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REGULATION OF LACTATE/PYRUVATE RATIOS BY CYCLIC AMP IN NEUROSPORA CRASSA

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Cyclic AMP is thought to have a general role in stimulating the breakdown of carbohydrate reserves and subsequent glycolytic activity. This would be expected to increase the availability of reducing equivalents in the form of cytoplasmic NADH. The current study examines another potential reaction controlling cytoplasmic NADH in the fungus Neurospora crassa, that of lactate dehydrogenase, to determine whether it is also regulated by cyclic AMP. The cr-1, adenylate cyclase and cyclic AMP-deficient mutant, grown with and without exogenous cyclic AMP was compared with an isogenic wild type. The results show that cyclic AMP raises pyruvic acid pools and lowers both lactic acid pools and lactate/pyruvate ratios. It does that, in part or in whole, by lowering lactate dehydrogenase activity. The possibility that cytoplasmic NAD+/NADH is a major target of cyclic AMP control is discussed. The high performance liquid chromatography procedures used in these studies are applicable to the measurement of intracellular pools of tricarboxylic acid cycle and other organic acids.

Cyclic AMP in fungi has been reported to regulate a variety of metabolic processes (1,2). The only identified substrates of cyclic AMP - dependent protein kinase in fungi are either enzymes involved in metabolism or the protein kinase itself (1,2). This pattern has led to the suggestion that many of the effects of cyclic AMP may be direct or indirect consequences of cyclic AMP influences on metabolism. It is important, therefore, to have a variety of measures of cyclic AMP effects on metabolism in fungi.

Cyclic AMP appears to produce an evolutionarily conserved stimulation of the breakdown of carbohydrate reserves (see, for example, ref. 1-7) and subsequent stimulation of glycolysis (2,8,9). In Neurospora, the glycolytic stimulation appears to be associated with inhibition of gluconeogenesis (9).

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Because much of the documented influence of cyclic AMP centers on the increased availability and metabolism of carbohydrates, it is important to determine what consequence of this increase may be a main target of cyclic AMP regulation. Obviously, glycolytic stimulation can lead to an increase in energy metabolism, possibly reflected in ATP/ADP ratios; it can lead to the generation of reducing equivalents as reflected in NAD/NADH ratios; it can lead to increased availability of metabolic precursors for biosynthetic pathways.

The current study attempts to look at the second possibility by investigating another possible regulatory point which may regulate the cytoplasmic NAD/NADH ratios. Lactate dehydrogenase (L-lactate:NAD+ oxidoreductase, E. C. 1.1.1.27) regulates the oxidation-reduction of lactate and pyruvate and may, consequently, influence the cytoplasmic NAD/NADH ratio. In this study we show that lactate/pyruvate ratios are regulated by cyclic AMP in Neurospora crassa, that regulation being mediated by cyclic AMP regulation of lactate dehydrogenase.

MATERIALS AND METHODS

St. Lawrence wild type strain 74OR23-1A and an essentially isogenic (9) cr-1 mutant strain (backcrossed eight times to the St. Lawrence wild type) were used in this study. These were grown essentially as described earlier (9) in Vogel's medium N (10) containing 2% sucrose at either 30° or 25° to about 1 to 1.5 mg dry weight per ml of culture medium. Where cyclic AMP was to be used, it was added from 1M stock of the sodium salt of cyclic AMP, to a final concentration of 30mM in a 30° culture. Cyclic AMP fed cultures were harvested 3h after cyclic AMP addition.

100 to 150 mls of such cultures were harvested by rapid filtration on 47mm glass fiber filters. The mycelia were extracted for 10 min. at 0° in 1.1 ml of 1N $HClO_4$. The extract was centrifuged for 10 min. at 40,000xg and the supernatant was processed by a modification of the procedure Guerrant et al (11). 1 ml of extract was shaken with 5 mls of diethylether, 0.6g NaCl and 25 µl of CH_3CN for about 1 minute and centrifuged in a clinical centrifuge to separate the phases. The organic phase was removed and extracted with 0.6 ml of 0.1 N NaOH. After centrifugation, the aqueous phase was acidified with 1 drop of 1N H_2SO_4 from a Pasteur pipet and passed through a 0.2 µm pore syringe filter before being analyzed by high performance liquid chromatography.

The samples were analyzed using an Interaction Chemicals ION-300 (0.78 x 30 cm bed) column with column jacket maintained at about 40° with a mobile phase of 0.01 N H_2SO_4 flowing at 0.5 ml/min. The exact column temperature was adjusted to optimize resolution. The apparatus used was described earlier (9) except that the absorbance was measured at 214 nm at a sensitivity of 0.05 AUFS. Quantitation was by a Shimadzu C-R3A electronic integrator using external standards taken through the same extraction procedure as used for the samples. Where peaks were poorly resolved, they were enlarged on a photocopier and cut and weighed.

