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Journal of Molecular Catalysis B: Enzymatic 4 (1998) 205–209

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

Production of L-carnitine from D-carnitine by partially purified D- and L-carnitine dehydrogenase of *Agrobacterium* sp. 525a

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Received 4 September 1997; revised 13 November 1997; accepted 1 December 1997

Abstract

D-Carnitine was converted to L-carnitine with partially purified D- and L-carnitine dehydrogenases from *Agrobacterium* sp. 525a. Using partially purified D- and L-carnitine dehydrogenases (pH 8.5 Tris–HCl buffer, 100 mM D-carnitine, 5 mM NAD^+, 2 mM glutathione), about 64% of the D-carnitine could be converted into L-carnitine.

Keywords: Bioconversion; L-Carnitine; D-Carnitine; *Agrobacterium*; Carnitine dehydrogenase

1. Introduction

The physiological function of L-carnitine is an essential factor for the transport of long-chain fatty acids across mitochondrial inner membranes [1]. Recently, it is reported that carnitine may act as an acyl sink in order to maintain adequate cellular levels of free CoA [2,3] and interact with membranes to change their physicochemical properties [4,5]. L-Carnitine has been shown to be effective in the treatment of certain dysfunctions of skeletal muscle, some cases of acute onset of hypoglycemia, some abnormalities associated with chronic hemodialysis, and possibly ischemic heart disease and other cardiomycopathies [6–8]. As only the L-isomer of carnitine can act as a substrate for acyltransferase, and the D-isomer is an inactive isomer and an inhibitor of acyltransferase, L-carnitine should be used in the therapy for carnitine deficiency [9].

L-Carnitine can be produced by the carnitine dehydrogenase-catalyzed reduction of 3-dehydrocarnitine [10], the γ-butyrobetaine hydroxylase-catalyzed hydroxylation of γ-butyrobetaine [11], stereospecific hydroxylation of γ-butyrobetaine in a β-oxidation process analogous to fatty acid degradation [12], and the carnitine dehydratase-catalyzed hydration of crotonobetaine [13,14]. Furthermore, L-carnitine is produced by L-specific acylcarnitine hydrolase-catalyzed hydrolysis of acetyl-DL-carnitine [15]. On the other hand, there are a number of chemical methods for the synthesis of L-carnitine, but these methods may involve complicated processes such as optical resolution of the L-isomer from the obtained DL-carnitine [16,17] or preparation of optically active precursors, e.g., L-4-

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1381-1177/98/$19.00
PII S1381-1177(97)00037-4

chloro-3-hydroxybutyrate for the synthesis of L-carnitine [18,19]. Although several chemical and biological procedures for L-carnitine production have been proposed, the main procedure of L-carnitine production is the optical resolution of racemic D,L-carnitine. This method provides D-carnitine as a waste product in equimolar amounts to the L-enantiomer.

Chemical racemization of the D-isomer to DL-carnitine is very difficult, unlike that of amino acids. Biological racemization of D-isomer to DL-carnitine is very limited. Whole cell of *Escherichia coli* 044 K74 grown under anaerobic conditions in the presence of L-carnitine is able to convert D-carnitine into the L-enantiomer [20]. A carnitine racemase activity is detected in cell-free extracts of *Pseudomonas* sp. AK 1 grown on D-carnitine under aerobic conditions [21]. The mechanism of carnitine racemization is still unknown.

Agrobacterium sp. 525a utilizes D-carnitine as sole source of carbon and nitrogen. D-Carnitine dehydrogenase was induced during growth on D-carnitine, and was purified and characterized [22]. During the investigation of the time course of D-carnitine dehydrogenase production, we found the accumulation of L-carnitine in the cultural broth at 12 h cultivation. Very recently, Hanschmann and Kleber [23] reported the conversion of L-carnitine into D-carnitine with stereospecific carnitine dehydrogenases. In this report, we describe the essential conditions of the conversion reaction of D-carnitine into L-carnitine by dried cell of *Agrobacterium* sp. 525a and partially purified D- and L-carnitine dehydrogenase.

