Carnitine Biosynthesis

HYDROXYLATION OF *N*⁶-TRIMETHYL-LYSINE TO 3-HYDROXY-*N*⁶-TRIMETHYL-LYSINE

Dileep S. SACHAN* and Charles L. HOPPEL

Veterans Administration Medical Center and Departments of Pharmacology and Medicine, School of Medicine, Case Western Reserve University, Cleveland, OH 44106, U.S.A.

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Rat kidney homogenates metabolize *N*⁶-trimethyl-lysine to *N*-trimethylammonio-butyrate, but not to carnitine. The first step in this conversion is the hydroxylation of trimethyl-lysine to form 3-hydroxy-*N*⁶-trimethyl-lysine. An assay system was developed in which hydroxylation of trimethyl-lysine is linear with respect to both time and homogenate protein concentration. The rate is 5 nmol of 3-hydroxy-*N*⁶-trimethyl-lysine formed/min per mg of homogenate protein. The cofactors required are ascorbate, α -oxoglutarate, FeSO₄, and O₂. Catalase and dithiothreitol give a 20% stimulation. Ca²⁺ produces a 2-fold increase in specific activity and cannot be replaced by Mg²⁺, Mn²⁺ or Zn²⁺. These last three bivalent cations lead to a decreased activity. Subcellular distribution studies demonstrate that trimethyl-lysine hydroxylase activity parallels the distribution profile of succinate dehydrogenase and citrate synthase. Thus trimethyl-lysine hydroxylase has a mitochondrial localization. Distribution of trimethyl-lysine hydroxylase activity between cortex and medulla of kidney is 67 and 33% respectively, similar to mitochondrial distribution.

Carnitine (3-hydroxy-4-trimethylammonio-butyrate) is synthesized from the essential amino acids lysine and methionine in the rat (Cox & Hoppel, 1973a; Tanphaichitr & Broquist, 1973) and in *Neurospora crassa* (Horne & Broquist, 1973; Rebouche & Broquist, 1973). The first sequence of reactions in mammalian species is: lysine → protein-bound lysine → protein-bound trimethyl-lysine → free trimethyl-lysine (Cox & Hoppel, 1973b); in *Neurospora*, lysine can be directly methylated without incorporation into protein (Rebouche & Broquist, 1973). The initial step in the biosynthesis of carnitine from trimethyl-lysine is hydroxylation to form 3-hydroxy-*N*⁶-trimethyl-lysine (Hoppel *et al.*, 1976; 1980; Novak *et al.*, 1980). Hoppel *et al.* (1976) showed that after injection of trimethyl-lysine in the rat, hydroxy-trimethyl-lysine was excreted in the urine. All tissues examined convert trimethyl-lysine into hydroxy-trimethyl-lysine; however, in tracer studies the highest conversion occurred in the kidney (Cox & Hoppel, 1974a).

Thus, using kidney, we have developed an enzymic assay for the hydroxylation of trimethyl-

lysine and have examined some of the properties of the activity and determined its intracellular localization in mitochondria. Hulse *et al.* (1978) suggested a mitochondrial localization for the enzymic activity in rat liver, although they neither used a kinetically determined assay nor examined its subcellular distribution.

Experimental

Kidney homogenates

Male Sprague–Dawley rats (200–400 g; Zivic Miller, Allison Park, PA, U.S.A.) were used in all experiments. The rats were maintained on Purina rat chow and water *ad libitum*. Rats were stunned by a blow to the head and decapitated, and the kidneys were removed; the capsules were peeled off and discarded. The kidneys were rinsed, blotted and weighed. All subsequent procedures were carried out on ice. The kidneys were then homogenized in 3 vol. of 220 mM-mannitol/70 mM-sucrose/5 mM-4-morpholinepropanesulphonic acid (pH 7.4) (MSM buffer) in a Potter–Elvehjem homogenizer with a tight-fitting pestle.

