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INVOLVEMENT OF CYCLIC AMP IN CAROTENOGENESIS AND CELL DIFFERENTIATION IN *BLAKESLEA TRISPORA*

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Summary

Cyclic adenosine 3′,5′-monophosphate (cyclic AMP) was detected in single and mated cultures of *Blakeslea trispora.*

Cyclic AMP levels increased on mating the plus and minus mycelia.

Trisporic acid and β-carotene levels were much higher in synthetic mucor medium supplemented with glycerol as compared to that with glucose.

Cyclic AMP induced significant morphological changes in plus and minus strains were comparable to those induced by mating event.

Introduction

Blakeslea trispora is a heterothallic mould of order mucorales. The two mating types plus and minus, are primarily distinguished by the fact that both are required for the collaborative formation of a group of hormone substances, the trisporic acids [1,2]. Although, the major function of trisporic acid is to induce sexual reproduction, its ability to derepress the carotene biosynthetic pathway has attracted the attention of most workers in this field [3,4]. We showed earlier that activation of carotenogenesis by trisporic acid molecule was by derepression of the enzyme(s) catalyzing conversion of 5-phosphomevalonate to dimethylallyl pyrophosphate [5]. Single cultures of *B. trispora* seem to contain all the genes required for trisporic acid synthesis since they spontaneously produce about 0.1 percent or less trisporic acid of the amount produced by mated cultures and therefore, are considered as non-producers for most purposes [6]. It is implied that some or all genes of trisporic acid synthesis are repressed in both the single cultures and that in the mated cultures all are derepressed.

From the evidences so far available, β-carotene is the probable precursor of trisporic acid [7,8]. Thus the system seems to be self-amplifying. Knowledge of factors controlling trisporic acid synthesis is essential for a biochemical understanding of carotenogenesis as well as sexual phenomenon. In the heterothallic system, catabolite repression mechanism has been linked with the whole spectrum of non-vegetative processes including so-called secondary metabolism. But no such factors or controls have been demonstrated with regard to trisporic acid formation in mucorales.

The objectives of present investigation were to elucidate the control of trisporic acid and β-carotene synthesis and to explore the possible involvement of cyclic AMP in control mechanisms. Experiments were carried out to demonstrate the presence of cyclic AMP in the mould and its role in regulating trisporic acid and β-carotene synthesis.

Materials and Methods

Mould strains. The plus (NRRL 2895) and minus (NRRL 2896) strains of *Blakeslea trispora* were obtained from Dr. A. Ciegler, U.S. Department of Agriculture Peoria, IL.

Maintenance. The cultures were maintained on slants containing synthetic mucor medium solidified with 1.8 per cent Difco agar [9]. Slants were kept at 20° C for 4—5 days and stored at 0—4° C. Transfers were made once a month.

Media for cultivation.

A. Synthetic mucor medium described by Hesseltine and Anderson [10], was used for most of the experiments. It contained glucose, 40.0 g; asparagine, 2.0 g; KH_2PO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.25 g; and thiamine HCl, 10.0 mg in 1000 ml of glass distilled water, pH of the medium was adjusted to 6.2. 250-ml Erlenmeyer flasks containing 100 ml of the medium were autoclaved at 10 lbs for 15 min.

B. Potato glucose thiamine medium described by Sutter [11] was used for trisporic acid production. Potato extract, 12.5 g; glucose, 20.0 g and thiamine HCl, 0.002 g were dissolved in 1000 ml of glass distilled water. Portions of 100 ml media were distributed in 250-ml Erlenmeyer flasks and autoclaved at 10 lbs for 15 min.

Inoculum preparation and cultivation. Mycelia from 4—5 days old agar slants were inoculated into 100 ml synthetic mucor medium containing 1.0 percent malt extract and were incubated for 2 days at 28 ± 2° C on a gyratory shaker (185 rev./min). Under these conditions, the mycelium formed one solid mass. Inoculum for the fermentation was prepared by aseptically transferring the thoroughly washed mycelial mass to Sorvall omnimixer and macerating it to make a homogeneous suspension. Equal volumes of the homogenate were used in all flasks for a given set of fermentations.

