Pure & Appl. Chem., Vol. 57, No. 5, pp. 667–669, 1985.
Printed in Great Britain.

Carotenogenic enzymes from *Neurospora*

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Abstract - In the fungus Neurospora crassa, the carotenogenic enzyme system, catalyzing both the synthesis of phytoene and that of more unsaturated carotenoids, is membrane-bound. Using geranylgeranyl pyrophosphate as a substrate no co-factors are required for the conversion to carotenoids, but Mg^{2+}ions stimulate the activity. Carotenogenic enzymes have been solubilized partly from membranes by detergent treatment. Only by treatment with sodium cholate and CHAPS was most of the enzyme activity maintained, whereas almost full activity could be restored only by the addition of lipids. Photoinduction of de novo synthesis of carotenogenic enzymes is indicated by studies using inhibitors of protein synthesis and by light-mediated changes in the pattern of membrane proteins.

INTRODUCTION

Although the biosynthesis of carotenoids and its regulation by light have been intensively studied, we are still far from a complete understanding (for review Ref. 1 and also Rau this vol.). In particular, our knowledge of carotenogenic enzymes is very limited. In the fungus Neurospora crassa - as in some other microorganisms - carotenoid formation in the mycelium is light dependent (Ref. 1). Consequently it has been concluded that the enzymes might be absent in dark-grown mycelia and present only after photoinduction. Therefore, these organisms seem to be well suited not only for investigations on the mechanism of photoregulation but also for studies on the properties of the carotenogenic enzymes. Cell-free systems from Neurospora capable of in vitro carotenogenesis have been elaborated (Ref. 3-5). This report presents more recent findings on carotenogenic enzymes from Neurospora. Most of the methods and experimental procedures used have been described elsewhere (Ref. 2 & 5).

LOCALISATION OF CAROTENOGENIC ENZYME ACTIVITY

Subcellular fractionation of crude cell extracts has shown that in fungi the steps of the carotenoid biosynthesis pathway are compartmentalised: The enzyme(s) converting mevalonic acid to geranylgeranyl pyrophosphate (prenyltransferase(s)) are soluble, whereas carotenogenic enzymes, i.e. phytoene synthase, isomerase, dehydrogenase(s) and cyclase(s) are membrane-bound (Ref. 3). Maximum carotenogenic activity (ca. 80 %) was localized in two membrane fractions shown to contain plasma membranes and in particular membranes of the endoplasmic reticulum (Ref. 6). These two membrane fractions also contain some carotenoid pigments, whereas the bulk of the carotenoid is in lipid globules (Ref. 6).

PROPERTIES OF THE MEMBRANE-BOUND ENZYME SYSTEM

For further purification and characterization of membrane-bound carotenogenic enzymes it seemed necessary to optimize the in vitro assay for carotenoid synthesis. Therefore, several of the conditions, including the illumination regime for photoinduction of carotenogenic enzymes, centrifugation procedures, mevalonic acid concentration, requirement and concentrations of cofactors, have been varied (Ref. 2 & unpublished results).

Using [2-^{14}C] mevalonic acid and a geranylgeranyl pyrophosphate-synthesizing system from Cucurbita endosperm as a source of geranylgeranyl pyrophosphate (Ref. 5 & 7), the following characteristics for carotenoid formation have been obtained. Saturation for mevalonic acid was achieved at a concentration of approximately 60 /uM. The incorporation of radioactivity into both phytoene and more unsaturated carotenoids proved to be linear up to 0.75 mg protein per incubation and up to an incubation time of 30 min. The conversion rate of mevalonic acid to phytoene could be improved up to 2.0, to the more unsaturated carotenoids up to 0.25 nmoles/mg protein/min.

When [2-^{14}C] geranylgeranyl pyrophosphate was used as a substrate the apparent K_m for GGPP was determined as 10/uM. The cell-free system incorporated significantly only during an incubation period of up to 10 min. The conversion of geranylgeranyl pyrophosphate to phytoene and more unsaturated carotenoids did not require additional cofactors (e.g. iron ions, pyridine nucleotides). However, the formation of phytoene was drastically increased in the presence of Mg^{2+}ions in concentrations higher than 10 mM. The stimulating effect of Mg ions could not be replaced by Mn.

