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SYNTHESIS OF CARNITINE FROM ε-N-TRIMETHYLLYSINE IN POST MITOCHONDRIAL FRACTIONS OF Neurospora crassa

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SUMMARY. An enzyme system in the post mitochondrial fraction of Neurospora crassa when supplemented with appropriate cofactors formed carnitine from ε-N-trimethyllysine. These findings together with previous studies of ε-N-lysine methylation in this fungi, illustrate that carnitine synthesis in Neurospora differs markedly in certain features from mammalian systems in that the entire synthesis is carried out employing free intermediates and cytosolic enzymes.

INTRODUCTION. The pathway of carnitine biosynthesis from lysine and methionine involves the same intermediates in Neurospora crassa and the rat, but differs in some important features with respect to free or protein-bound intermediates and in the cellular location of the relevant enzymes. For example, in N. crassa, a cytosolic enzyme, S-adenosylmethionine-ε-N-L-lysine methyltransferase, carries out the step-wise ε-N-methylation of free lysine (1) yielding ε-N-trimethyllysine, an efficient carnitine precursor. But in the rat it appears that trimethyllysine arises from the turnover of proteins containing this amino acid (2,3). Latter steps of carnitine biosynthesis then proceed as follows: trimethyllysine $\xrightarrow{a}$ β-hydroxytrimethyllysine $\xrightarrow{b}$ γ-N-trimethylaminobutyraldehyde $\xrightarrow{c}$ γ-butyrobetaine $\xrightarrow{d}$ carnitine. A unique finding relative to these transformations is that reactions (a) and (d) above are both α-ketoglutarate dependent dioxygenases but (a) is present in the mitochondria [e.g. rat liver (4) and rat kidney (5)] whereas (d) is exclusively in the cytosol [e.g. rat liver (6), monkey kidney (7), hyman kidney (7,8)].

In this communication we report the formation of β-hydroxytrimethyllysine, γ-butyrobetaine, and carnitine from trimethyllysine in cell-free

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extracts of N. crassa under kinetically defined conditions and show specifically that essentially all of such enzymatic activity is located in the post-microsomal supernatant.

EXPERIMENTAL PROCEDURE.

Materials: ε-N-[CH_3-^{3}H]trimethyllysine, β-hydroxy-ε-N-trimethyllysine, and γ-butyrobetaine, were synthesized by published procedures, namely (9), (10) and (9) respectively. All other chemicals and materials were available commercially.

Preparation of Cell Homogenate: Neurospora crassa lysine auxotroph (Fungal Genetic Stock Center No. 33933) was grown in 15 l batches of Vogel's medium (11) supplemented with 0.5 mm L-lysine under conditions previously described (1). The mycelia were harvested by filtration, pressed dry, frozen in liquid nitrogen and pulverized to a powder. Approximately 3 volumes of 0.25 M sucrose-MOPS (2-(-morpholino) propanesulfonic acid) buffer, pH 7.4 was added, the powder stirred, and allowed to stand on ice bath for a brief period. This suspension was then homogenized (3 passes) in a Potter-Elvehjem homogenizer (glass-glass) and used directly for enzyme studies or fractionated into subcellular fractions.

Subcellular Fractionation: These procedures followed established centrifugal methods for fractionating mammalian tissue (12) and Neurospora cells (13) at 2°C. The homogenate was centrifuged at 2000 x g, for 15 min, to obtain the pellet, nuclear fraction (N) and supernatant, cytoplasmic extract (E). Fraction (E) was further centrifuged at 14,500 x g, for 20 min, to obtain the mitochondrial fraction (M) and the supernatant. The latter supernatant was separated into microsomal fraction (P) and final supernatant or post microsomal supernatant (S) by centrifugation at 145,000 x g for 45 min. Both mitochondrial and microsomal pellets were suspended in sucrose-Tris buffer, pH 7.4. Proteins in all fractions were determined by a microbiuret procedure (14).