Single and mated cultures were incubated for 5 days at 28° C on a Gyratory shaker for the time specified. For analytical and preparative work on trisporic acid, the cultures were incubated in dark.

Growth determination. After the extraction of carotenoids, lipid-free mycelia were dried at 50° C in an oven and weighed. In the experiments where carotenoids were not extracted, mycelial growth was harvested by filtration,

pressed dry and weighed unless specified otherwise.

Extraction and estimation of carotenes. The cultures were filtered through muslin in a Buchner funnel, washed thoroughly with water and pressed as dry as possible. The mycelium was cut into small pieces and ground in acetone/diethyl ether (1 : 1, v/v) mixture. The mixture was filtered and the lipid-free residue re-extracted until the filtrate was colorless. The residue was collected, dried at 50°C and weighed. The solvent extract was washed with cold water and dried over anhydrous sodium sulphate. Total carotene content was estimated as β-carotene by $E^{1\%}_{1cm}$ value of 2500 at 450 nm as described by Davies [12].

Extraction and estimation of trisporic acids. Trisporic acid was extracted from the culture medium of mated cultures by the method described by Sutter [11]. The hormone was first extracted with chloroform. The chloroform fraction was extracted with 4 percent sodium bicarbonate, and the bicarbonate or acid fraction after acidification was extracted again with chloroform. The chloroform was evaporated to dryness and the residue was dissolved in 0.1 M Tris-sulphate buffer, pH 7.5. The trisporic acid content was calculated using $E^{1\%}_{1cm}$ value of 700 at 325 nm. All operations were performed under diffused light at 0—4°C.

Mating under different nutritional conditions. Plus and minus cultures were grown for 48 h in 100 ml liquid synthetic mucor medium separately. Mycelia, after washing in sterile distilled water, were transferred to fresh synthetic mucor medium containing glucose or glycerol as carbon source and incubated for 48 h or 72 h further as specified in the text. Carotene and trisporic acid from the mycelia were extracted and estimated.

Isolation of cyclic AMP from Blakeslea trispora. As controls, plus and minus cultures were grown for 72 h and cyclic AMP was extracted [13]. For cyclic AMP determination in mated cultures, single strains, grown for 66 h, were mated and incubated for 6 h to complete 72 h growth period. The mycelia were harvested by filtering under vacuum through several layers of a nylon cloth supported on a pyrex 47 mm filter holder. The mycelia were transferred to 7.5 percent trichloroacetic acid containing a known amount of ^{3}H-labelled cyclic AMP (spec. act. 20.8 Ci/mmol) as internal standard and homogenised in a teflon homogeniser. The homogenate was centrifuged at 19 000 × *g* for 15 min at 0°C. Pellet was saved for protein estimation. The supernatant was decanted into a separatory funnel and was extracted with cold water-saturated diethyl ether until the pH of aqueous phase was above 4. The aqueous phase was then heated to 80°C to remove the residual ether. It was further purified by adding Norit A (2 mg/ml). The charcoal was recovered by filtration through two layers of Whatman No. 1 paper and cyclic AMP was eluted with 2 percent NH_3 in 50 percent ethanol. The eluate was lyophilized, dissolved in 2 ml of glass distilled water and chromatographed on Biorad aminex MS cation exchange resin of mesh size 200—400 (packed volume 2 ml). The column was eluted with distilled water and 2-ml fractions were collected. The fractions containing ^{3}H-labelled cyclic AMP were pooled and lyophilised. For further purification, it was chromatographed on thin layer plates of Silica gel G using solvent system (*n*-butanol/methanol/ethyl acetate/ammonia, 7 : 3 : 4 : 4, v/v). After the chromatographic separation was over, the area corresponding to

cyclic AMP was scraped and eluted with 50 percent ethanol. The eluate was lyophilised and dry material was dissolved in glass distilled water for the determination of cyclic AMP.