Subcellular fractionation

The procedure for preparing subcellular fractions from liver (de Duve *et al.*, 1955; Norum & Bremer,

* Present address: Department of Food Science, Nutrition and Food Systems Administration, University of Tennessee, Knoxville, TN 37916, U.S.A.

1967) was followed. Kidneys were homogenized in MSM buffer in the presence or absence of 2 mM-EDTA. An RC-5 Sorvall (rotor SS-34) centrifuge was used to prepare the nuclear and heavy and light mitochondrial fractions and a Beckman L-50 ultracentrifuge (Rotor TI 50.2) was used for isolation of the microsomal fraction. The sum of the values for the low-speed pellet (N, nuclear fraction) and the supernatant (E, cytoplasmic extract) was used to represent the total homogenate fraction as discussed by de Duve *et al.* (1955). Proteins were determined by using the biuret method after solubilization with deoxycholate (Gornall *et al.*, 1949).

Enzyme assays

(a) *Trimethyl-lysine hydroxylase*. The enzymic activity was measured by determining the amount of [$Me-^{14}C$]trimethyl-lysine converted into hydroxytrimethyl-lysine. The incubation medium was 75 mM-Tris/HCl (pH 8.0)/2.5 mM-ascorbate/2.5 mM-2-oxoglutarate/0.5 mM-FeSO₄/0.5 mM-dithiothreitol/catalase (0.5 mg/ml)/5 mM-trimethyl-lysine (1 μ Ci of $^{14}C/\mu$ mol of trimethyl-lysine). The final volume was 2 ml and the temperature was 37°C. After a 5 min preincubation, the reaction was initiated by adding approx. 2 mg of homogenate protein. Flasks were sealed with serum caps and continuously flushed with O₂/CO₂ (19:1) with shaking at 100 strokes/min. The reaction was terminated by addition of 0.2 ml of 60% (w/v) HClO₄. The flask and its contents were then chilled, transferred to test tubes and centrifuged at 2000 rev./min for 5 min. After this, the supernatant was collected and the pellet was washed with 0.5 ml of water. The combined wash and supernatant were neutralized with 10 M-KOH and kept on ice; the KClO₄ was removed by centrifugation, and the supernatant was chromatographed on a column (1 cm $\times$ 50 cm) of AG-50 (X8; H⁺ form; 200–400 mesh) and eluted with a gradient of increasing HCl concentration (150 ml each of 1.5 M-HCl and 3 M-HCl) at a flow rate of 0.5 ml/min. Fractions (2 ml) were collected, and 0.2 ml of each fraction was counted for radioactivity in 4 ml of toluene/Triton X-100 (2:1, v/v) scintillant in a Packard 3375 scintillation counter. The product was identified as 3-hydroxytrimethyl-lysine by t.l.c. (Cox & Hoppel, 1973b) and high-pressure liquid chromatography (C. L. Hoppel, unpublished work) and by conversion into 4-trimethylammonio-butyrate and carnitine in liver slices (Cox & Hoppel, 1974b).

(b) *Subcellular fractionation markers*. Fractions [except for the high-speed supernatant (Hoppel & Tomec, 1972) obtained in subcellular distribution studies] were treated with Triton X-100, 1 mg/ml final concentration, for assaying total acid phosphatase, glucose 6-phosphatase and 5'-nucleotidase. Acid phosphatase (free and total) and glucose

6-phosphatase were determined by the method of de Duve *et al.* (1955), and 5'-nucleotidase by the method of Emmelot *et al.* (1964). Succinate dehydrogenase (Hoppel & Copper, 1968) and citrate synthase (Srere, 1969) were assayed spectrophotometrically by published methods. For these spectrophotometric assays, the fractions were diluted to 1 mg of protein/ml in 0.1% cholic acid and 0.05 M-phosphate buffer, pH 7.2. All the above assays were done at 37°C. Catalase was measured spectrophotometrically at 25°C, by the method of Luck (1965).