Lactate dehydrogenase assays were made using buffer A composed of 50mM

MOPS, pH 7.3, 5μM pepstatin, 1 μg/ml leupeptin, 2mM dithiothreitol, 10mM NaF and 0.5mM phenylmethylsulfonyl fluoride. 25 mls of culture was collected onto a 25 mm Whatman GF/A filter and quickly placed into 2ml of ice cold buffer A in a 16 x 150 mm tube. This was homogenized for 30 seconds at top speed using a Brinkmann Polytron homogenizer with PT10 ST probe generator. After high speed centrifugation at 4°, 10 to 100 μl of supernatant was assayed in a final volume of 1ml of buffer A containing 0.1mM NADH and 1mM pyruvate. The enzyme activity was measured by measuring the change in NADH absorbance at 340 nm at 22° using a Beckman DU-8 spectrophotometer. Protein in the extract was measured by the Lowry procedure.

Statistical differences between means were analyzed by Student's t test.

RESULTS AND DISCUSSION

This study was initiated using two approaches. Firstly organic acid pools, including pools of lactic and pyruvic acids, were measured by high performance liquid chromatography. A chromatogram of such organic acid pools from Neurospora is shown in Fig. 1. Several tricarboxylic acid cycle organic acids as well as lactate and pyruvate from cell extracts are well separated by this procedure (Fig. 1).

The second approach used here was to compare the properties of the cr-1

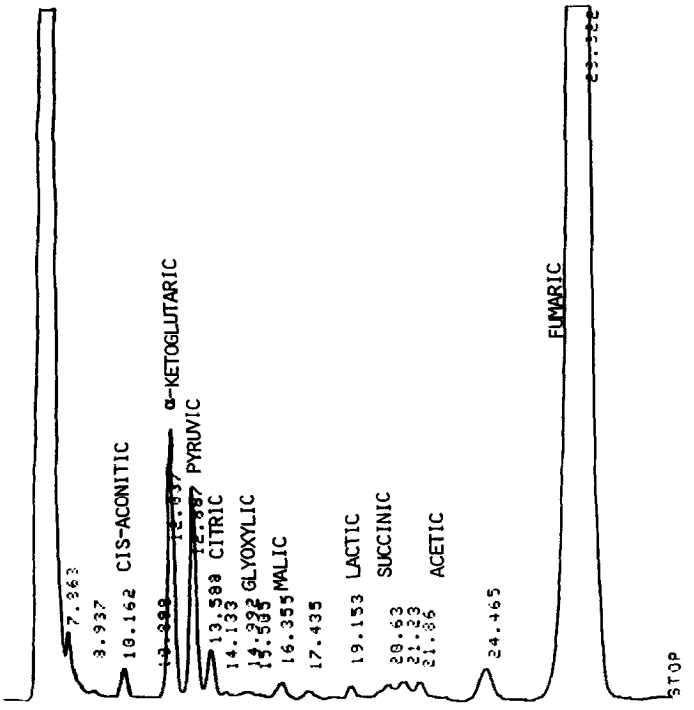

Figure 1. Organic Acid Chromatogram of Neurospora Extract.

An extract of Neurospora crassa wild type 74OR23-1A was analyzed by high performance liquid chromatography on an ION-300 column. Peak identification was made by comparing the elution times with those of standards.

adenylate cyclase and cyclic AMP-deficient mutant of Neurospora crassa (12-15) with those of an isogenic wild type strain. Aberrations in the cr-1 strain when compared with wild type is presumed to be due to its cyclic AMP deficiency. The cr-1 strain is responsive to exogenous cyclic AMP when it is grown at elevated (30-37°) temperatures (9). Consequently, a second prediction is that aberrations of the cr-1 strain due to its lack of cyclic AMP should be reversed in part or in whole by growth in cyclic AMP medium.

As shown in Table I, pyruvic acid pools in the cr-1 mutant are lower than those found in wild type and the pyruvic acid pools in the mutant are elevated by growth in the presence of cyclic AMP. Lactic acid pools show the converse pattern. Lactic acid pools are elevated in the mutant as compared with the wild type and lactic acid pools in the mutant decline when the mutant is grown with cyclic AMP in the medium (Table I). Thus cyclic AMP increases pyruvic acid pools and decreases lactic acid pools. The lactate/pyruvate ratios which reflect the activity of lactate dehydrogenase are much higher in the mutant grown in the absence of cyclic AMP than they are in wild type or in the mutant with cyclic AMP in the medium (Table I).