Synthesis of [γ-^{32}P]ATP for protein kinase assay. [γ-^{32}P]ATP was prepared according to the procedure of Post and Sen [14]. After stopping the chain reactions in the process to incorporate ^{32}P in ATP, reaction mixture was loaded on DEAE Sephadex A-25 to purify ATP from unicorporated ^{32}P [15]. The column was eluted successively with 0.01, 0.1, 0.23 and 0.4 N NH_4HCO_3. Fractions containing [γ-^{32}P]ATP were pooled and desalted by adsorption to 200 mg of purified Norit A. cAMP was eluted from the charcoal with 2 percent NH_3 in 50 percent ethanol. The eluate was lyophilised and was dissolved in glass distilled water.

The radiopurity of each batch of ATP was established by chromatography on Whatman No. 1 paper in solvent system (isobutyric acid/ammonia/water (6 : 6 : 33, v/v). Product of the reaction mixture gave a single radioactive spot which co-chromatographed with authentic ATP.

Purification of cyclic AMP. Purified cyclic AMP fraction was obtained in four steps during the isolation which were trichloroacetic acid extraction, charcoal adsorption, chromatography on Biorad Aminex resin and thin layer chromatography. These steps led to a fairly purified cyclic AMP preparation which was able to stimulate cyclic AMP dependent protein kinase. During chromatographic separation fraction which cochromatographed with standard ^{3}H-labelled cyclic AMP was isolated. Contamination of cyclic GMP and other cyclic nucleotides was ruled out as they all showed different R_F values from that of cyclic AMP in solvent system used for the TLC. Charcoal adsorption and chromatography on Biorad Aminex resin removed most of purine and pyrimidines.

Preparation of cyclic AMP-dependent protein kinase. The protein kinase was partially purified from rabbit skeletal muscle as described by Wastila et al. [16]. All operations were carried out at 4° C.

Protein kinase assay. Cyclic AMP was determined as described by Kuo and Greengard [17] with some modification. The reaction volume of 200 μl contained 0.05 M sodium acetate buffer (pH 6.0); magnesium acetate, 2 μmol; histone mixture, 40 μg; cyclic AMP or mold extract; 100 μl and 25 μl of cyclic AMP-dependent protein kinase. Tubes were kept in ice during the addition of enzyme. The reaction was initiated with the addition of [γ-^{32}P]ATP, 100 pmol and tubes were incubated for 5 min at 30° C with shaking. The reaction was terminated by adding 2 ml of ice cold trichloroacetic acid-tungstate-sulphuric acid (protein precipitating solution). Bovine serum albumin was added as a carrier protein (0.2 ml of 0.6% solution) and the contents of the tube were mixed by vigorous addition of another 2 ml of precipitating solution. The mixture was centrifuged and the supernatant was removed by aspiration. The precipitate was redissolved in 0.1 ml of 1 N NaOH and 2 ml of precipitating solution was added and centrifuged. This procedure was repeated once more and the protein was finally collected by centrifugation. The pellet was dissolved in 0.1 ml of 1 N NaOH and resuspended in 10 ml of Bray's scintillation fluid [18] for determining the radioactivity.

Determination of protein. Protein content of samples was estimated accord-

ing to the procedure described by Lowry et al. [19].

Morphology of the mould in the presence of cyclic AMP and phosphodiesterase inhibitors. Morphological study of the mould was carried out in petri dishes containing synthetic mucor medium solidified with 1.8 percent agar (Difco). Cyclic AMP, aminophylline and theophylline (Sigma) were dissolved in glass distilled water whereas 3-isobutylmethylxanthine was made soluble in dilute NaOH. The solutions were sterilised by membrane filtration (0.45 nm). Petri dishes containing synthetic mucor medium with different concentrations of each effector individually, were inoculated with small pieces of mycelium of plus or minus strain and incubated at 25° C in dark for 48 h. The growth of the mycelium was observed microscopically at regular intervals during the incubation period.