Separation of cortex and medulla

The method of Reimer & Jennings (1971) was followed to separate kidney cortex and medulla. Kidney slices were made by using a Stadie-Riggs tissue slicer and cortical and medullary regions were separated by dissection under a dissecting microscope. After separation, homogenization and assays were carried out similarly to that for whole kidney.

Results

Assay requirements

Homogenates of rat kidney are active in converting substrate amounts of trimethyl-lysine into hydroxytrimethyl-lysine and were used as a source of enzyme. The cofactors essential for enzymic assay were determined (Table 1). When ascorbate or α -oxoglutarate was deleted from the assay, only 10% of activity remained. If the system was not purged with O₂, only 33% of activity was expressed. The absence of FeSO₄ led to a 50% loss in activity, whereas absence of catalase or dithiothreitol produced a slight decrease in activity. Glucose was not required. The effect of 0.5 mM-FeSO₄ was maximal,

Table 1. *Requirements for hydroxylation of N⁶-trimethyl-lysine in rat kidney homogenate*

Complete incubation system consisted of 75 mM-Tris buffer (pH 8.0)/2.5 mM-ascorbate/2.5 mM-2-oxoglutarate/0.5 mM-dithiothreitol/50 mM-glucose/5 mM-FeSO₄/5 mM-N⁶-trimethyl-lysine/catalase (0.5 mg/ml)/homogenate protein (2.9 g/ml). An activity of 100% equals 158 nmol of 3-hydroxy-N⁶-trimethyl-lysine per 5.8 mg of protein in 10 min at 37°C.

Conditions	Activity (%)
Complete	100.00
Minus FeSO ₄	52.4
Minus glucose	92.3
Minus catalase	78.4
Minus dithiothreitol	79.1
Minus 2-oxoglutarate	9.2
Minus ascorbate	10.6
Minus O ₂	32.9

Table 2. *Effect of EDTA and Ca²⁺ on hydroxylation of N⁶-trimethyl-lysine in rat kidney homogenate*

The homogenate was prepared and assayed for enzymatic activity as described in the Experimental section. The incubation was carried out for 3 min with 2 mg of homogenate protein. Specific activity refers to nmol of 3-hydroxy-N⁶-trimethyl-lysine formed/min per mg of protein.

Addition to incubation	Specific activity	Change (%)
—	1.54	100
0.03 mM-EDTA	1.16	75
0.20 mM-EDTA	0.33	21
5.00 mM-CaCl ₂	3.39	220

and a concentration of 5 mM-trimethyl-lysine was saturating. The apparent K_m for trimethyl-lysine is 1.6 mM.

By using rat kidney homogenates prepared in the absence of EDTA, a 2-fold increase in specific activity was observed when Ca²⁺ was added to the incubation medium (Table 2). The addition of EDTA to the incubation medium produced an inhibition of trimethyl-lysine hydroxylation. This decrease was concentration-dependent, with 80% of enzymic activity lost at 0.2 mM-EDTA (Table 2). All subsequent experiments were done in the presence of 5 mM-CaCl₂.

When kidney homogenates were prepared by using MSM buffer containing a final concentration of 2 mM-EDTA, a 50% decrease was observed in the activity for hydroxylation of trimethyl-lysine (0.35 compared with 0.64 μ mol/min per g of kidney, with and without EDTA respectively; assay contained 5 mM-CaCl₂ for both determinations). Thus the loss was not completely restored by the addition of Ca²⁺ to the incubation medium.

Time and protein curves

By using rat kidney homogenates prepared in the absence of EDTA and with CaCl₂ added to the incubation medium, we determined the dependence of the reaction on time and protein. The initial rate of product formation was proportional to the homogenate protein concentration up to 1.5 mg/ml and linear with time for 4 min (Fig. 1).