Assay of Enzymatic Activity: The enzymatic activity of the cell fractions was measured by determining the amount of radioactive ε-N-[CH_3-^{3}H]trimethyllysine converted to β-[CH_3-^{3}H]-hydroxytrimethyllysine, γ-N-[CH_3-^{3}H]-butyrobetaine, and γ-N-[CH_3-^{3}H]-carnitine. The incubation medium consisted of Tris HCl, 100 mM: pH 8:ascorbate, 5 mM; α-ketoglutarate, 5 mM; dithiothreitol 0.5 mM; catalase, 1 mg/ml; ferrous sulphate, 1 mM; calcium chloride, 5 mM; ε-N-trimethyllysine, (4.85 μC^{3}H/μmole trimethyllysine), 5.4 mM. The total volume was 1 ml with a few exceptions where it was 2 ml.

Following a 5 min preincubation, the reaction was started by adding ε-N-[CH_3-^{3}H]trimethyllysine. Flasks were sealed with serum caps and continuously flushed with 95% O_2:5% CO_2 gas mixture with shaking at 100 strokes/min, at 37° for 10 min. The reaction was terminated by addition of 0.1 ml (or 0.2 ml) 60% (w/v) perchloric acid. The flasks and the contents were then chilled, transferred to test tubes and centrifuged at 2000 rpm for 5 min. The supernatant was collected and the pellet resuspended in 0.5 ml of distilled water and recentrifuged. The combined supernatant and wash were neutralized with 10 N KOH and kept on ice. The precipitate (potassium perchlorate) was removed by centrifugation. The supernatant was chromatographed on a column (1 cm x 50 cm) packed with Bio-Rad (AG-50W-x 8, 200-400 mesh resin, H^+ form). The column was eluted with 80 ml of 1.5 N HCl, followed by a linear gradient of increasing HCl concentration (150 ml each of 1.5 N HCl and 3 N HCl) at a flow rate of 0.5 ml/min. Two ml fractions were collected, and 0.4 ml of each fraction was counted for determination of radioactivity in 4.5 ml of ACS ™, aqueous counting scintillant (Amersham Corp.) on a Packard 3255 scintillation counter. The products were identified by comparison with previously established elution patterns for trimethyllysine and its metabolites.

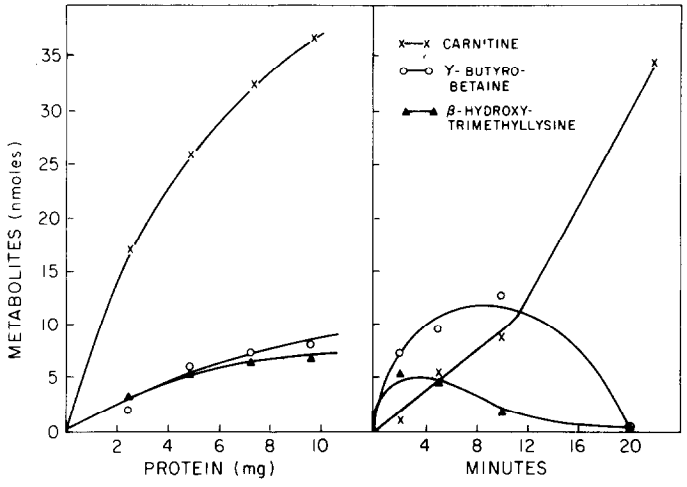

Figure 1. ε-N-Trimethyllysine metabolism in Neurospora crassa cell homogenates.
A. Dependence on protein
B. Time course of reaction

RESULTS AND DISCUSSION. Transformations (a)——→(d) above have been established in appropriate isotope experiments in growing N. crassa cultures (15). Attempts were made to demonstrate such events in N. crassa enzyme systems under conditions paralleling relevant mammalian studies (4,5). It soon became evident that trimethyllysine was readily metabolized by crude N. crassa homogenate and cell free extracts of N. crassa. The predominant product was carnitine with only small accumulations of β-hydroxytrimethyllysine and γ-butyrobetaine, The formation of these latter metabolites could be studied under kinetically defined conditions. This is illustrated in Fig. 1A and 1B which shows that the formation of these metabolites was dependent on homogenate protein (Fig. 1A) and time of incubation (Fig. 1B). An incubation time of 10 minutes was employed in all subsequent experiments to quantitate overall trimethyllysine metabolism, as the sum of the respective rates was nearly linear at 10 min incubation time.

Fig. 1B provides additional evidence for carnitine precursor-product relationships. Thus it is evident that β-hydroxytrimethyllysine and γ-butyrobetaine are formed initially from trimethyllysine metabolism and then are utilized with the concommitant formation of carnitine, γ-N-trimethylamino-

TABLE I

Subcellular Distribution of ε-N-Trimethyllysine (TML) Metabolizing Enzyme System in Terms of TML Metabolites: β-Hydroxytrimethyllysine (β-OH-TML), γ-Butyrobetaine (γ-BB) and Carnitine.