Assay of mevalonate kinase. Mevalonate kinase (ATP : mevalonate 5-phosphotransferase) was assayed by the procedure of Levy and Popjak [20]. The assay system contained: Tris-HCl buffer (pH 8.0), 100 μmol; sodium mevalonate (prepared from mevalonic acid lactone by treating it with $NaHCO_3$ and incubating for 30 min at 37° C), 5 μmol; cysteine hydrochloride (pH 7.0), 2.5 μmol; $MgCl_2$, 5 μmol; ATP, 10 μmol; NADH, 0.1 μmol; phospho*enol*-pyruvate, 0.15 μmol; lactate dehydrogenase (Sigma type I L 2375, 40—100 units/mg protein), 25 μg; pyruvate kinase, (Sigma type I P 1381, 40—80 units/mg protein), 15 μg and appropriate amount of enzyme in a final volume of enzyme which caused a change of 0.001 absorbance unit per min at 340 nm at 25° C under the conditions of assay.

Results and Discussion

Following the time course after mating for the levels of β-carotene and trisporic acid, it was found that the synthesis of both these metabolites increased linearly between 24 to 120 h, reaching maximum at about 120 h of fermentation (Fig. 1). Further incubation resulted in decreased amounts of both the secondary metabolites. A gradual fall in the glucose concentration in the culture medium was observed during this incubation time. Only 30 percent (w/v) of the initial sugar supplied was remaining at the time of maximal production of trisporic acid and β-carotene. To ascertain whether or not the concentrations of glucose in the culture medium had any effect on the synthesis of β-carotene and trisporic acid, mated cultures were grown in synthetic mucor medium for 72 h and the mycelia were transferred into fresh synthetic mucor medium containing glucose as carbon source in increasing concentrations. After incubating them for 48 h β-carotene and trisporic acid contents were estimated. It was found that levels of β-carotene and trisporic acid dropped drastically with increasing amounts of glucose in the culture medium. At 0.3 M glucose concentration, about 94 percent decrease in trisporic acid synthesis and 80 percent decrease in β-carotene synthesis occurred as compared to that at 0.1 M glucose level.

To study the effect of glucose on trisporic acid formation and derepressed carotene synthesis, plus and minus cultures were grown separately in mucor medium for 48 h. The mycelia were picked and mated in fresh medium which had either glucose or glycerol as carbon source. We shall refer to these media as

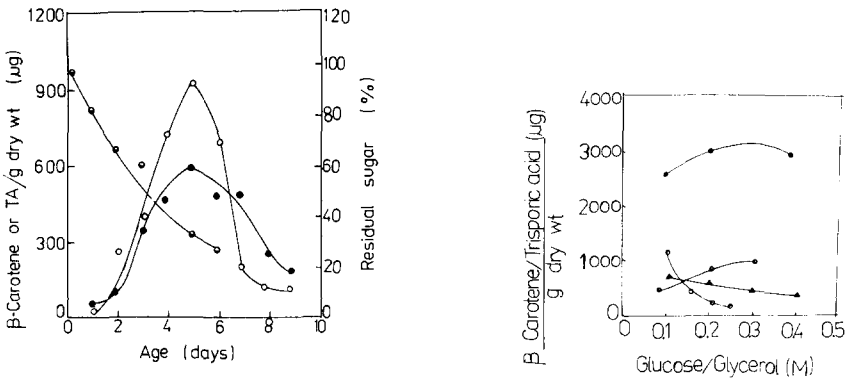

Fig. 1. Time course of β-carotene and trisporic acid (TA) production and glucose utilization by *B. trispora* (±). ●———●, β-carotene; ○———○, trisporic acid; ⊖———⊖, residual glucose.

Fig. 2. Effect of glucose and glycerol on β-carotene and trisporic acid synthesis by mated cultures of *B. trispora*. Trisporic acid: ○———○, glucose; ●———●, glycerol. β-Carotene: ▲———▲, glucose; ⊖———⊖, glycerol.

