A Structural Gene for *Neurospora crassa* Isocitrate Lyase

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SUMMARY

A purification procedure for *Neurospora crassa* isocitrate lyase (ICL) is described. Thirteen mutants at the *acu-3* locus produced no detectable ICL activity either on sucrose as sole carbon source or in the presence of acetate. These mutants also produced little or no protein able to neutralize anti-ICL antibodies. Selection of revertants, able to grow on acetate as sole carbon source, from one of the *acu-3* mutants gave one class with moderately thermolabile and one with highly thermolabile ICL as well as apparently true revertants with ICL of wild-type stability. The mutants with highly labile ICL grew on acetate at 25° but not at 37°. One of the temperature-sensitive revertants was analysed genetically and shown to be due to further mutation within or close to *acu-3* itself.

INTRODUCTION

Neurospora crassa can grow on acetate as sole carbon source (Flavell & Fincham, 1968*a*). During growth on acetate, the glyoxylate cycle replenishes the tricarboxylic acid (TCA) cycle which generates energy and supplies metabolites to the biosynthetic pathways of the cell. The glyoxylate cycle enzymes, isocitrate lyase (ICL) and malate synthase, are present only in low (repressed) amounts during growth on sucrose, but on transfer of the mycelium to acetate, the specific activity of both enzymes increases to a higher (derepressed) level.

Fourteen mutants of *Neurospora crassa* which fail to grow on acetate and which lack ICL activity have been isolated (Flavell & Fincham, 1968*a, b*). Malate synthase is present in these mutants. They form a single complementation group which maps at a locus designated *acu-3* between *asp* and *inos* on linkage group V of the genome. The absence of ICL activity could be due to a defect in a structural or a regulator gene for ICL, or in a gene necessary for the proper integration of ICL into a cell structure (Woodward, 1968). If *acu-3* is a structural gene, *acu-3* mutants may produce altered forms of ICL recognizable through changes in thermostability or kinetic properties or, in the absence of enzyme activity, as immunologically cross-reacting material (CRM). Mutation in an ICL regulator gene would alter the amount of ICL present without affecting its structure. Attempts to discover whether *acu-3* is the structural gene for ICL are described below.

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METHODS

Neurospora crassa strains. The wild-type strains used were St Lawrence's *stA* (74*A*) and *sta*, a wild-type strain highly inbred with 74*A*; *inos-6-8 A* had been obtained after six successive backcrosses to *stA* (Fincham, 1967). The *acu-3* mutants were induced in *inos-6-8 A* or *stA* (Flavell & Fincham, 1968*a*). They are identified in this paper by their original isolation numbers placed in parentheses after the *acu-3* symbol.

Media. Vogel's minimal medium (Vogel, 1956) was supplemented with 1% sucrose (sucrose medium) or with 1 M-sodium acetate solution to a final concentration of 40 mM-acetate (acetate medium). Solid medium contained 1.5% agar. Other supplements were added as necessary: amino acids at a concentration of 50 mg./100 ml. and inositol at 0.2 mg./100 ml. To induce colonial growth on solid medium, 0.2% sucrose and 0.9% sorbose or 0.2% sorbose and 40 mM sodium acetate, was added to the Vogel's salt solution. Crosses were made on Westergaard & Mitchell's crossing medium (Westergaard & Mitchell, 1947).

N-methyl-N'-nitro-N-nitrosoguanidine (NTG) treatment and isolation of revertants. Five to 7 day conidia of an *acu-3 inos* strain were suspended in sterile water at a concentration of between 10^6 and 10^7 conidia/ml. The suspension was shaken with 0.1 mg./ml. of NTG at 30° for 12 min. This killed about 50% of the conidia. The conidia were centrifuged, washed in water, and resuspended in acetate medium + inositol + 0.1% agar to give 10 times the original volume of suspension. Two ml. aliquots were pipetted into tubes, incubated at 25°, and the cultures which grew tested for growth on inositol; *inos*⁺ cultures were discarded. The remainder were backcrossed to an *acu-3* strain of opposite mating type. Single ascospores which grew on acetate were isolated, one spore being selected from each of the original revertant colonies.

Crossing procedure. One strain was allowed to grow by itself on Petri dishes for 6 days after which the resulting protoperithecia were fertilized with about 1 ml. of a conidial suspension of the other strain.

Growth of Neurospora crassa. Mycelium for enzyme assays was grown in 100 ml. shake cultures of sucrose medium and derepressed in the same volume of acetate medium (Fincham & Flavell, 1968*a*). Inositol (1 mg./100 ml.) was added if required.

