JOURNAL OF BACTERIOLOGY, July 1990, p. 4103–4105
0021-9193/90/074103-03$02.00/0

Vol. 172, No. 7

In Vitro Characterization of Two Different *Phycomyces blakesleeanus* Mutants with Impaired Phytoene Desaturation

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Received 27 December 1989/Accepted 9 April 1990

In vitro phytoene desaturation was investigated in two *Phycomyces blakesleeanus* mutants, C5 and S442, in which phytoene is accumulated instead of β-carotene. For strain C5 but not strain S442 the phenotypic block of phytoene conversion could be overcome in vitro by the addition of Tween 40. Immunodetection of phytoene desaturase revealed in all cases the presence of a 40-kilodalton protein.

The first desaturation step in the carotenogenic pathway, the introduction of two additional double bonds into the phytoene molecule, has been focused on in recent years because of the development of herbicidal inhibitors which target phytoene desaturase (see reference 14 for a review). This reaction, catalyzed by phytoene desaturase, has been characterized enzymatically to a certain extent (8, 15), and the corresponding gene has been isolated from *Rhodobacter capsulatus* and sequenced (1). Mutants with an inhibited phytoene desaturase reaction have been generated and selected from many prokaryotic and eukaryotic organisms (10, 11, 13), including the mucoraceous fungus *Phycomyces blakesleeanus* (7). Only a few of them have been characterized in vitro, and very little is known about the nature of their mutation. In general, the stalled desaturation of lipophilic phytoene to ζ-carotene can result from an effect of the mutation either on the catalytic reaction or on substrate transfer from phytoene synthase to phytoene desaturase. In this study, we investigated the latter possibility by in vitro assays of phytoene conversion with two phytoene-accumulating mutants of *P. blakesleeanus*, C5 and S442.

The mutants of *P. blakesleeanus*, C5 (*carB10*), formerly designated Alb 10 (12), and S442 (*carB* [3]), and the wild-type strain NRRL 1555, all from the culture collection of the Departamento de Genetica, Universidad de Sevilla, were grown for 3 days as described by Than et al. (17). Extracts from freeze-dried mycelia were incubated with (*R*)-[2-^{14}C]mevalonate for 2 h as previously described (4). After termination, the mixture was partitioned against diethyl ether and the organic layer was collected and subjected to high-pressure liquid chromatography (HPLC) separation (Spherisorb ODS-1 5 μ column; acetonitrile–methanol–2-propanol [85:10:5]) (9). Radioactivity was monitored by an on-line radioactivity flow detector (Raytest Ramona LS). In some experiments, the carotene reaction products were prepurified by thin-layer chromatography (TLC) on silica gel with 15% toluene in hexane. For electrophoresis and Western blotting (immunoblotting), the freshly harvested mycelia were ground with four times the amount (weight/volume) of a 0.4 M Tris hydrochloride buffer (pH 8.0) containing 5 mM dithiothreitol; the reaction mixture contained a total of 0.2 mg of protein. The details for sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunodetection of phytoene desaturase were described previously (16). The antiserum was purified by affinity chromatography on CNBr-activated Sepharose coupled to β-galactosidase.

Figure 1 shows the HPLC separation of carotenes formed during incubations with [^{14}C]mevalonate. The marker carotenes in Fig. 1A determined the positions of ζ-carotene, β-carotene, and phytoene. The in vitro reactions were carried out in the presence of 1% Tween 40. The distribution of radioactivity in Fig. 1B represents the lipophilic reaction products after partitioning into diethyl ether. Prior to the run shown in Fig. 1C, a hydrocarbon fraction including all of the carotenes was obtained by an additional TLC step. Besides phytoene, which is formed from [^{14}C]mevalonate, ζ-carotene could also be identified as a reaction product. The corresponding peak at a retention time of about 16 min could only be seen as a shoulder of the pre-phytoene alcohol peak (retention time, 14 min) when prepurification by TLC was not done. However, this additional TLC step separates pre-phytoene alcohol from carotenes and therefore allowed the detection of ζ-carotene and the quantitation of the radioactivity in the corresponding peak in the HPLC run shown in Fig. 1C.