Exper.	Cell fraction	Activity expressed as	TML Metabolites β-OH-TML	γ-BB	Carnitine
1	Homogenate	nmoles/g cells/min	15.8	12.4	8.5
1	N	% of E+N activity	4.3%	4.2%	0.8%
1	M	" " "	2.0	1.4	1.8
1	P	" " "	9.7	17.5	10.5
1	S	" " "	66.6	82.5	89.5
		ΣN+M+P+S	82.6	105.6	102.6
2	Homogenate	nmoles/g cells/min	18.4	15.4	47.6
2	N	% of E+N activity	8.1%	19.5%	46.8%
2	M	" " "	2.1	0.1	0.8
2	S[b]	" " "	93.5	74.6	36.1
		ΣN+M+S	103.7	94.2	83.7
3	M[c]	% of M+S	5.4%	9.9%	17.2%
3	S	% of M+S	94.6	90.1	82.8

[a] As described in EXPERIMENTAL PROCEDURE, Subcellular Fractionation
E = cytoplasmic extract, N = nuclear fraction, M = mitochondrial fraction, P = microsomal fraction, S = post microsomal supernatant.

[b] The S fraction, Exper. 2, represents post mitochondrial supernatant.

[c] The M fraction, Exper. 3, is the pellet following centrifugation of fraction E at 145,000 x g and hence contains mitochondria plus microsomes.

butyraldehyde could not be detected in this crude enzyme system however, although there is good evidence for its involvement in carnitine biosynthesis in Neurospora crassa (15). A subcellular distribution of the activity of the trimethyllysine metabolizing enzyme system in N. crassa was examined in three separate experiments, Table 1. Most of the enzyme activity measured in terms of the trimethyllysine metabolites formed, was located in the post-microsomal supernatant (Exp 1), but also in the post mitochondrial supernatant (Exp 2), and in a high speed supernatant devoid of both mitochondria and microsomes

TABLE II

Cofactor Requirements for ε-N-Trimethyllysine Metabolism in Homogenates of Neurospora crassa.

Deletions from complete system[a]	Products		
	β-Hydroxy-trimethyllysine	γ-Butyro-betaine	Carnitine
	nmoles		
None	2.2	3.6	12.2
Minus ascorbate	1.2	1.9	10.6
Minus α-ketoglutarate	0.0	6.9	6.6
Minus $FeSO_4$ + α, α-dipyridyl (2mM)	1.0	6.3	7.6

[a]Incubations were carried out as described in the experimental procedure. 4 mg crude Neurospora crassa homogenate protein was used in 1 ml total volume of incubation mixture. Products of trimethyllysine metabolism were identified and measured following cation exchange chromatography of reaction mixtures as described in the text.

(Exp 3). The table clearly shows that trimethyllysine hydroxylase is a soluble enzyme present predominantly in the cytosol of N. crassa thus differing markedly from the rat where it is localized in kidney (5) and liver mitochondria (4).

It is known that ascorbate, α-ketoglutarate, and Fe^{++} are essential cofactors for trimethyllysine hydroxylase (4,5) and γ-butyrobetaine hydrosylase (6). The data in Table 2 show that these cofactors stimulate in varying degrees the trimethyllysine metabolizing system in homogenates of N. crassa. For example, deletion of ascorbate decreased the formation of all three products, β-hydroxytrimethyllysine, γ-butyrobetaine, and carnitine. But deletion of α-ketoglutarate and Fe^{++} only appeared to significantly reduce the formation of β-hydroxytrimethyllysine and carnitine. Indeed, in

these instances γ-butyrobetaine levels were higher than those in the complete system. Conceivably this reflects a greater dependence of γ-butyrobetaine hydroxylase for α-ketoglutarate and Fe^{++} and hence it accumulates. This would argue that the dioxygenases catalyzing steps (a) and (d) are different enzymes; but obviously purification of the enzymes involved is essential to answer these and other questions about transformations (a)——→(d). The fact that all of these enzymes are in the N. crassa cytosol as demonstrated herein should aid materially in such investigations.

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