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

RATE OF ENZYME SYNTHESIS IN THE PRESENCE AND ABSENCE OF ACETATE MEASURED BY [³⁵S]METHIONINE LABELLING AND IMMUNOPRECIPITATION

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The rate of increase of isocitrate lyase activity was measured in darkened *Chlorella fusca* var. *vacuolata* cultures in the presence and absence of acetate and compared with the rate of incorporation of [³⁵S]methionine into isocitrate lyase enzyme protein under the same conditions. Isocitrate lyase enzyme protein was isolated for this purpose by specific immunoprecipitation and sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. After 4 h in the dark, in the presence of acetate the rate of increase of isocitrate lyase activity was 75 times that in the absence of acetate. Incorporation of [³⁵S]methionine into isocitrate lyase was 140 times greater in the presence of acetate. Incorporation of [³⁵S]methionine into the trichloroacetic acid-insoluble fraction overall was about five times as fast in the presence of acetate. These data are not consistent with an increased turnover of isocitrate lyase enzyme molecules, sufficient to account for the low rate of increase of isocitrate lyase activity in the absence of acetate. The greater rate of enzyme synthesis in the presence of acetate must therefore be due to some effect of this metabolite on the processing or translation of isocitrate lyase mRNA.

The control of isocitrate lyase (*threo*-D₅-isocitrate glyoxylate-lyase, EC 4.1.3.1) synthesis in the green alga *Chlorella fusca* var. *vacuolata* has two distinguishable components. By using a translation assay *in vitro* it has been shown that mRNA for isocitrate lyase is not detectable in *C. fusca* cells while they are growing exponentially in the light, but when photosynthesis becomes light-limited or in the dark when acetate is supplied, mRNA for isocitrate lyase has been detected in amounts which were not detectably different (Scragg *et al.*, 1975). However, the fully induced amount of isocitrate lyase activity is only obtained when cells are provided with acetate as sole carbon source in the dark (Syrett *et al.*, 1963; Syrett, 1966). When an autotrophic *C. fusca* culture is darkened without addition of acetate, an increase in isocitrate lyase activity occurs which is very small by comparison with the increase in activity when acetate is present (Thurston, 1977). Thus isocitrate lyase mRNA synthesis is derepressed when high rates of photosynthesis are prevented, but a high rate of isocitrate lyase enzyme synthesis apparently requires the presence of acetate as well.

It is possible that acetate increases the rate of isocitrate lyase synthesis through (direct or indirect) activation of mRNA processing or translation. However, it is equally likely that isocitrate lyase mRNA is always translated when present in *C. fusca* cells, but isocitrate lyase enzyme protein only accumulates in the presence of acetate because it is otherwise rapidly degraded. In other words acetate

(directly or indirectly) stabilizes the newly formed enzyme molecules. This mechanism predicts that the rate of isocitrate lyase polypeptide-chain synthesis will be the same in two darkened *C. fusca* cultures, where only one is supplied with acetate. Conversely, if acetate controls the translation of isocitrate lyase mRNA, or any step between transcription and translation, then the low activity of isocitrate lyase present in darkened cultures that are not supplied with acetate reflects a low rate of synthesis of isocitrate lyase protein, compared with the rate of enzyme synthesis when acetate is present.

This paper describes an experiment which attempts to distinguish between these two alternatives by measuring the short-term incorporation of a radioactive amino acid into isocitrate lyase.

Experimental

Culture of the organism

Chlorella fusca var. *vacuolata* Cambridge Culture Collection of Algae and Protozoa strain 211/8p was grown in the light at 25°C in a mineral salts medium as described by John *et al.* (1970).

Incorporation of [³⁵S]methionine and preparation of cell-free extracts

[³⁵S]Methionine (5mCi/μmol) was added to cultures to give a final concentration of 0.13 μM.

Samples (200 ml) were withdrawn at timed intervals and quickly poured into centrifuge bottles which already contained 525 μ g of cycloheximide dissolved in 10 ml of the resuspension buffer described below. The bottles were capped and rapidly cooled by partial immersion in a solid CO₂/ethanol mixture until the sample began to freeze. All subsequent operations were carried out at 0–4°C. Cells were harvested by centrifugation, washed and resuspended in 4 ml of 14 mM-Tris/HCl/1 mM-EDTA/1 mM-dithiothreitol, pH 7.7. The concentrated cell suspensions were broken by two passages through a French pressure cell at 1.35×10^8 Pa and centrifuged for 1.5 h at 120 000 g. The supernatant extracts were stored at –20°C. Protein concentration was measured by the method of Lowry *et al.* (1951) with dry crystalline bovine plasma albumin as standard.