Table 1 quantitates the ability of Tween 40 to overcome the block of phytoene desaturation in membranes of mutant C5. With 1% Tween 40 present in the reaction mixture, the maximum rate of conversion of phytoene to ζ-carotene was 20%. This value is about half of that determined in wild-type *P. blakesleeanus* with intact carotenogenic capacity under the reaction conditions used here. Tween 40, which is necessary to obtain desaturation of phytoene to ζ-carotene, could be partially replaced by NaCl. Together with the white phytoene desaturase mutant C5, S442, another phytoene-accumulating *P. blakesleeanus* mutant which additionally accumulates small amounts of ζ-carotene, was assayed for stimulatory effects of Tween 40 and NaCl on phytoene desaturation. In contrast to mutant C5, mutant S442 showed a basic in vitro rate of conversion of phytoene into ζ-carotene of about 10%. The addition of neither Tween 40 nor NaCl had any effect on phytoene desaturation by S442 membranes. The comparably high basal rate reflects the leakiness of this mutation (3).

Figure 2 shows the synthesis of phytoene desaturase in the wild-type strain as well as in the mutants. Solubilized proteins from mycelia of wild-type *P. blakesleeanus*, C5, and S442 were separated by electrophoresis on sodium dodecyl sulfate-polyacrylamide gels. Immunodetection with an antiserum against phytoene desaturase revealed in all cases a single immunoreactive polypeptide of 40 kilodaltons

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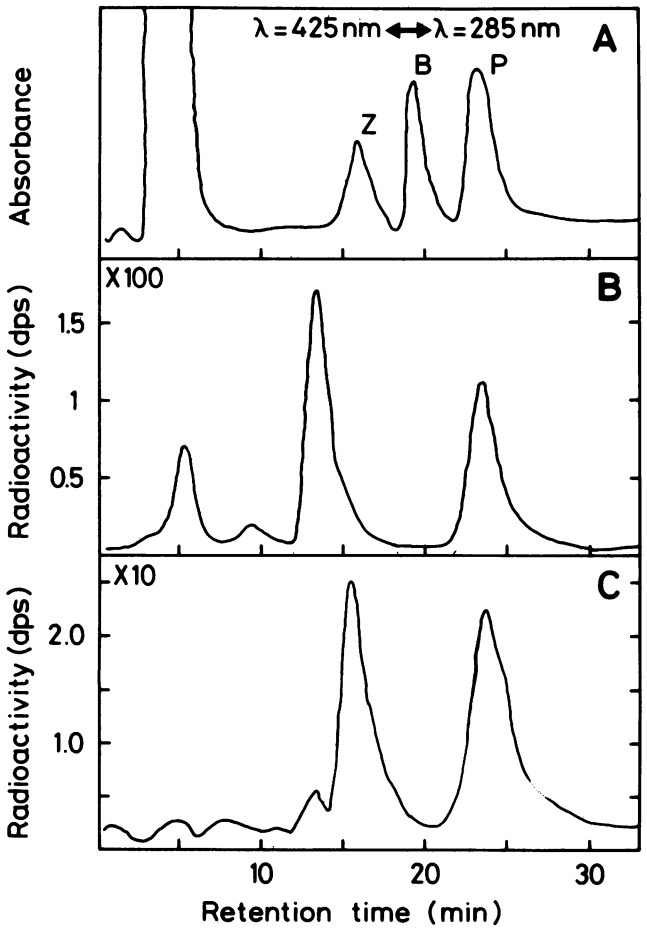

FIG. 1. In vitro formation of phytoene and ζ-carotene from [^{14}C] mevalonate by preparations of the C5 mutant of *P. blakesleeanus* in the presence of Tween 40. (Panel A) HPLC trace of marker carotenes phytoene (P), β-carotene (B), and ζ-carotene (Z). (Panel B) HPLC trace of reaction products without prepurification of carotenes. (Panel C) HPLC trace of ^{14}C-labeled carotenes after a TLC prepurification step.

which was less pronounced in both mutants. This result provides only limited information on a possibly mutated phytoene desaturase, but a mutation leading to inhibition of expression or premature termination of translation of the protein in the mutants can be excluded. Nevertheless, the molecular mass of phytoene desaturase from a fungus was determined for the first time and shown to be smaller than those of phytoene desaturases from photosynthetic organisms (1, 16).

