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CYCLIZATION OF LYCOPENE IN THE BIOSYNTHESIS OF β-CAROTENE

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Key Word Index—*Mycobacterium marinum*; Bacteria; biosynthesis; β-carotene; cyclization of lycopene; inhibition by nicotine and CPTA.

Abstract—*Mycobacterium marinum* produces carotenoids when exposed to light or when antimycin A is added. Although the major pigment synthesized is β-carotene, lycopene is accumulated when the induced bacteria are incubated in the presence of nicotine (5 mM) or 2-(4-chlorophenylthio)-triethylamine hydrochloride (CPTA) (50 μM). Both of these compounds inhibit β-carotene synthesis by blocking the cyclization of lycopene. When nicotine is removed by washing the cells, the accumulated lycopene is cyclized to form β-carotene. The cyclization of lycopene is not an energy-requiring reaction and, furthermore, does not require oxygen or any other electron acceptor. Chloramphenicol addition also does not inhibit the conversion of lycopene to β-carotene indicating that no *de novo* protein synthesis is involved. Nicotine appears to act by inhibiting the activity of the enzyme required for the cyclization of lycopene.

Although the mode of action of CPTA is similar to nicotine, it cannot be removed by washing once the cells have been incubated in its presence, suggesting that the molecule is tightly bound to the enzyme. The possible active molecular sites of nicotine and CPTA are discussed.

INTRODUCTION

FOR THE past several years, our research efforts have been directed towards elucidating the mechanism of light-induced carotenoid synthesis in non-photosynthetic plants.[1] *Mycobacterium marinum* and *Mycobacterium* sp. are two such organisms which show this light-triggered carotenoid synthesis.[2–8] Studies have shown that carotenoid synthesis in these and other organisms consists of two phases.[3,5] First is a photochemical reaction which is temperature-independent and requires oxygen; oxygen appears to participate directly in the photochemical reaction rather than acting as an electron acceptor.[9] This photochemical reaction is followed by a series of dark reactions, which eventually result in the formation of substantial amounts of carotenoids. In the dark reactions, carotenoid synthesis is preceded by *de novo* protein synthesis.[2,4,5,10] These proteins perhaps are carotenogenic enzymes. Thus, it might be expected that the photochemical reaction triggers the synthesis of proteins required for carotenogenesis.

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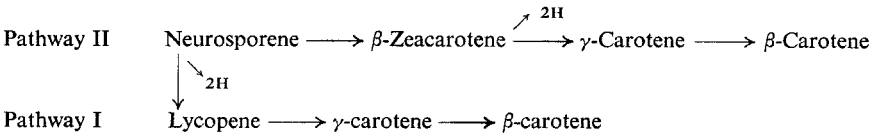
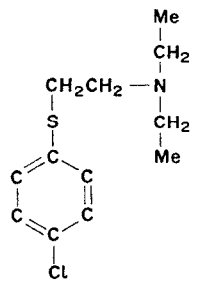
Further studies have revealed that antimycin A can also trigger the synthesis of carotenoids in the absence of light in *M. marinum*[2,11] and that it does so not through its usual interference in the electron transport system but, paradoxically, through its effect on protein synthesis.[12]

During the course of these investigations with *M. marinum* and *Mycobacterium* sp., it was found that nicotine is a potent inhibitor of the cyclization reaction in the synthesis of cyclic carotenes.[13] Addition of nicotine to the light- or antimycin A-induced bacteria results in the formation of lycopene rather than β-carotene and when nicotine is removed by washing the cells, the concentration of lycopene falls, with a concomitant increase in the concentration of β-carotene. During this conversion, γ-carotene is detected as an intermediate. These observations were interesting since they provided evidence that lycopene is the substrate for cyclization, and not neurosporene as suggested by some investigators.[14,15] The two alternate pathways of β-carotene formation are outlined in Scheme 1. A similar conclusion that lycopene is the substrate for cyclization has been arrived at by Kushwaha *et al.*[16,17] using tomato fruit plastids.

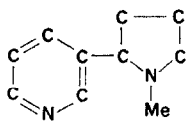


SCHEME. 1. TWO PROPOSED ALTERNATE PATHWAYS FOR β-CAROTENE FORMATION.

Recently, Coggins *et al.*[18] have reported that 2-(4-chlorophenylthio)-triethylamine hydrochloride (I, CPTA) addition results in the accumulation of lycopene in a number of organisms including the tomato fruit, although the mode of action of CPTA was not suggested. Similarly, it was not reported whether the formation of cyclic carotenes occurs after CPTA is removed.