Intracellular distribution of trimethyl-lysine hydroxylase activity

The distribution pattern of trimethyl-lysine hydroxylase activity in the subcellular fractions of kidney (homogenized in 2 mM-EDTA) was compared with the distribution of marker enzymes (Table 3). The distribution of marker enzymes, i.e. citrate synthase (mitochondrial matrix), succinate dehydrogenase (mitochondrial inner-membrane), 5'-

Fig. 1. *Time- (●) and protein- (○) dependent formation of 3-hydroxy-N⁶-trimethyl-lysine by rat kidney homogenates*

The assay was done under standard conditions described in the Experimental section. To determine the linearity with time, the homogenate protein concentration was 1.5 mg/ml; to determine linearity with protein concentration the time was 3 min. Both experiments contained 5 mM-CaCl₂.

nucleotidase (plasma membrane), glucose 6-phosphatase (microsomal), acid phosphatase (lysosomal) and catalase (peroxisomal) corresponded to that from studies in liver (Hoppel & Tomec, 1972) and kidney (de Duve & Baudhuin, 1966; George & Kenny, 1973). On the basis of marker enzymes, contamination between fractions was low except that acid phosphatase (total) activity was in the nuclear fraction. Free acid phosphatase activity, an indicator of damage done during homogenization and fractionation, was about 36% of the total. This value is about 14% higher than that reported for liver; however, in the absence of comparative data for kidney, the operation should be considered gentle and differences attributed to two types of tissues. The recovery of enzymic activity and protein was satisfactory.

On the basis of differential centrifugation, two populations of mitochondria (heavy and light) in kidney were demonstrated by these data (Table 3) and are consistent with indirect evidence available in the literature (Ch'ih & Devlin, 1971; Bondi *et al.*, 1972). Trimethyl-lysine hydroxylase activity was distributed in parallel with succinate dehydrogenase and citrate synthase (Table 3), suggesting a mitochondrial location of this enzymic activity.

When fractionation was carried out in the absence of EDTA, the activity of trimethyl-lysine hydroxylase was 2-fold higher than in the presence of 2 mM-EDTA. The distribution of succinate dehydro-

Table 3. *Subcellular distribution of N^6 -trimethyl-lysine hydroxylase and some marker enzymes in rat kidney*

The enzymes and proteins were assayed as described in the Experimental section. Proteins were expressed in mg/g of kidney (wet wt.). The absolute values in the total homogenate (fractions E + N) are given in μmol of substrates metabolized or product formed/g of kidney (wet wt.) per min. Fractions are defined as follows: E, cytoplasmic extract; N, nuclear fraction; M, heavy mitochondrial fraction; L, light mitochondrial fraction; P, microsomal fraction; S, the final supernatant. Values for acid phosphatase (free activity) are given as the percentage of acid phosphatase (total activity) in the homogenate (E + N).

Enzyme	Total homogenate	Recovery (%)						
		E + N	N	M	L	P	S	Total
N^6 -Trimethyl-lysine hydroxylase	0.35	100	9.4	42.8	33.9	8.0	5.0	99.1
Citrate synthase	31.35	100	19.1	26.7	32.8	4.9	7.8	91.3
Succinate dehydrogenase	6.66	100	14.9	40.5	29.8	7.7	4.2	97.1
5'-Nucleotidase	10.53	100	21.5	7.8	14.9	58.9	1.1	104.2
Glucose 6-phosphatase	21.49	100	18.6	1.9	3.7	54.6	0.3	79.1
Acid phosphatase (total)	5.39	100	37.5	18.5	12.5	22.1	2.1	91.7
Acid phosphatase (free)	1.90	(35.8)	(4.4)	(8.7)	(8.8)	(16.1)	(0)	(38.0)
Catalase	4.94	100	3.7	2.2	4.4	7.5	68.2	86.0
Protein	157.34	100	20.3	10.9	9.1	21.1	37.7	99.1

Table 4. *Effect of bivalent cations on hydroxylation of N^6 -trimethyl-lysine by rat kidney mitochondria*

Mitochondria were prepared and assayed for enzymic activity as described in the Experimental section. The incubation was carried out for 3 min with 0.5 mg of mitochondrial protein. Specific activity refers to nmol of 3-hydroxy- N^6 -trimethyl-lysine formed/min per mg of mitochondrial protein.