Isolation of isocitrate lyase by immunoprecipitation

As the amount of isocitrate lyase in the radioactive extracts was very small, to allow immune precipitation purified non-radioactive isocitrate lyase was added as carrier. Non-radioactive isocitrate lyase was purified to homogeneity as described by John & Syrett (1967), except that gel filtration was performed with Sephacryl S-200 (Pharmacia, London W.5, U.K.) instead of Sephadex G-100. The precipitation mixtures contained 1–2.5 mg of radioactive extract protein, 50 μ g of carrier isocitrate lyase and 40 μ l of anti-(isocitrate lyase) serum in a total volume of 0.5–0.8 ml. After standing for 16 h at 4°C precipitates were recovered by centrifugation, washed and dissolved for electrophoresis as described by Scragg *et al.* (1975), except that 5 min at 100°C rather than 2 min was used to achieve more complete dissociation of isocitrate lyase into its monomeric form. The rabbit anti-(isocitrate lyase) serum gave no precipitin lines when challenged with a cell-free extract from an autotrophic *C. fusca* culture in an agar double-diffusion assay. It was established that the ratio of antigen to antiserum described above gave optimum precipitation.

Polyacrylamide-gel electrophoresis

For this, 10% polyacrylamide gels containing sodium dodecyl sulphate were prepared and run as described by Laemmli (1970), except that 0.5% (v/v) ethylene diacrylate was used in place of *NN'*-methylene bisacrylamide to cross-link the polymer. After electrophoresis gels were fixed by soaking overnight in 15% (w/v) trichloroacetic acid and twice washed for 1 h in 10% (v/v) acetic acid to remove sodium dodecyl sulphate (Bertolini *et al.*, 1976). Protein bands, stained with Amido Black, were recorded by scanning densitometry at 600 nm by using an SP.1700

spectrophotometer with an SP.1809 scanning accessory (Pye–Unicam, Cambridge, U.K.).

Measurement of radioactivity

All measurements were made with samples in 1 ml of aqueous solution mixed with 10 ml of toluene/Triton X-100/butyl-PBD [5-(4-biphenyl)-2-(4-*t*-butylphenyl)-1-oxa-3,4-diazole] (250:125:2, v/v/w) in a liquid-scintillation spectrometer at 10°C. Total radioactivity incorporated was measured in 50 μ l amounts of cell-free extract. Trichloroacetic acid-insoluble radioactivity was determined in 100 μ l amounts of cell-free extract co-precipitated with 0.1 mg of bovine plasma albumin by addition of an equal volume of 10% (w/v) trichloroacetic acid. The precipitates were washed twice with 5% (w/v) trichloroacetic acid and dissolved in 100 μ l of 1 M-NaOH. Polyacrylamide gels were frozen on solid CO₂ and cut into 2 mm slices which were digested with 0.5 ml of 20-vol. H₂O₂ for 36 h at 45°C. The dry residues were dissolved in 1 ml of water before scintillation mixture was added. To obtain representative background counts when measuring radioactivity in gel slices, each set of determinations included six slices from a gel to which no sample had been applied. Counts were converted into d.p.m. by using an external-standard channels-ratio method and corrected for decay by relating all values to the radioactivity date given by the manufacturer for each batch of [³⁵S]methionine.

Assay of isocitrate lyase activity

Enzyme activity was measured in samples of *C. fusca* cells whose semi-permeability had been destroyed by freezing and thawing as previously described (Thurston, 1977). The method is based on the procedure devised by Syrett (1966). Enzyme activity was expressed as μ mol of glyoxylate formed/min per 10¹⁰ cells.

Chemicals

[³⁵S]Methionine was supplied by The Radiochemical Centre, Amersham, Bucks., U.K. Butyl-PBD was from Intertechnique, Uxbridge, Middx., U.K. and crystalline bovine plasma albumin was from Armour Pharmaceutical Co., Eastbourne, Sussex, U.K. Ethylene diacrylate was from Monomer-Polymer Laboratories, Philadelphia, PA, U.S.A.