(I) CPTA



(II) Nicotine

In the present paper, we report further investigations on various facets of the cyclization of lycopene including a comparative study of the effects of nicotine (II) and CPTA on carotenogenesis in *M. marinum*.

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RESULTS AND DISCUSSION

Features of the Cyclization Reaction

Nicotine addition to the induced *M. marinum* cells results in the accumulation of lycopene, and when nicotine is removed lycopene is converted to β-carotene. We have investigated certain features of this conversion (Table 1).

The conversion of lycopene does not require oxygen, since under anaerobic conditions β-carotene is formed at the expense of lycopene. The rate of conversion is the same regardless of whether an electron acceptor (e.g. ferricyanide, a suitable electron acceptor in mycobacteria) is present or not under anaerobic conditions. This indicates that the scheme for the conversion of lycopene to β-carotene does not involve an oxidation reaction.

TABLE 1. EFFECT OF VARIOUS CONDITIONS ON LYCOPENE CYCLIZATION

	Additions		Phytoene	Phyto-fluene	β-Carotene	Lycopene	γ-Carotene	Total
	During first incubation	During second incubation	μg/g Bacteria dry wt					
1	—	—	11·3	1·5	195 9	—	—	208·7
2	Nicotine	Nicotine	9·4	0·6	—	190·8	—	200·8
3	Nicotine	—	13·6	1·2	103·5	73·2	1·0	192·5
4	Nicotine	N_2	15·3	1·9	98·4	80·6	0·8	197·0
5	Nicotine	N_2 and $K_3Fe(CH)_6$	14·1	1·1	96·5	75·6	1·3	198·6
6	Nicotine	Chloramphenicol	10·7	2·3	101·3	77·1	1·5	192·9
7	Nicotine	Puromycin	9·5	1·9	105·3	72·1	0·8	189·7
8	Nicotine	Dinitrophenol	12·5	1·0	96·3	72·5	0·4	182·7

Dark grown *M. marinum* cells, suspended in 50 mM phosphate buffer (pH 8·0), 50 mM glycerol and 3 mM $(NH_4)_2SO_4$, were induced with light and antimycin A. To all flasks, except 1, was added 5 mM nicotine. The bacterial suspensions were then incubated for 24 hr. Bacteria in flasks 3 to 8 were washed four times with 200 ml of 50 mM phosphate buffer (pH 8·0) to remove nicotine and were reincubated under varying conditions for an additional period of 24 hr. Carotenoids were then extracted and chromatographed.

The conversion of lycopene to β-carotene also occurs when the bacterial cells are incubated in the presence of dinitrophenol. This indicates that the formation of β-carotene from lycopene is not an energy-requiring reaction.

After nicotine is removed, the conversion of lycopene to β-carotene also occurs in the presence of chloramphenicol and puromycin, potent inhibitors of protein synthesis. This would imply that the mode of action of nicotine is not an inhibition of the synthesis of the enzyme required for cyclization, but rather that nicotine inhibits the activity of the cyclizing enzyme.

Comparative Effect of CPTA and Nicotine

It was of interest to study the effect of CPTA on mycobacteria to compare the results with those obtained using nicotine. Initial experiments indicated that CPTA inhibited β-carotene synthesis while lycopene accumulated in light-induced and antimycin A-induced *M. marinum* (Table 2). The optimal concentration of CPTA for maximal lycopene accumulation was 50 μM, whereas the optimal concentration of nicotine was 5 mM.[13] Thus, CPTA is much more effective in the accumulation of lycopene than is nicotine.

Previous studies[13] have indicated that nicotine may be washed from the bacterial cells after 24 hr and that subsequently β-carotene is synthesized at the expense of lycopene. Several attempts were made to wash CPTA in a similar fashion from the bacteria after 24 hr of incubation with no success (Table 2). This suggests that CPTA binds very strongly to its site of action.

TABLE 2. EFFECT OF CPTA ON CAROTENOID SYNTHESIS

Flask	Condition	Phytoene	Phytofluene	β-Carotene	Lycopene	γ-Carotene	Total
		μg/g Bacteria dry wt					
1	Uninduced bacteria	18·5	1·1	—	—	—	19·6
2	Induced bacteria	24 3	3·0	289·1	Trace	Trace	316·4
3	Induced bacteria + CPTA	14 4	2·8	—	243 7	—	260·9
4	Induced bacteria + CPTA; wash after 24 hr	18 9	3·2	—	257·9	—	280·0

90 ml of a 7% bacterial suspension were induced with light and antimycin A (70 μM) and incubated with 5 mM glycerol and 3 mM $(NH_4)_2SO_4$ for 48 hr. CPTA, where added was at a concentration of 50 μM. Bacteria in flask 4 were washed 4 × with 50 mM phosphate buffer (pH 8·0) after the first 24 hr of incubation and then were reincubated for an additional period of 24 hr. Carotenoids were extracted and chromatographed.