Cations	Concentration (mM)	Specific activity	Activity (%)
None	—	8.92	100.0
CaCl_2	3.0	15.76	177.7
CaCl_2	5.0	16.32	184.0
MgCl_2	0.5	6.24	70.0
MgCl_2	3.0	3.21	36.0
MnCl_2	1.0	0.0	0
ZnCl_2	1.0	0.0	0

genase, citrate synthase and trimethyl-lysine hydroxylase shifted in the absence of EDTA. About 10% more of the activity for all three enzymes remained in the nuclear fraction. The migration of mitochondrial enzyme activity into the nuclear fraction was accompanied by an equivalent decreased activity in the heavy mitochondrial fraction without affecting the activity in the light mitochondria. This migration of enzymic activity may be due to swelling of mitochondria in the absence of EDTA and settling with the nuclear pellet during differential centrifugation.

Effect of metal ions

The effect of bivalent cations on the activity of trimethyl-lysine hydroxylase present in the combined light and heavy mitochondrial fractions was

Table 5. *Distribution of enzymic activities between cortex and medulla of rat kidney*

Cortex and medulla were isolated and enzymic activity was measured by the procedure described in the Experimental section and Table 1. The activity refers to μmol of product formed/min per g of kidney (wet wt.).

Enzyme or protein	Activity	
	Cortex	Medulla
Protein	107.2	54.7
N^6 -Trimethyl-lysine hydroxylase	0.36	0.17
Citrate synthase	23.60	12.30
Succinate dehydrogenase	7.60	3.40

examined (Table 4). As observed in homogenate (Table 2), Ca^{2+} enhanced the activity almost 2-fold in mitochondria. There was no significant difference between 3 mM- and 5 mM- Ca^{2+} in the incubation medium. On the contrary, Mg^{2+} , Mn^{2+} and Zn^{2+} inhibited enzymic activity. Whereas 0.5 and 3.0 mM- Mg^{2+} inhibited basic activity by 36 and 70% respectively, 1 mM- Mn^{2+} or Zn^{2+} was 100% inhibitory.

Enzymic activity in cortex and medulla

The distribution of protein, N^6 -trimethyl-lysine hydroxylase, citrate synthase and succinate dehydrogenase in cortical and medullary homogenates of kidney was examined (Table 5). There was twice as much activity in cortex as in medulla in all the above enzymes and in protein. These observations are consistent with those reported in the literature for proteins and succinate dehydrogenase (Ch'ih & Devlin, 1971; Bondi *et al.*, 1972).

Discussion

Kidney was used for studying the enzymic activity responsible for hydroxylation of trimethyl-lysine to form hydroxytrimethyl-lysine. Subcellular fractionation studies showed that trimethyl-lysine hydroxylase was located in the mitochondrial fractions (both heavy and light) and paralleled the distribution profile of succinate dehydrogenase and citrate synthase. The light mitochondrial fraction of kidney contained almost as much of the marker enzymes (also trimethyl-lysine hydroxylase) as did the heavy mitochondrial fraction. In liver, the light mitochondrial fraction has only traces of mitochondrial marker enzymes (Hoppel & Tomec, 1972). Because of the inhibitory effect of EDTA on trimethyl-lysine hydroxylase, subcellular fractionation was carried out both with and without EDTA. The recovery of activity after subcellular fractionation was 100%, on the basis of homogenate activity whether with or without EDTA. This suggests that EDTA is affecting the hydroxylase, and this effect is not totally reversed by washing without EDTA or by adding Ca^{2+} to the incubation.