'glucose' or 'glycerol' media and the cultures mated in these media as 'glucose-supplemented cultures' and 'glycerol-supplemented cultures'. After mating, the cultures were incubated for 48 h in dark. Active synthesis of trisporic acid would be expected to start only when plus and minus mycelia are combined.

It was observed that cultures mated in 'glycerol' medium produced much higher yields of trisporic acid and β-carotene than those mated in 'glucose' media as shown in Fig. 2. In the presence of 0.2 M glucose concentration, almost 90 percent inhibition of trisporic acid synthesis was obtained. There was a rapid decline in trisporic acid content in presence of higher glucose concentrations whereas in glycerol supplemented cultures, the level remained nearly same. The difference in carotene levels between glucose and glycerol supplemented cultures increased with increase in the amounts of glucose and glycerol. 0.3 M glucose supplemented cultures contained about 61 percent less β-carotene than in 0.3 M glycerol-supplemented cultures.

Also when plus and minus strains were grown separately in glycerol and then mated in glucose containing media, it was found that carotene production was sensitive to glucose concentrations above 0.2 M.

Desai and Modi [21] reported that on mating, mevalonate kinase activity increases. This increase was cycloheximide sensitive. They concluded that formation of trisporic acid in mated cultures caused derepression of this enzyme synthesis. We studied the mevalonate kinase activity in the cultures mated in glucose media. It was observed that 0.2 M and 0.3 M glucose supplemented cultures had much lower enzyme activity as compared to that in 0.1 M glucose-supplemented cultures. These results suggested that glucose inhibition on carotenogenesis during mating may be due to catabolite repression.

Cyclic AMP has been shown to remove catabolite repression of many repressible enzyme synthesis in bacteria. Its presence was reported in a few mould species [22—26]. Experiments were therefore carried out to see the effect of cyclic AMP on carotenogenesis and trisporic acid synthesis in glucose-supple-

mented cultures. Addition of 1.0 mM cyclic AMP to the cultures which were mated in 0.1, 0.2 and 0.3 M glucose media elevated carotene production. Effect of cyclic AMP in reversing glucose effect on carotenogenesis was more pronounced at higher glucose concentrations. No significant change was observed in carotene production on supplying cyclic AMP to single plus and minus cultures. Cyclic AMP seemed to affect trisporic acid synthesis specifically. For the detection and measurements of cyclic AMP, we used the cyclic AMP-dependent protein kinase assay as described in the Materials and Methods.

A linear relationship between the amount of cyclic AMP and activation of protein kinase was used to derive values of cyclic AMP from samples. Purified cyclic AMP-containing fractions obtained after TLC were added to the assay system. This caused a marked increase in the protein kinase activity (Table I).

Cyclic AMP contents of plus, minus and mated cultures are tabulated in Table II. Plus strain contained two times more cyclic AMP than the minus strain. Mated cultures showed high levels of cyclic AMP which were about 100 percent more compared to plus and 450 percent as compared to minus cultures. This increase on mating single cultures for 6 h might be significant for trisporic acid synthesis. The conspicuous rise in cyclic AMP content on mating could have an important role to play in trisporic acid synthesis. It is therefore suggested that trisporic acid synthesis is under catabolite repression which is removed in presence of cyclic AMP.

Strains of *Phycomyces blakesleeanus*, *Mucor mucedo* and *B. trispora* reproduce asexually by sporangiophore formation in absence of the opposite mating partner [1]. But plus and minus when grown together, reproduce sexually by producing zygospores. Trisporic acid induces zygophore formation in single cultures, a prerequisite for mating process. Cyclic AMP is implicated in regulating asexual reproduction in *P. blakesleeanus* [26,28,29] and sexual reproductive in *Coprinus macrorhizus* [23] and *Fusarium roseum* [25] by inducing sporangiophore and basidiocarp formation. In three mucoraceous fungi namely, *M. mucedo*, *Mucor hiemalis* and *Mucor racemosus*, cyclic AMP induced cell differentiation [28]. This interested us to study whether cyclic AMP could induce any morphological changes in *B. trispora*. It was observed that addition of cyclic AMP in the growth medium resulted in significant change in pattern of growth and mycelial morphology of plus and minus strains as well. Jones and Bu'Lock [30] have studied the effect of dibutyryl cyclic AMP on morphogenesis in 5 spp. of Mucorales. *M. hiemalis* and *M. mucedo* were sensitive to