As the carotenogenic pathway from phytoene to β-carotene is catalyzed by membrane-bound enzymes and the intermediates are all highly lipophilic compounds, the association of several enzymes in a functional complex which works as an assembly line has been suggested (5, 6). Therefore, mutants with blocked phytoene desaturation could either possess an inactive phytoene desaturase or contain a carotenogenic complex with a disturbed organization. The latter possibility might be caused by a mutation in phytoene desaturase affecting membrane association as well as by a mutation in another protein of the carotenogenic complex. The results presented indicate that mutant C5 possesses a functional phytoene desaturase. The in vitro activation by Tween 40 can then be explained either by a spatial rearrangement of the carotenogenic complex or by detergent-mediated transfer of the lipophilic phytoene molecules from phytoene synthase to phytoene desaturase.

TABLE 1. In vitro conversion of phytoene in *P. blakesleeanus* carotenogenic mutants C5 and S442

Mutant	Incubation conditions[a]	Radioactivity (dpm/mg of protein) in:		% Conversion by phytoene desaturase[b]
		Phytoene	ζ-Carotene	
S442[c]	No additions	13,980	1,586	10.2
	1% Tween 40	17,286	2,136	10.9
	0.1% Tween 40 + 0.5 M NaCl	12,552	1,401	10.0
C5[c]	No additions	56,180	Tr	<0.1
	1% Tween 40	53,107	12,985	19.7
	0.1% Tween 40	55,515	326	0.6
	0.1% Tween 40 + 0.5 M NaCl	55,247	1,867	3.3

[a] Incubation was done for 2 h with 0.5 μCi of (*R*)-[2-^{14}C]mevalonate.
[b] The conversion of phytoene by phytoene desaturase in wild-type strain NRRL 1555 is about 30 to 40%.
[c] The carotene contents in the mutants after cultivation were as follows: for S442, 2.82 and 0.17 mg of phytoene and ζ-carotene per g (dry weight), respectively; for C5, 3.27 mg of phytoene per g (dry weight) and no detectable ζ-carotene.

In contrast to the phytoene desaturase in C5, the phytoene desaturase in S442 is directly affected by the mutation. Despite the differential biochemical features of phytoene desaturases from the mutants, genetic analysis indicates lesions in the same allele (3). As phytoene desaturase genes have been cloned and sequenced recently (1, 2), subsequent analysis of the mutations of C5 and S442 at the gene level may soon be possible and will certainly improve our understanding of the nature of the mutations affecting phytoene desaturation.

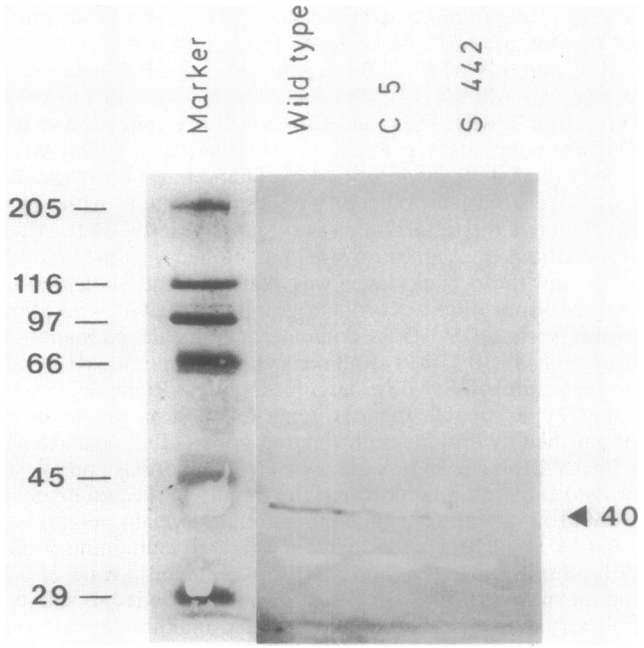

FIG. 2. Immunodetection of phytoene desaturase on sodium dodecyl sulfate-polyacrylamide gels after electrophoresis of solubilized proteins from wild-type strain NRRL 1555 and mutants C5 and S442. Numbers are masses in kilodaltons.

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