The rates of hydroxylation of trimethyl-lysine were measured under optimal assay conditions to obtain maximal activity. The kinetic criteria for an assay were met with a saturating concentration of substrates and linearity with respect to time and protein concentration. The mitochondrial activity under these conditions was 16.8 nmol/min per mg when isolated from homogenates prepared in EDTA and 30.2 nmol/min per mg without EDTA. These rates, under kinetically acceptable conditions, are 500–1000-fold higher than those reported (0.03 nmol/min per mg of protein) by Hulse *et al.* (1978) for rat liver mitochondria. The kidney enzyme perhaps is more active than the hepatic enzyme, as Cox & Hoppel (1974a), by using tissue slices, noted greater incorporation of trace radioactive trimethyl-lysine in kidney relative to liver.

The mitochondrial localization of trimethyl-lysine hydroxylase is unusual. Proline hydroxylase and lysine hydroxylase from rat liver and chick-embryo cartilage are located in the endoplasmic reticulum (microsomal fraction) (Peterkofsky & Assad, 1976; Oikarinen *et al.*, 1976; Guzman *et al.*, 1976; Risteli *et al.*, 1976), and butyrobetaine hydroxylase and tryptophan hydroxylase are soluble enzymes (Lindstedt, 1967; Lindstedt & Lindstedt, 1970; Hosoda *et al.*, 1977). Trimethyl-lysine hydroxylase bears similarity to aryl-hydrocarbon hydroxylase (Uemura & Chiesara, 1976) and 25-hydroxycholecalciferol 1-hydroxylase (DeLuca, 1974) in that all have a mitochondrial distribution and require molecular O_2 . Whether cytochrome *P*-450 is involved in hydroxylation of trimethyl-lysine was not tested in the studies reported in the present paper.

The kidney cortex contained twice as much protein, trimethyl-lysine hydroxylase, succinate dehydrogenase and citrate synthase (per g weight) as did the medulla. There is some evidence in the literature that supports the partitioning of proteins and succinate dehydrogenase between kidney cortex and medulla (Ch'ih & Devlin, 1971; Bondi *et al.*, 1972).

Ascorbate, α -oxoglutarate, FeSO_4 and molecular O_2 are essential for maximal activity. These co-factor requirements of trimethyl-lysine hydroxylase are similar to those of proline hydroxylase, lysine hydroxylase and butyrobetaine hydroxylase (Peterkofsky & Assad, 1976; Oikarinen *et al.*, 1976; Guzman *et al.*, 1976; Risteli *et al.*, 1976; Lindstedt, 1967; Lindstedt & Lindstedt, 1970). Bovine serum albumin is an integral part of lysine hydroxylase and proline hydroxylase assay, but was without effect on trimethyl-lysine hydroxylase in the present study (results not shown).

The effect of Ca^{2+} in enhancing hydroxylation appears to be unique to trimethyl-lysine hydroxylase. The basal mitochondrial activity is enhanced 2-fold by Ca^{2+} . The requirement for Ca^{2+} appeared to be specific, as other bivalent cations, namely Mg^{2+} , Mn^{2+} and Zn^{2+} , were unable to replace Ca^{2+} , but instead were inhibitory. Mn^{2+} and Zn^{2+} were more potent inhibitors than was Mg^{2+} . Bivalent cations have been reported to inhibit proline hydroxylase and lysine hydroxylase *in vitro* by competing for Fe^{2+} ions (Ryhanen, 1976).

The role of Ca^{2+} in stimulating hydroxylation is unknown. It is possible that Ca^{2+} is an integral part of trimethyl-lysine hydroxylase or simply facilitates transport of trimethyl-lysine or α -oxoglutarate across the mitochondrial membrane. Ca^{2+} -mediated transport of α -oxoglutarate in rat and rabbit kidney mitochondria has been suggested by Mason *et al.* (1977). This alternative is interesting, as α -oxoglutarate is an obligatory requirement for trimethyl-lysine hydroxylase.

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