Although cultures grown at lower temperatures do not respond well to exogenous cyclic AMP (9), it is still possible to compare the cr-1 mutant with the wild type. For cultures grown at 25°, the lactate/pyruvate ratios of the cr-1 mutant were 5.1±1.1 as compared with the wild type ratio of 2.8±1.2

Table I

Cyclic AMP and Lactic Acid/Pyruvic Acid Pools in Neurospora

	wild type	cr-1	cr-1 (30mM cyclic AMP)
pyruvic acid (N=8)	1.16Δ ±0.46	0.81 ±0.47	1.63** ±0.40
lactic acid (N=8)	1.32* ±0.58	2.76 ±2.10	1.36* ±0.50
lactic acid/ pyruvic acid ratio (N=8)	1.14** ±0.20	3.26 ±1.23	0.83** ±0.19

Cultures were grown at 30°. Pyruvic acid and lactic acid pools are expressed as micromoles per ml of cell water.
Δ $p<0.10$ when compared with cr-1 control grown without cyclic AMP
* $p<0.05$ when compared with cr-1 control grown without cyclic AMP
** $p<0.005$ when compared with cr-1 control grown without cyclic AMP

(N=6 in both cases, $p<0.005$). At 25° then, the mutant also shows an elevated ratio over the wild type, providing further evidence for cyclic AMP control of pyruvic and lactic acid pools. Both mutant and wild type ratios are higher in 25° grown cultures than in 30° grown cultures (compare with Table I).

The pyruvic acid here is presumably generated by the glycolytic pathway with lactic acid coming from pyruvate via lactate dehydrogenase.

$$\text{pyruvic acid} + \text{NADH} + \text{H+} \xleftrightarrow[\text{dehydrogenase}]{\text{lactate}} \text{lactic acid} + \text{NAD+}$$

The decrease in lactate/pyruvate ratios produced by cyclic AMP can be explained in two alternative ways: 1. The lactate dehydrogenase reaction in Neurospora is essentially in equilibrium as it is thought to be in rat liver (16,17). In this case a decrease in lactate/pyruvate ratio must be accompanied by an increase in NAD/NADH ratio. This follows from the K_{eq} of the lactate dehydrogenase reaction:

$$Keq = \frac{(\text{lactate})\ (\text{NAD+})}{(\text{pyruvate})\ (\text{NADH})}$$

Thus if the reaction is in equilibrium, our results suggest that cyclic AMP produces increases in cytoplasmic NAD/NADH ratio. This is opposite what would be predicted from the discussion in the introduction.

The alternative hypothesis is that the lactate dehydrogenase reaction is not in equilibrium and that cyclic AMP lowers the lactate dehydrogenase activity. Such lowered lactate dehydrogenase activity would be expected to raise raise NADH levels by lowering the rate of NADH oxidation.

Levels of lactate dehydrogenase were measured in extracts of wild type and cr-1 mutant (grown with and without cyclic AMP in the medium). The mutant grown without cyclic AMP is found to have much higher levels of lactate dehydrogenase than either wild type or mutant grown with cyclic AMP (Table II). These results support the second hypothesis. They suggest that the lactate dehydrogenase reaction is not in equilibrium and show that it is regulated by cyclic AMP. A cyclic AMP-deficiency produces an increased activity of lactate dehydrogenase which, in turn, oxidizes NADH and increases lactate/pyruvate ratios.

One difficulty in pursuing these studies derives from the presence of two

Table II

Lactate Dehydrogenase Activity in Wild Type and cr-1

Wild type	cr-1	cr-1 (grown in 30mM cAMP)
44.3±16.8* (N=6)	134.1±32.3 (N=6)	86.9±20.0* (N=5)

Cultures were grown at 30°. Values are expressed as micromoles of NAD synthesized/min/mg protein.
* $p<0.01$ when compared with cr-1 control grown without cyclic AMP.

different cellular pools of NAD/NADH making it difficult to measure the cytoplasmic pools of these pyridine nucleotides (see, for example refs. 16,17). It is not possible, therefore, to directly measure cytoplasmic NAD+ and NADH pools.

The high performance liquid chromatography procedures described here may be useful in measuring intracellular pools of lactate, pyruvate and a variety of tricarboxylic acid cycle intermediates. The ION-300 column has good resolution for these acids when initially used although its resolution deteriorates rapidly as samples are run on it.

ACKNOWLEDGEMENT

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