Results and Discussion

Effect of acetate on isocitrate lyase activity

An autotrophic *C. fusca* culture was divided into two batches which were placed in the dark (without

otherwise changing culture conditions), and one batch was at the same time supplied with a sterile solution of sodium acetate to give a final concentration of 25 mM. Both cultures stopped growing when placed in the dark as, in the absence of acetate, the organisms were starved of carbon and energy and, in the presence of acetate, adaptation to its use as sole carbon and energy source would take 24 h before cell division began again (Syrett *et al.*, 1963). Fig. 1 shows isocitrate lyase activity which was measured in samples taken at hourly intervals. After 4 h in the dark the rate of increase of isocitrate lyase activity was 0.0187 unit (μmol of glyoxylate formed/min)/min per 10^{10} cells in the presence of acetate and 0.00025 unit/min per 10^{10} cells in the absence of acetate.

Incorporation of [^{35}S]methionine by C. fusca cultures in the dark

At 4 h after darkening the above cultures [^{35}S]-methionine was added, and samples for analysis of incorporated radioactivity were withdrawn 10, 20 and 30 min later. Total radioactivity taken up by the two cultures was similar (Fig. 2a), but incorporation into the trichloroacetic acid-insoluble fraction was about 5-fold greater in the presence of acetate (Fig. 2b). In both cultures incorporation into this fraction was linear with time after a lag of about 4 min.

Incorporation of [^{35}S]methionine into isocitrate lyase

Incorporation of [^{35}S]methionine into isocitrate lyase was measured in immunoprecipitates and in the

isocitrate lyase band after electrophoresis of the dissociated immunoprecipitates on sodium dodecyl sulphate/polyacrylamide gels (Table 1 and Fig. 3). Both types of measurement show linear incorporation with time, but only a proportion of the precipitated radioactivity migrated with the isocitrate lyase band during electrophoresis and the proportion was much smaller in samples from the culture which lacked acetate. This was probably due to non-specific adsorption or trapping of proteins other than isocitrate lyase during the formation of immunoprecipitates. From the data obtained after electrophoretic separation, the rate of incorporation into isocitrate lyase was 1540 d.p.m./min per mg of cell-free extract protein in the presence of acetate and 11 d.p.m./min per mg of cell-free extract protein in the absence of acetate.

Comparison of the effect of acetate on the rate of incorporation of [^{35}S]methionine and the rate of increase of isocitrate lyase activity

If the presence of acetate had no effect on the specific radioactivity of the methionine precursor pool(s) or on the overall rate of protein synthesis in *C. fusca*, the increase in isocitrate lyase activity (Fig. 1) could be compared directly with the observed incorporation of [^{35}S]methionine into the enzyme (Table 1). As the ratio of rate of increase of enzyme activity in the absence of acetate (hereafter '–acetate') to the rate of increase of enzyme activity in the presence of acetate (hereafter '+acetate') was $0.00025/0.0187 = 0.0134$, then if the enzyme was stable '–acetate' the expected rate of incorporation

Fig. 1. Effect of acetate on the increase in isocitrate lyase activity when *C. fusca* cultures were transferred to the dark. An autotrophic *C. fusca* culture (1.34×10^7 cells/ml) was divided into two 850 ml portions and placed in the dark. One batch was immediately given 25 mM-sodium acetate (a) and the other was not (b). Isocitrate lyase activity was assayed by the frozen cell method. Note the difference in ordinate scales.

Fig. 2. Uptake of [³⁵S]methionine by *C. fusca* cultures in the dark

The cultures described in the legend of Fig. 1 were supplied with 0.5 mCi of [³⁵S]methionine after 4 h in the dark, when the volume of each culture was 770 ml. Total uptake into cell-free extracts is shown in graph (a) and uptake into the trichloroacetic acid-insoluble fraction in graph (b), for cultures in the presence (○) and absence (●) of 25 mM-sodium acetate.

