenous metabolism. This correlates with the observation of Atkinson *et al.* (1972) that starch stored during the light-period in light-dark-synchronized *C. fusca* cells was consumed after 8–9 h in the dark.

The lag of induced isocitrate lyase synthesis after addition of acetate to *C. fusca* cultures (Fig. 3) was not eliminated by conditions which minimized the repressive effect of prior growth conditions. This is consistent with the idea that acetate induces isocitrate lyase by a mechanism which is distinct from the repression control resulting from photosynthetic carbon metabolism, as we have previously proposed (Scragg *et al.*, 1975).

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