SOLUBILIZATION OF CAROTENOGENIC ENZYMES

In order to solubilize carotenogenic enzymes we treated the membrane fraction with different detergents such as Tween 20, Tween 80, Triton X-100, sodium cholate and sodium desoxycholate (Ref. 2 & 8). After centrifugation (115.000 g for 2.5 hr) the supernatant was used as soluble enzyme fraction for determinations of enzymic activity. The results indicated that a general loss of carotenogenic activity was effected by the presence of detergents. This decrease of enzyme activities was dependent on both the type and the concentration of detergents. Carotenogenic activity could not be restored by subsequent dialysis. Sodium cholate at a final concentration of 0.2 % (corresponding to 1 mg/ 10 mg protein) solubilized 20-30 % of the membrane protein. In this solubilized fraction the specific carotenogenic activity was reduced compared to the specific activity of the membrane-bound enzymes; it could be almost fully restored by the addition of total lipids which had been extracted from membranes of Neurospora to the incubation mixture (Table 1).

Table 1: Solubilization of carotenogenic activities from membranes of Neurospora by sodium cholate (1 mg/10 mg protein) and the effect of lipid addition on carotenogenic activity in the soluble enzyme fraction. Incubations were for 10 min in the presence of [2-^{14}C] MVA (493 nmol; 83.6 KBq) and a GGPP-synthesizing system from Cucurbita endosperm (2.5 hr preincubation).

Fraction	Spec. incorporation (cpm/mg prot.)		Spec. activity[a] (nmol/mg/min)		Total activity[a] (nmol MVA inc./min)	
	Phytoene	unsat. Carot.	Phytoene	unsat. Carot.	Phytoene	unsat. Carot.
Membrane fraction	177 980	22 960	1.78	0.230	136.6	17.6
Soluble enzyme fraction	163 330	13 370	1.64	0.134	53.3	4.4
S.e.-fract.+lipids	166 800	20 360	1.67	0.204	54.4	6.6

[a], activity refers to the incorporation of mevalonic acid into phytoene and more unsaturated carotenoids.

Corresponding results have been also obtained with CHAPS (1 mg/ 7.5 mg protein), a zwitterionic detergent which might become more useful for further investigations.

PHOTOREGULATION OF MEMBRANE PROTEINS

In previous investigations with crude carotenogenic enzyme systems (Ref. 4 & 5) and in particular with membrane fractions (Ref. 5) evidence for a light-induced de novo synthesis of carotenogenic enzymes in Neurospora was found. As an approach towards an unequivocal proof we have compared the composition of membrane proteins from dark-grown and illuminated mycelia. In order to selectively intensify light regulated proteins we used additionally labelling in vivo with [^{35}S] methionine during a period in which carotenogenic enzymes should appear. After isolation and extraction the labelled membrane polypeptides were separated according to their isoelectric points and molecular masses by two-dimensional polyacrylamide gel electrophoresis and visualized by fluorography. At least 4 polypeptides were found to be conspicuously increased in response to photoinduction of carotenogenesis (Ref. 9 & 10).

In order to assay these polypeptides for carotenogenic activity we tried to enrich solubilized light-induced proteins in a native form by chromatofocusing. So far, however, the carotenogenic enzymes have not been enriched by this method. This might be due to a loss of carotenogenic activity by both removal of lipids and the long period of the procedure (Mitzka-Schnabel and Seigner, unpublished results).

Acknowledgement - I would like to thank Prof. Dr. Rau for stimulating discussions and his generous help throughout these studies.

REFERENCES

1. W. Rau in: Biosynthesis of Isoprenoid Compounds, (J.W. Porter and S.L. Spurgeon eds.) Vol. 2, 123-157, Wiley and Sons, New York (1983).

2. W. Rau and U. Mitzka-Schnabel, in: Methods in Enzymology, Steroids and Isoprenoids, (J.H. Law and H.C. Rilling eds.) 110, 253-267 (1985).

3. G.S. Bobowski, W.G. Barker and R.E. Subden, Canad. J. Bot. 55, 2137-2141 (1977).

4. S.L. Spurgeon, R.V. Turner and R.W. Harding, Arch. Biochem. Biophys. 195, 23-29 (1979).

5. U. Mitzka-Schnabel and W. Rau, Phytochemistry 20, 63-69 (1981).

6. U. Mitzka-Schnabel and W. Rau, Phytochemistry 19, 1409-1413 (1980).

7. A. Steiger, U. Mitzka-Schnabel, W. Rau, J. Soll and W. Rüdiger, Phytochemistry in the press (1985).

8. U. Mitzka-Schnabel and W. Rau, Carotenoid Chem. Biochem., Proc. Int. Symp. Carotenoids, 6th, 1981, Abstracts (1982).

9. U. Mitzka-Schnabel, E. Warm and W. Rau, Abstr. 7th Int. Symp. on Carotenoids, p. 245 München, (1984).

10. U. Mitzka-Schnabel, E. Warm and W. Rau, in: Blue Light Effects in Biological Systems (H. Senger ed.) p. 264-269, Springer-Verlag, Berlin (1984).