Table 1. Incorporation of [³⁵S]methionine into isocitrate lyase protein by darkened *C. fusca* cultures in the presence and absence of acetate

[³⁵S]Methionine was added to the cultures described in the legend to Fig. 1 after 4 h in the dark. Total incorporation of [³⁵S]methionine and incorporation into the trichloroacetic acid-insoluble fraction are shown in Fig. 2. Methods of immunoprecipitation and electrophoresis are described in the Experimental section. Radioactivity in immunoprecipitates and in the isocitrate lyase band after electrophoresis is expressed as d.p.m. per mg of cell-free extract protein from which these were obtained.

Time after addition of [³⁵ S]methionine (min)	Incorporation of [³⁵ S]methionine into isocitrate lyase (d.p.m./mg)			
	Acetate present		Acetate absent	
	Immunoprecipitated radioactivity	Radioactivity in the isocitrate lyase band on gels	Immunoprecipitated radioactivity	Radioactivity in the isocitrate lyase band on gels
10	21 770	7975	807	57.3
	19 330	8362*	653	43.5*
20	46 880	26 320	1462	181.3
	45 750	24 620*	1090	160.4*
30	73 210	38 800	2230	272.6
	62 030	40 020*	2303	281.3*
Rate of incorporation (d.p.m./min per mg of protein)		1540		.11

* These values are from the polyacrylamide-gel analyses shown in Fig. 3.

would have been (rate of incorporation '+acetate' = 1540) $\times$ 0.0134 = 21 d.p.m./min per mg of extract protein. This is about twice the observed value.

The about 5-fold greater rate of incorporation of radioactivity into the trichloroacetic acid-insoluble fraction '+acetate' (Fig. 2b) suggests that the above

Fig. 3. Electrophoretic analysis of $[^{35}\text{S}]$ methionine-labelled isocitrate lyase immunoprecipitates

Isocitrate lyase was precipitated from *C. fusca* cell-free extracts with anti-(isocitrate lyase) serum. The dissociated precipitates were run on sodium dodecyl sulphate/polyacrylamide gels, which were scanned for protein after staining with Amido Black, and cut into 2mm slices for measurement of radioactivity. Radioactivity in samples taken 10 (a), 20 (b) and 30 (c) min after addition of $[^{35}\text{S}]$ methionine to *C. fusca* cultures in the presence and absence of acetate (as in Fig. 2) is shown together with densitometer traces of protein on the gels of the samples taken at 30min. The amount of radioactivity attributed to isocitrate lyase is indicated by the solid part of each histogram.

interpretation is untenable. Either the specific radioactivity of the methionine precursor pool(s) supplying protein synthesis was lower '—acetate', or the overall rate of protein synthesis was lower. The latter could be due to the greater availability of energy in the presence of acetate, which is readily oxidized by *C. fusca* (Merrett & Syrett, 1960). Where the difference in incorporation into the trichloroacetic acid-insoluble fraction is attributed to a difference in the overall rate of protein synthesis, if isocitrate lyase was stable in the absence of acetate the expected rate of incorporation of radioactivity '—acetate' would remain 21 d.p.m./min per mg of extract protein, calculated as above. Alternatively, when all of the difference in incorporation is attributed to a difference in pool specific radioactivity, the expected rate of incorporation into isocitrate lyase in the absence of acetate becomes (rate of incorporation '+acetate' = 1540) $\times$ (ratio of rates of increase of isocitrate lyase activity '—acetate'/' +acetate' = 0.0134) $\times$ (ratio of rates of incorporation into the trichloroacetic acid-insoluble fraction '—ac-

tate'/' +acetate' = 0.2) = 4 d.p.m./min per mg of extract protein. The observed value of 11 d.p.m. (Table 1) lies between these two estimates.

The alternative hypothesis, that the difference between the rates of increase of isocitrate lyase activity was due to turnover of the enzyme '—acetate', gave the predicted curves in Fig. 4. This demonstrates that the observed incorporation of radioactivity into the enzyme '—acetate' was very different from the incorporation kinetics expected if turnover of the enzyme was entirely responsible for the lower rate of increase of enzyme activity in the absence of acetate. The predicted curves were constructed by assuming either that the overall rate of protein synthesis and the precursor pool specific radioactivity were the same in the presence and absence of acetate (curve *a*), or that one of these was 5-fold less in the absence of