There appeared to be two possibilities for the accumulation of lycopene in the CPTA-treated cells. CPTA either inhibits the activity of the enzyme required for the cyclization of lycopene or acts at the level of protein synthesis, inhibiting the formation of the cyclizing enzyme. To evaluate these possibilities, *M. marinum* cells were induced with light and antimycin *A* and then incubated for 4 hr. This is the time interval during which the caro-

TABLE 3. EFFECT OF DELAYED ADDITION OF CPTA ON LYCOPENE ACCUMULATION

Flask	Addition	β-Carotene	Lycopene
		μg/g Bacteria dry wt	
1	None	248·3	—
2	CPTA	—	233·1
3	CPTA + Chloramphenicol	—	199·8

90 ml of a 7% bacterial suspension were prepared as indicated in Table 2. Induction was with light *plus* antimycin *A*. Following 4 hr of incubation, CPTA (50 μM) was added to flasks 2 and 3. Chloramphenical (20 μM) was also added to flask 3 at this time. All bacterial suspensions were incubated for an additional period of 20 hr

tenogenic enzymes including the cyclizing enzyme (but not carotenes) are synthesized.[2] Thus, if CPTA were to act by inhibiting the synthesis of the cyclizing enzyme, CPTA addition at the end of the four-hour incubation should have little effect on β-carotene formation inasmuch as the enzyme has already been synthesized by the cells. Yet, the experiments (Table 3, Flask 2) show that no β-carotene formation occurs when CPTA is

added at the end of the 4-hr incubation. The most likely explanation is that CPTA inhibited the activity of the cyclizing enzyme previously synthesized during the 4-hr incubation. To exclude the possibility that perhaps CPTA was initiating a new pathway of lycopene formation by inducing the synthesis of a specific enzyme, chloramphenicol was added along with CPTA at the end of the 4-hr incubation (Table 3; Flask 3). Under these conditions, lycopene accumulation still occurred thereby showing that no new pathway is initiated and that lycopene is a direct intermediate in the biosynthetic pathway of β-carotene (Scheme 1). Thus, we conclude that the effect of CPTA must be through its inhibition of the activity of the enzyme required for the cyclization of lycopene. The inability to remove CPTA once the cells have been incubated may be due to its tight binding with the enzyme.

Apparently then, the mode of action of CPTA and nicotine is similar. In an attempt to obtain a better understanding of the mode of action of these compounds, molecular models were constructed. The most striking similarities in the two molecules after observing various configurations were the following corresponding positions: (a) sulfur of CPTA (I) and nitrogen of the pyridine ring of nicotine (II); and (b) nitrogen of CPTA and the nitrogen of the *N*-methylpyrrolidine ring of nicotine. If the sulfur of CPTA and the nitrogen of the pyridine ring of nicotine are active sites in the inhibition of the cyclization reaction, it may explain why nicotine, but not CPTA, can be removed by washing the cells. The sulfur atom of CPTA, being more electron dense than the nitrogen of the pyridine ring of nicotine, may result in an irreversible binding of the CPTA molecule to the cyclizing enzyme.

EXPERIMENTAL

Mycobacterium marinum (ATCC 927) was used in all studies. The cells were cultured in the dark as previously reported.[2] Bacteria were harvested by centrifugation at the end of the logarithmic phase of growth and frozen until needed. The frozen bacteria were thawed, washed twice with 20 × their weight of 50 mM phosphate buffer, pH 8·0, and then suspended in the same buffer to obtain a 3·3% suspension. Carotenogenesis was induced with 15 min of light from two cool white fluorescent tubes and 70 μM antimycin *A*. Under these conditions, *M. marinum* is maximally induced.[2,4] Following induction, the bacteria were incubated with 50 mM glycerol and 5 mM ammonium sulfate for 24 hr in the dark. The incubation was terminated by the addition of two vols. of 7:2, acetone–methanol. Carotenoids were extracted and chromatographed on a column of alumina (Woelm grade II) as previously reported.[2,13]

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