Enzyme assays. Extracts for assay were made by grinding mycelium with sufficient acid-washed sand and ice-cold ICL assay buffer to make a slurry. The slurry was centrifuged at 23,000 *g* for 30 min. and the supernatant used as a crude extract.

ICL and malate synthase were measured at 30° by the methods of Dixon & Kornberg (1959). For ICL, the reaction mixture contained, in 2.6 ml., 5 μ moles DL-isocitrate, 10 μ moles phenylhydrazine HCl, 0.1 ml. enzyme extract and 0.1 M, pH 6.8, sodium phosphate buffer (ICL assay buffer) containing 1 mM-EDTA, 5 mM-MgCl₂ and 0.28 ml./l. of mercaptoethanol (Sigma type 1). The increase in optical density at 324 nm. was measured. For the malate synthase assay the reaction mixture contained, in 0.4 ml.: 20 μ moles sodium phosphate buffer, pH 7.1; 2 μ moles MgCl₂, 0.025 μ mole acetyl CoA and 5 to 20 μ l. enzyme extract. The fall in optical density at 232 nm. was measured. Units of enzyme were expressed as μ moles product per h. Specific activity of enzyme = μ moles product/h./mg. protein.

Protein was measured by a micro-biuret method (Itzhaki & Gill, 1964).

Identification of the ICL band in electrophoresis gels. Acrylamide gel electrophoresis was carried out by the method of Davis (1964). Crude extracts of wild-type myce-

lium, after derepression of the glyoxylate cycle enzymes by transfer to acetate, showed the appearance of a strong protein band which was identified as ICL by running adjacent to purified ICL in a split polyacrylamide electrophoresis gel. Similarly derepressed extracts of an *acu-7* mutant, thought to be defective in the α -oxoglutarate oxidase system (Flavell & Fincham, 1968*b*), gave an even stronger ICL band after similar derepression; here the ICL was the most conspicuous protein in the electrophoretic pattern. Four *acu-3* mutants, numbers 11, 21, 23 and 53, were tested in a similar manner but none produced any detectable protein band in the ICL position.

CRM tests. Antibodies were induced in a New Zealand White rabbit. An extract of acetate-derepressed *Neurospora crassa* in 0.02 M-sodium phosphate buffer (pH 7.4) + complete Freund's adjuvant was injected intrascapularly, followed by two more weekly injections of extract + incomplete Freund's adjuvant. The rabbit was bled a week after the last injection. Non-immune serum was obtained from the rabbit before the first injection. The blood was stood at room temperature for 12 h. and the serum was decanted, and centrifuged. It was concentrated by dialysing overnight against 24 % saturated $(\text{NH}_4)_2\text{SO}_4$; the precipitate was dissolved in the minimum amount of 0.1 M phosphate buffer (pH 7.0) and dialysed against the same buffer.

The ability of the antiserum to inhibit ICL was demonstrated by incubating serum and ICL assay buffer in a total volume of 1.6 ml. with 0.025 ml. of an extract of acetate-derepressed *STA* at 30° for 30 min. It was found that 0.05 ml. of serum inhibited 2.2 units of ICL by 40 %. No inhibition was observed when extracts were incubated alone or with non-immune serum.

The ability of extracts of *acu-3* mutants to protect ICL against anti-ICL serum was tested as follows. The strain under test was grown on sucrose medium and derepressed by transfer to acetate medium. The 0.1 ml. samples of extracts which showed at least a tenfold derepression of malate synthase were each incubated with 0.05 ml. of antiserum. Then 0.025 ml. of test ICL was added, and the mixtures were reincubated and finally assayed for ICL. The remaining activity of the test ICL was compared with the activity of ICL incubated for 30 min. at 30° with (a) 0.05 ml. antiserum and 0.1 ml. buffer, (b) 0.05 ml. non-immune serum and 0.1 ml. buffer, (c) 0.15 ml. buffer. The amount of CRM in the extracts was expressed as units of test ICL protected/mg. of protein in the CRM extracts.

The amount of CRM in extracts of sucrose-grown and acetate-derepressed wild-type *STA* was also found. Amounts of *STA* extract were chosen so that their ICL activity was completely inhibited during the preincubation and so did not interfere with the assay of the test ICL.

Double-diffusion plates. The method of Ouchterlony (1953) was used.

