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# Pyruvate carboxylase and N. Crassa suc mutants

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## Abstract

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The enzyme lesion in succinate-requiring (*suc*) mutants was investigated by Strauss (1957 J. Biol. Chem. 225:535) who concluded that these mutants were deficient in a carboxylating enzyme identified as 'oxalacetic acid carboxylase.' From current understanding of the carboxylating enzymes (see reviews in P. D. Boyer (ed.) 1972 The enzymes, Vol. 6, Academic Press, New York), it is probable that Strauss's assay systems measured the activity of the enzyme now termed phosphoenolpyruvate (PEP) carboxykinase (EC. 4. 1. 1. a). This enzyme is implicated in PEP synthesis during gluconeogenesis in vertebrate liver tissue. Convincing evidence that this enzyme also serves this gluconeogenic role in *N. crassa* comes from the observation that *acu*-6 mutants, which are PEP carboxykinase deficient, are unable to grow on acetate but are normal in their growth on sucrose (Flavell and Fincham 1968 J. Bacteriol. 95: 1063). In the course of a study of *N. crassa* PEP carboxykinase it was decided to re-examine the enzyme complement of *suc* mutants, as it seemed probable that the *in vitro* differences in carboxylating activity observed by Strauss reflected meta-

bolic regulation of PEP carboxykinase rather than the absence of a carboxylating enzyme. It was found that suc mutants grow as well as wild type on acetate and under these conditions have high levels of PEP carboxykinase, which is thus clearly not the enzyme deficient in these strains.

As concluded by Strauss, the growth properties of suc mutants suggest that they lack an 'anaplerotic' carbon dioxide fixing enzyme making oxaloacetate in vivo. In vertebrates, yeast and Aspergillus, the enzyme pyruvate carboxylase (EC 6.4.1.1) has been identified as carrying out this role (see the review by Scrutton and Young 1972 in The enzymes, vol. 6 and Skinner and Armitt 1972 FEBS Lett. 20:16) and it is this enzyme that is the most reasonable candidate for the enzyme lesion of suc mutants. Assays have been developed that demonstrate the presence of this enzyme in extracts from wild type and show that it is absent from extracts of suc strains. Some properties of the enzyme have been investigated.

Mycelium was grown in liquid shake cultures in 2 liter Erlenmeyer flasks with 1200 ml of Vogel's medium N plus 2% sucrose plus 4.3 mM Na succinate. Conidia were inoculated to give $10^5$/ml and mycelium was harvested during the phase of rapid growth when the fresh weight yield was about 0.8g/100 ml (24 hr for STA4 and 48 hr for the suc strains.) Mycelium was harvested by filtration and frozen at -15°C. Extracts were made by grinding frozen mycelium with glass beads (Ballotini No. 11) and 0.05 M tris-HCl buffer pH 7.4 containing 1 mM EDTA (1/1.5/6; w/w/v). The homogenate was centrifuged for 20 minutes at 30,000 x g and the supernatant used as 'crude extract'. Two continuous spectrophotometric assays, essentially the same as those described by Payne and Morris (1969 J. Gen Microbiol. 59: 97), were used (Table 1). The pyruvate-dependent rate of absorbance increase at 412 nm and 25°C was followed for assay (a) and the rate of decrease at 340 nm and 30°C followed for assay (b). Endogenous citrate synthetase and malate dehydrogenase were in excess in all extracts. Rates of absorbance change were linear for at least two minutes.

Table 1. Assay of pyruvate carboxylase in wild-type N. crassa.

| Assay components | Specific activity of pyruvate carboxylase (μmoles product/min/mg protein)* | | |
|---|---|---|---|
| | Crude extract Assay (a) | Fractionated extract** Assay (a) | Assay (b) |
| Complete system | 0.009 | 0.014 | 0.022 |
| Minus $KHCO_3$ | 0.000 | 0.005 | 0.008 |
| Minus $MgCl_2$ | 0.005 | 0.000 | 0.000 |
| Minus ATP | 0.000 | 0.000 | - |

*Assay (a) mixture contained in 0.5 ml final vol: 0.25 ml 0.2M tris-HCl buffer pH 8.0; $KHCO_3$ 5 μmole; $MgCl_2$ 5 μmole; ATP 2.5 μmole; acetyl-CoA 0.05 μmole; DTNB 0.5 μmole; pyruvate 1 μmole; crude enzyme extract 50 μl.
Assay (b) mixture contained in 3 ml: 2 ml 0.1M tris-HCl buffer pH 8.0; $KHCO_3$ 20 μmole; $MgCl_2$ 20 μmole; ATP 10 μmole; $NADH_2$ 0.28 μmole; pyruvate 6 μmole; enzyme extract 0.2 ml. The rate of absorbance change without pyruvate was about half that with pyruvate for both assay systems using crude enzyme extracts, Using fractionated enzyme the 'background' was zero.
**The 30-40% $(NH_4)_2SO_4$ fraction of a sample of crude extract was redissolved in extraction buffer.

Table 1 summarises results of assays with wild type extracts confirming the dependence of activity on bicarbonate, $Mg^{++}$ and ATP. Activity was also shown to be proportional to the volume of extract added. Both assays are rather insensitive in relation to the activities measured, and the precision of the values given is estimated at about ± 0.002 units. Ammonium sulfate fractionation allowed more reliable estimates to be made because of the removal of 'background'. Adding aspartate to a level of 1 mM reduced the activity to about 10% of the control. Acetyl-CoA, added to the 0.1 mM level, did not stimulate the enzyme. In these latter two properties the enzyme more closely resembles the yeast and Aspergillus enzyme than that from vertebrate liver, which is inactive without acetyl-CoA and is not inhibited by aspartate. The conclusion that acetyl-CoA is not essential for activity is tentative as significant levels of acetyl-CoA may be present in the crude extracts. The specific activities of pyruvate carboxylase of wild type after growth on glutamate, aspartate or ethanol were only about 50% of the level after growth on sucrose. This contrasts with the behavior of PEP carboxykinase, which is derepressed 10- to 100-fold after growth on these carbon sources. The presence of PEP carboxylase (EC 4.1.1.31) in crude extracts of wild type was tested for by substituting PEP for pyruvate plus ATP in assay (b). No activity was detected.

No pyruvate carboxylase was found in the two suc strains examined (alleles 39311 and 66702; strains FGSC #1248 and 1214). An activity of less than about 20% of the wild type level would not, however, have been detected. Nevertheless, these results provide some direct support for the suggestion that pyruvate carboxylase is the enzyme deficient in suc mutants.

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