2. Experimental

2.1. Materials

L-Carnitine hydrochloride was purchased from Sigma Chemicals, St. Louis, USA. D-Carnitine hydrochloride used for the cultivation and conversion reaction was a gift from Ajinomoto, Tokyo, Japan. L-Carnitine dehydrogenase from *Alcaligenes* sp. was a gift from Asahi Chemical Industry, Tokyo, Japan. All other reagents were commercial products of analytical grade.

2.2. Microorganisms and culture conditions

Agrobacterium sp. 525a was used. The culture medium at pH 7.0 contained 0.5% D-carnitine hydrochloride, 0.2% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, and 0.05% yeast extract. Culture was done at 30°C for 48 h with reciprocal shaking.

2.3. Preparation of partially purified enzymes

Dried cells of *Agrobacterium* sp. 525a were prepared by drying of D-carnitine grown cells with an electric fan at room temperature overnight. For the preparation of partially purified enzyme, the washed cells (41 g as wet weight) harvested from 10 l of the medium were suspended in 300 ml of 70 mM triethanolamine buffer, pH 7.5, containing 2 mM glutathione and disrupted for 30 min with a sonicator. The cell-free extract was obtained by centrifugation as supernatant. Ammonium sulfate was added to the cell-free extract to 35% saturation, and the precipitate formed was discarded. The precipitate formed by further addition of ammonium sulfate to 55% saturation was centrifuged and collected. The precipitate was dissolved in a minimum volume of the buffer and dialysis against the buffer for 18 h. Glycerol was added to the dialyzed preparation at 50% and stored at −20°C before use. Ratio of D- and L-carnitine dehydrogenase activity is about 0.8–1.1 in the enzyme preparation.

2.4. Assay of D-/L-carnitine dehydrogenase activities

D- and L-carnitine dehydrogenase activities were measured from the increase in absorbance at 340 nm at 30°C. The reaction mixture (1.5

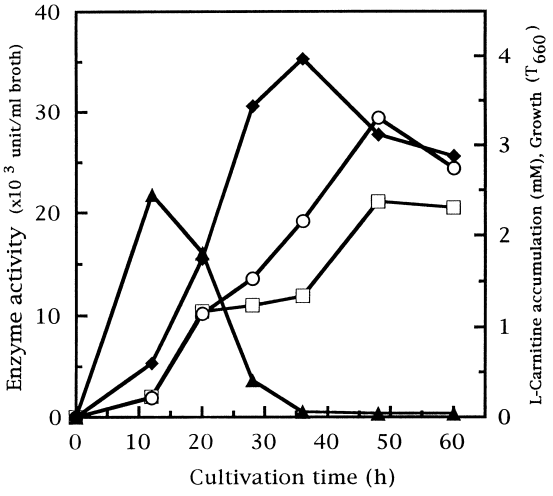


Fig. 1. Time course of L-carnitine accumulation, D- and L-carnitine dehydrogenase formation, and growth of *Agrobacterium* sp. 525a. The cultivation was carried out as experimental. The determination of enzyme activities and L-carnitine are described in the text. (▲) L-carnitine, (♦) growth, (○) L-carnitine dehydrogenase activity, (□) D-carnitine dehydrogenase activity.

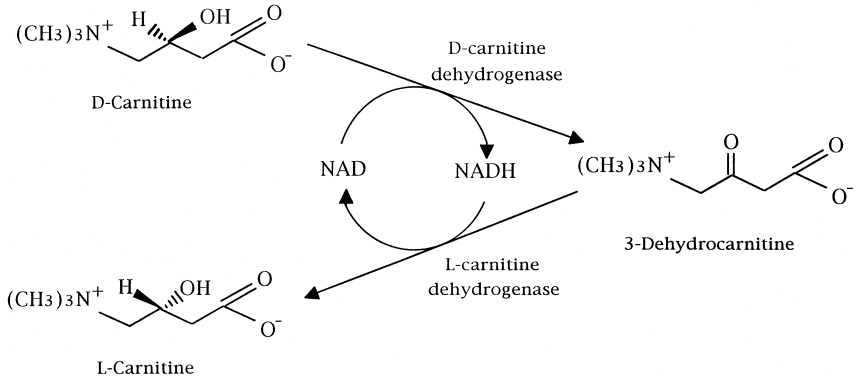
ml) contained 0.1 M glycine–NaOH buffer (pH 9.5), 15 mM D- or L-carnitine hydrochloride, which had been adjusted to pH 7.2 with NaOH, 0.5 mM NAD^+, and an appropriate amount of the enzyme. The activity was calculated using an extinction coefficient of 6220 M^{-1} cm^{-1} for NADH. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the formation of 1 μmol of NADH per min under the assay conditions.