Purification of ICL

(1) **Preparation of extract.** *STA* was grown from a heavy conidial inoculum on sucrose medium in a 101 New Brunswick 'Microferm' fermentor jar, collected by filtration through muslin and then transferred to acetate medium for 6 h. before being harvested. The mycelium was frozen in liquid N_2 , homogenized in a Waring blender, and the frozen powder stirred into cold ICL assay buffer. The mixture was centrifuged and the supernatant was decanted and used as the crude extract. All subsequent operations were carried out at 4°.

(2) **$(\text{NH}_4)_2\text{SO}_4$ precipitation.** Solid $(\text{NH}_4)_2\text{SO}_4$ was added to the extract to 45 %

saturation, the precipitate was discarded and the supernatant brought to 35% saturation. The resulting precipitate was dissolved in a buffer similar to that used in the ICL assay, except that the phosphate was reduced to 0.01 M and dialysed overnight against the same buffer.

(3) *DEAE chromatography*. The dialysed $(\text{NH}_4)_2\text{SO}_4$ fraction was applied to a column of DEAE cellulose equilibrated with the 0.01 M-phosphate buffer and eluted with the same buffer. The breakthrough fractions contained ICL and were dialysed against the 0.1 M-ICL assay buffer with the addition of 5 M-glycerol.

(4) *Sephadex gel*. The active fractions from the DEAE column were applied to the top of a Sephadex G200 column equilibrated with the 0.1 M assay buffer and were eluted with the same buffer. Fractions containing ICL were run through the column again; and the ICL-containing fractions from the second column were concentrated by pressure dialysis.

Studies on temperature sensitivity of revertants. Growth rates were measured at 25° and 37° in 1 cm. × 30 cm. growth tubes (Ryan, Beadle & Tatum, 1943) each containing 10 ml. of acetate or sucrose medium. To test the heat-sensitivity of ICL *in vitro*, extracts containing 2 to 3 mg./ml. protein were made from acetate-derepressed *stA* and revertants. Aliquots of 0.1 ml. were heated in a water bath for 8 min. Then 1.6 ml. of assay buffer at 30° was added and the mixture assayed for ICL.

RESULTS

ICL purification

The progress of the purification is shown in Table 1. The purified ICL gave a single band on polyacrylamide gel electrophoresis and a single band when run on a double-diffusion plate against antiserum to a crude extract of acetate-derepressed *stA*. It lost activity on freezing and thawing, and at 4° one preparation of the enzyme lost half of its activity in 14 days.

Table 1. *Progress of ICL purification*

Fraction	Volume (ml.)	ICL units/ml.	Protein (mg.)	(%) Recovery of units	(%) Recovery of protein	Specific activity	Purification factor
(1) Crude extract	1,000	90,600	6,500	100	100.0	15	1.0
(2) 45 to 65 % $(\text{NH}_4)_2\text{SO}_4$ precip. dialysed	70	88,800	1,620	83	25.0	54	3.6
(3) DEAE column	128	44,400	142	46	2.2	310	20.6
(4) Dialysis	34	37,200	142	39	2.2	260	—
(5) Sephadex:							
Col. 1	115	32,400	67	34	1.2	480	32.0
Col. 2	93	18,300	30	19	0.4	610	41.0
(6) Concentration	14	7,440	24	8	0.37	310	—

CRM tests

Table 2 shows the ability of extracts of *stA* and *acu-3* to protect ICL from anti-ICL serum. The derepressed *stA* extract contained 10 times as much CRM as the extract from sucrose-grown mycelium. This agrees with the fact that *stA* derepressed extract shows about 10 times as much ICL activity as sucrose-grown extract. Most *acu-3*

mutants gave no detectable CRM, or a level of CRM no higher than that found in sucrose-grown *stA*. These results were confirmed by double-diffusion plates. When extracts of acetate-derepressed *stA acu-3* and pure ICL were placed in alternate wells round a central well containing anti-ICL serum, a continuous precipitin band indicating a component common to *stA* extract and pure ICL was observed. No band was observed with wells containing the same amount of *acu-3* extract; indicating that the amount of CRM in *acu-3* is less than the amount in acetate-derepressed *stA*.