Fig. 4. Comparison of the incorporation of radioactivity into isocitrate lyase in the absence of acetate with the incorporation predicted if degradation of the enzyme accounted completely for the difference in rate of increase of isocitrate lyase activity in the presence and absence of acetate

Observed incorporation into isocitrate lyase in the absence of acetate (●) are the data in Table 1. The amount of radioactivity incorporated into isocitrate lyase '—acetate' was predicted from the incorporation '+acetate' as follows. For curve (*a*), $A_n = A_{n-1} + \alpha - \beta(A_{n-1}/E_{n-1})$, where A_{n-1} was the amount of radioactivity in, and E_{n-1} was the amount of activity of, isocitrate lyase at the end of the previous minute, α was the gross amount of radioactivity incorporated in 1 min, taken as the incorporation rate in the presence of acetate (1540 d.p.m./min per mg of cell-free extract protein, from Table 1) and β was the rate of degradation of isocitrate lyase (the rate of increase of isocitrate lyase activity '+acetate' minus the rate of increase of enzyme activity '—acetate', i.e. $0.0187 - 0.00025 = 0.01845$ unit/min per 10^{10} cells, from the data in Fig. 1). The amount of isocitrate lyase present '—acetate' at the start of the period of incorporation (0.06 unit/ 10^{10} cells) was also taken from the data in Fig. 1. This predicted curve was based on the assumption that the overall rate of protein synthesis and the specific radioactivity of the methionine precursor pool(s) was the same in the presence and in the absence of acetate. For curve (*b*), the value of α was 308 d.p.m., which shows the effect of decreasing the specific radioactivity of the precursor pool '—acetate' to one-fifth of the value '+acetate', which is discussed further in the text. For curve (*c*), α was 308 d.p.m. and β was 0.00374, which shows the effect of decreasing the overall rate of protein synthesis '—acetate' to one-fifth of the rate '+acetate', that is, the gross rate of incorporation was taken as one-fifth of the rate '+acetate' and the degradation rate was taken as one-fifth of the rate of increase of enzyme activity '+acetate' minus the rate of increase of activity '—acetate'. For the first 1 min of incorporation, the degradation component was ignored for all three predicted curves.

acetate (curves *b* and *c*). As discussed above, the difference in overall incorporation of radioactivity into protein (Fig. 2*b*) strongly suggested that curve (*a*) is unrepresentative, but comparison of this curve with curves (*b*) and (*c*) demonstrates that either the rate of protein synthesis or the precursor pool specific radioactivity would have to be less than in the presence of acetate by very much more than 5-fold to give an incorporation curve similar to the observed result. This in turn could not be reconciled with the overall incorporation of radioactivity into protein (Fig. 2*b*). The same conclusion was reached where, for instance, the precursor pool specific radioactivity was decreased to one-tenth of the '+acetate' value and the overall rate of protein synthesis doubled, so that the overall incorporation into protein '-acetate' was held at one-fifth of the '+acetate' value (the curve for this is not shown) or vice versa. It should be noted, however, that the predicted curves are approximate; the calculations ignore the amount of radioactivity corresponding to degradation within the first minute of incorporation, which if included would marginally lower the amount of incorporation, and degradation was computed from the amount of radioactivity present at the beginning of each minute (rather than from an average value), which would also result in a marginal error. A rather larger error was probably introduced by ignoring the lag in incorporation which was apparent both in the data for overall incorporation of radioactivity into protein (Fig. 2*b*) and incorporation into isocitrate lyase (Table 1 and Fig. 4), but this delay would not radically alter the predicted curves other than by shifting them about 4 min later with respect to the observed result.

It should also be noted that our data do not show that isocitrate lyase was absolutely stable in the absence of acetate, or for that matter when acetate

was present, as absolute rates of enzyme synthesis cannot be measured without precise measurement of precursor pool specific radioactivity (Koch, 1962). Rather, by comparison of rates of increase of enzyme activity with rates of incorporation of radioactivity we conclude that the rate of isocitrate lyase synthesis was less when acetate was absent. Similar results were obtained when [³⁵S]methionine was replaced by [³H]leucine (results not shown), but with this radioisotope label recovery of radioactivity after immunoprecipitation and after electrophoresis was not quantitative.

The greater rate of increase of isocitrate lyase activity in the presence of acetate therefore reflected an increase in translation of isocitrate lyase mRNA.

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