2.5. Conversion reaction by dried cells and partially purified D- and L-carnitine dehydrogenases

Standard reaction mixture (3.0 ml) contained 66.7 mM Tris–HCl buffer (pH 8.5), 50 mM D-carnitine, 5 mM NAD, 1 mM glutathione, and 50 mg of the dried cells or the partially purified enzyme (1 unit as D-carnitine dehydrogenase activity) at 30°C. In the case of dried cells reaction, the reaction mixture was shaken. An aliquot of the reaction mixture (0.5 ml) was added to 0.5 ml of 1.0 M HCl at different time intervals.

2.6. Enzymatic determination of L-carnitine and NAD

The concentration of L-carnitine was enzymatically determined with L-carnitine dehydrogenase. The reaction mixture (1 ml) contained 0.2 M glycine–NaOH buffer (pH 9.5), 5 mM NAD, 0.3 mM nitroblue tetrazolium, 1% Tween 80, 5 units diaphorase, 3.5 units L-carnitine dehydrogenase, and an appropriate amount of sample. The reaction was carried out at 30°C for 60 min. The reaction was stopped by the addition of 2 ml of 0.1 M HCl and measured by spectrophotometrically at 530 nm. The concentration of NAD in reaction mixture of L-carnitine conversion was determined with alcohol dehydrogenase [24].



Scheme 1. Pathway of D-carnitine into L-carnitine by carnitine dehydrogenases.

3. Results and discussion

During the investigation of the time course of D-carnitine dehydrogenase production, we found that 2.5 mM L-carnitine was accumulated in the culture broth for 12 h of the cultivation time and also the L-carnitine dehydrogenase existed in the cell (Fig. 1). It is expected that D-carnitine can be converted into L-carnitine by coupling reactions of D- and L-carnitine dehydrogenase (Scheme 1). Hanschmann et al. also reported during growth on D-carnitine as sole source of carbon and nitrogen, two carnitine dehydrogenases with different stereospecificities were induced in *Agrobacterium* sp. and both of the enzyme were purified and characterized [25–27].

With dried cells of *Agrobacterium* sp. 525a, D-carnitine was observed to convert to L-carnitine: 28% of initial D-carnitine was converted to L-carnitine in 10 h reaction and 28 mM of L-carnitine was accumulated. Hanschmann and Kleber [23] reported that very low yield of L-carnitine was observed in resting cell reaction, and that 11% of the initial D-carnitine was converted to L-carnitine in permeabilized cell reaction. Generally, optimum pH of the oxidation reaction by dehydrogenase is alkaline range, and optimum pH of the reduction reaction by dehydrogenase is acidic range. In this experiment, the effect of pH on the conversion reaction were examined using various buffers. The highest L-carnitine formed was obtained at pH 8.5 (Fig. 2). Various NAD concentrations were tested to obtain the highest formation of L-carnitine (Table 1). Effective concentration of NAD on L-carnitine formation were found at 5 or 10 mM NAD. In the case of no addition of NAD, no formation of L-carnitine was observed. High concentration of NAD inhibited the formation of L-carnitine from D-carnitine. The addition of NADH was not effective for L-carnitine formation. Optimal temperature of the conversion reaction was at 30°C. High concentration of D-carnitine dehydrogenase (3 U) increased L-carnitine formation to 1.5-fold as compared with standard conditions.