Table 2. Tests for CRM in *acu-3* mutants grown on sucrose and acetate medium

Strain	ICL specific activity		Malate synthase specific activity		Protein tested (mg.)	Amount CRM
	Sucrose	Acetate	Sucrose	Acetate		
<i>stA</i> (wild-type)	—	9.1	—	32.0	0.03	12.0
<i>stA</i>	0.6	—	0.9	—	0.15	1.2
<i>acu-3</i> (33)	0	0	1.3	20.5	0.10	0
<i>acu-3</i> (46)	0	0	1.1	49.0	0.24	0
<i>acu-3</i> (22)	0	0	1.1	34.0	0.38	0.6
<i>acu-3</i> (53)*	0	0	0.8	20.0	0.13	0
<i>acu-3</i> (50)*	0	0	—	38.6	0.38	0
<i>acu-3</i> (23)	0	0	—	20.0	0.40	1.2
<i>acu-3</i> (178)*	0	0	—	20.0	0.48	0.3
<i>acu-3</i> (115)*	0	0	—	21.0	0.55	0
<i>acu-3</i> (11)	0	0	1.8	39.0	0.33	0
<i>acu-3</i> (52)*	0	0	1.0	24.0	0.40	0
<i>acu-3</i> (80)*	0	0	1.1	20.0	0.49	0
<i>acu-3</i> (121)*	0	0	1.7	20.0	0.44	0
<i>acu-3</i> (38)	0	0	2.3	23.0	0.39	0

* These strains were isolated by mutation from *inos-6-8A* and still carried *inos*; they were grown with an inositol supplement. The other mutants were isolated from *stA* and were grown on minimal medium.

Revertant studies

Since no altered ICL was detected in the original *acu-3* mutants, we decided to obtain revertants from them which might produce ICL differing from 'wild-type ICL in heat-sensitivity or other properties. One hundred and ten revertants were obtained from *acu-3* (115) and 29 of these were tested (a) for their heat-sensitivity at 25° and 37° when growing on sucrose and on acetate medium, and (b) for the heat-sensitivity of their ICL *in vitro* at 45° and 50°.

Three main types of revertant were obtained; two groups, numbering 15 and seven and typified by 115 R6 and 115 R20 respectively, with heat-sensitive forms of ICL; and one group, numbering only two and including R48, with ICL similar to the *stA* enzyme (Table 3).

The growth of 115 R20 was somewhat depressed on acetate medium at 37° while 115 R6 did not grow at all on acetate at this temperature. On sucrose 115 R6 and 115 R20 grew equally well at 25° and 37°. All media were supplemented with inositol since the original *acu-3* (115) strain carried *inos*. Most of the subsequent work was done on revertant R6.

Fig. 1 shows the rate of growth of *acu-3* (115 R6), *stA* and *acu-3* (115) on acetate, and Fig. 2 shows the heat-inactivation curves for ICL in extracts of R6, *stA* and in a mixture of R6 and *stA* extracts.

Complementation tests on a temperature-sensitive revertant

Forced heterokaryons were constructed between a double mutant *inos acu-3* (115 R6) *A* strain (isolated in the course of the genetic analysis described in the next section) and two different *acu-3* mutants of the original series, *acu-3* (22) *A* and *acu-3* (38) *A*. These heterokaryons grew on acetate medium without inositol supplement at 25° at the wild-type rate but failed to grow at all on the same medium at 37°. Thus *acu-3* (115 R6) shows no complementation with either of the ICR-negative *acu-3* mutants tested; neither is its expression altered in the heterokaryons.

Fig. 1

Fig. 2

Fig. 1. The growth rates on solid acetate medium at 25° and 37° of: *stA* (x), *acu-3* (115 R6) (○), *acu-3* (115) (●). Transfers of growth tubes between the 25° and 37° incubators were made as indicated.

Fig. 2. The heat-sensitivity at 45° of ICL from *stA* (○), *acu-3* (115 R6) (●), and a mixture of equal amounts of ICL units from *stA* and *acu-3* (115 R6) (◐). The line drawn for the mixture is that expected for independent behaviour of two enzyme species.

Table 3. *Growth of revertants at different temperatures and stability of their ICL in vitro*

Strain		Growth on acetate (mm/h.)		% Remaining ICL activity after 8 min. at	
		25°	37°	45°	50°
Revertants from <i>acu-3</i> (115)	<i>stA</i> (wild-type)	3.2	3.1	100	60
	<i>acu-3</i> (115 R6)	2.2	0	4	3
	<i>acu-3</i> (115 R20)	3.0	2.5	100	22
	<i>acu-3</i> (115 R48)	2.2	2.0	100	55

Genetic studies

If the revertants which produce temperature-sensitive forms of ICL carry suppressor mutations in genes other than *acu-3*, they should segregate the original *acu-3* mutant in 25 % of the progeny if the suppressor is not linked to *acu-3*, or in lower frequency if there is linkage. Genetic analysis was, however, hampered by the fact that *acu-3* ascospores show very poor germination even in sucrose. Even *R6* ascospores germinate somewhat less well than wild-type.