TABLE I

STIMULATION OF PROTEIN KINASE BY EXTRACTS OF SINGLE AND MATED *B. TRISPORA*

10 µl of plus, minus and mated extracts after TLC were added to the assay system; results are means of duplicate samples of 2 experiments.

Addition of mold extract	^{32}P incorporation (cpm)
None	97 119
plus	202 126
Minus	127 959
Mated	169 001

TABLE II

LEVELS OF CYCLIC AMP IN SINGLE AND MATED CULTURES OF *B. TRISPORA*

Age of the cultures was 72 h. Plus and minus cultures were mated for 6 h at the harvest time.

Culture	Cyclic AMP/g fresh weight (pmol)
Plus	956.26
Minus	436.93
Mated	1869.34

dibutyryl cyclic AMP effect and showed marked morphological changes. While *P. blakesleeanus*, *B. trispora* and *Zygohyricus moelleri* were insensitive to dibutyryl cyclic AMP.

When plus and minus cultures of *B. trispora* were grown separately on agar containing medium without the addition of cyclic AMP, the mycelial growth was rather fast and hyphae were thin.

The growth became dense and the mycelial mat increased in thickness as the concentration of cyclic AMP was increased. When mycelia of single strains grown on agar medium containing various cyclic AMP amounts were examined under the light microscope, it became apparent that the rate of mycelial elongation was reduced with increasing concentrations of cyclic AMP and the frequency of mycelial branching as well as the mycelial diameter were increased. The effect of the cyclic nucleotide was similar in both plus and minus strains. They were sensitive to as low as 0.15 μM cyclic AMP concentrations. The hyphal tips were differentiated into spherical dark globules. Mycelia gave an appearance of a string of dark regions.

Synthesis of cyclic AMP is catalysed by adenyl cyclase whereas the degradation by phosphodiesterase enzyme. Aminophylline, theophylline and 3-isobutylmethylxanthine are known inhibitors of the phosphodiesterase. It was checked if these inhibitors could mimick cyclic AMP effect on single cultures.

It was observed that plus and minus strains grown in the presence of aminophylline, theophylline and 3-isobutylmethylxanthine show marked morphological changes compared to control cultures. Extension growth of hyphae was inhibited immediately. Hyphae were thickened with swollen club-shaped tips. Whole length of mycelia appeared to get divided into many dark compartments. All three inhibitors were equally efficient in bringing about these changes which were similar to those induced by cyclic AMP. Thus, it was apparent that phosphodiesterase inhibitors could imitate cyclic AMP action. This indirectly showed that when intracellular cyclic AMP levels were increased in both *B. trispora* mating types, it resulted in hyphal differentiation. The phosphodiesterase inhibitors might have raised the levels of intracellular cyclic AMP by inhibiting its degradation.

It was interesting to note that trisporic acid induced morphological changes were comparable to those induced by cyclic AMP. Small pieces of plus and minus strains were inoculated on agar medium at two opposite ends of petri dish. The mycelia grew and spread towards each other. At one time, mycelia of both mating types came in close proximity. When this mating zone was

observed under microscope, zygophores and spores were clearly seen. These changes resembled cyclic AMP induced dark special differentiated portions markedly which led us to believe that cyclic AMP may also be involved in the sexual reproduction of *B. trispora.* So far, only trisporic acid is held responsible for the zygophore induction. But above data strengthenes the possibility of involvement of cyclic AMP together with trisporic acid in zygophore induction.

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