Table 1
Effect of NAD on the formation of L-carnitine

NAD (mM)	L-carnitine formation (mM)
0	0.4
1.7	6.9
5.0	12.7
10.0	13.2
16.7	12.2

The reaction was carried out for 12 h as described in the text except that the concentration of NAD was varied as indicated.

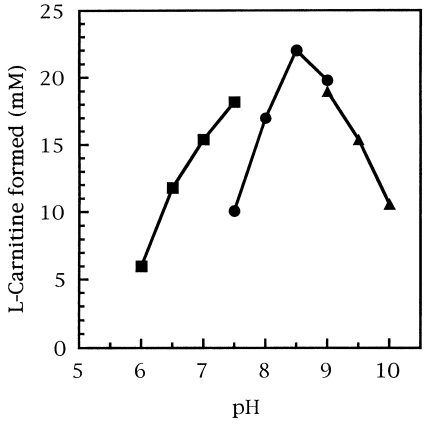


Fig. 2. Effect of pH on the formation of L-carnitine. The reaction was carried out at 30°C for 7 h as described in the text except that the buffer and pH were varied as indicated. (■) potassium phosphate buffer, (●) Tris–HCl buffer, (▲) glycine–NaOH buffer.

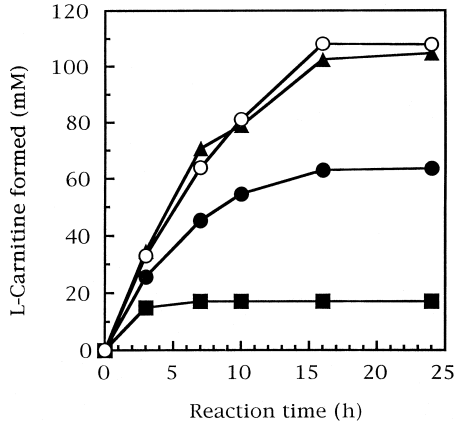


Fig. 3. Formation of L-carnitine with various concentrations of D-carnitine. The reaction mixture (3.0 ml) contained 66.7 mM Tris–HCl buffer (pH 8.5), 5 mM NAD, 1 mM glutathione, and the partially purified enzyme (1 unit as D-carnitine dehydrogenase activity), and reaction was carried out at 30°C. The concentration of D-carnitine were varied as indicated. (■) 0.05 M, (●) 0.1 M, (▲) 0.3 M, (○) 0.5 M.

Table 2
Disappearance of NAD during the conversion reaction

Time (h)	Residual NAD (mM)	L-carnitine formed (mM)
0	5.0	0
3.0	4.02	23.6
7.0	2.78	44.0
10.0	1.80	44.9
24.0	0.01	45.9

The reaction was carried out as described in the text except that the concentration of D-carnitine was 0.1 M.

The conversion of L-carnitine from D-carnitine at various concentration was examined (Fig. 3). The maximal yield was about 64% on a molar basis at 0.1 M of D-carnitine, and the maximal amount of L-carnitine formed was 108 mM from 0.5 M of D-carnitine. Hanschmann and Kleber [23] reported that using highly purified D- and L-carnitine dehydrogenase from *Agrobacterium* sp. almost 50% of D-carnitine could be converted into L-carnitine under the following reaction conditions; pH 8.5, 50 mM D-carnitine, 1 mM NAD, 0.1 mM NADH, 0.0104 U of D-carnitine dehydrogenase, 25-fold excess of L-carnitine dehydrogenase. Effect of ratio of D- and L-carnitine dehydrogenase on the formation of L-carnitine was examined. Addition of 15-fold excess of highly purified carnitine dehydrogenase of *Alcaligenes* sp. was not effective for the L-carnitine formation. NAD in the reaction mixture was decreased during the conversion reaction (Table 2). The NAD degrading activity may be contained in the partially purified carnitine dehydrogenase preparation. Further investigation of L-carnitine production by highly purified D- and L-carnitine dehydrogenase is in progress.

Acknowledgements

This work was supported in part by the Sasakawa Scientific Research Grant from The Japan Science Society.

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