Table 4. Genetic analysis of *acu-3* (115 *R6*) (*acu-3*^t)

Cross	Progeny genotypes	No. observed	% Recombination between
<i>inos acu-3</i> ^t <i>A</i> × wild-type <i>a</i>	<i>acu-3</i> ^t <i>inos</i>	13	<i>acu-3</i> ^t — <i>inos</i> = 13 %
	+ <i>inos</i>	4	
	<i>acu-3</i> ^t +	1	
	+ +	21	
<i>asp</i> ⁺ <i>acu-3</i> ^t <i>inos</i> ⁺ × <i>asp acu-3</i> ^t <i>inos</i>	<i>asp</i> + <i>inos</i>	90	<i>asp</i> — <i>acu-3</i> ^t = 8.3 % (<i>asp</i> — <i>acu-3</i> = 6.3 %)* <i>acu-3</i> ^t — <i>inos</i> = 20.8 (<i>acu-3</i> — <i>inos</i> = 20 %)* <i>asp</i> — <i>inos</i> = 25.9 %
	+ <i>acu-3</i> ^t +	84	
	<i>asp</i> + +	28	
	+ <i>acu-3</i> ^t <i>inos</i>	18	
	+ + <i>inos</i>	8	
	<i>asp</i> <i>acu-3</i> ^t +	8	
	+ + +	4	
	<i>asp</i> <i>acu-3</i> ^t <i>inos</i>	0	

* Values previously obtained for *acu-3* (Flavell & Fincham, 1968*a*).

The *R6* phenotype will be represented by symbol *acu-3*^t, a usage which receives some justification from the following results. Spores from *inos acu-3*^t × *STA* were germinated on supplemented sucrose medium at 25°, and 39 spores were tested to determine their genotype. An *acu-3*^t *inos*⁺ strain from this first cross was then mated with the double mutant *asp inos*; 240 germinated spores were picked at random and analysed for the presence of *acu-3*, *acu-3*^t, *inos* and *asp*. Germination from this cross was about 80 %. No *acu-3* cultures of the original non-temperature-sensitive type were obtained and nearly half the cultures were *acu-3*^t. The recombination between *asp/acu-3*^t, *acu-3*^t/*inos* and *asp/inos* may be compared with the recombination frequencies previously obtained (Flavell & Fincham, 1967*a*) between *acu-3*, *inos* and *asp*.

The results in Table 4 indicate that the further mutation in *acu-3*^t is at least close to the original *acu-3* mutation and, in all likelihood, within the same gene.

DISCUSSION

The production of an altered ICL by each of two kinds of *acu-3* revertants is strong evidence that *acu-3* is indeed the structural gene for ICL. Both the mapping and the complementation data are consistent with this view. It remains possible that the 'reverse' mutation in *R6* activated a normally inactive gene closely linked to *acu-3* coding for a separate and more labile species of ICL. It is also not formally ruled out that the *R6* mutant could carry a closely linked dominant (or *cis*-dominant) suppressor, perhaps resulting in the reading of a nonsense codon as a mis-sense codon, though the high efficiency of suppression which would have to be postulated in this case would be

unusual. Neither of these two alternative hypotheses seems at all likely. The temperature-sensitive nature of the *acu-3* + *acu-3*⁺ heterokaryons rules out the possibility that the temperature sensitivity could be due to a *recessive* suppressor mutation.

A surprising result was the failure to find CRM in significant amounts in any of 14 different *acu-3* mutants. These mutants probably represent many different sites within the gene since intercrosses among five members of the series gave low frequencies of wild-type recombinants in every cross except one (B.J. Leckie, unpublished). On the basis of results obtained with other structural genes in fungi (Roberts & Pateman, 1964) and bacteria (Margolies & Goldberger, 1968), we had expected that a proportion of the *acu-3* mutants would be mis-sense mutations producing CRM. Possibly many of them are the result of nonsense mutations producing drastically shortened (and CRM-negative) polypeptide chains. However, no evidence was found for suppression of any of the *acu-3* mutants by Seale's nonsense suppressor *su-am*¹⁷ (Seale, 1968).

Two forms of ICL have been reported from *Neurospora* (Sjogren & Romano, 1967) but only one is induced strongly by acetate. The *acu-3* gene appears to code for the acetate induced enzyme (ICL-1) and not for ICL-2, since *acu-3 arg-10* double mutants have been found to produce appreciable amounts of ICL-2 under the conditions of arginine limitation which have been reported to cause derepression of this enzyme (Flavell & Woodward, 1970; B. J. Leckie, unpublished).

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