Determination of the Isotope Effect of the Enzymatic Oxidation of (R)Carnitine by Displacement of the Equilibrium *via* Mass Action

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(R) Carnitine, ^{3}H-Isotope Effect, (R) Carnitine Dehydrogenase

An isotope effect of the dehydrogenation of (R) Carnitine [(R) 3-hydroxy-4-trimethylaminobutyric acid hydrochloride] catalyzed by (R) carnitine dehydrogenase [(R) carnitine: NAD oxidoreductase E.C. 1.1.1.108] from *Pseudomonas aeruginosa* was measured at different temperatures. It was found that k^1H/k^3H does not vary greatly with changes of temperature. The value of 3 for k^1H/k^3H measured at small initial conversions strongly indicated that the rate limiting step of the oxidation of (R) carnitine is the cleavage of the C–H bond at C_3.

We have recently reported on a new enzyme assay for (R) carnitine based on a stereospecific exchange between the C_3 hydrogen of (R) [3-^{3}H] carnitine and water using NAD as hydrogen carrier catalyzed by the coupled action of (R) carnitine dehydrogenase (R-CDH) of *P. aeruginosa* and α-lipoamide dehydrogenase of pig heart (diaphorase) [1]. In order to investigate the kinetic parameters governing this highly specific enzymatic exchange reaction [2, 3], the isotope effect of the NAD dependent oxidation of (R) carnitine needed to be investigated.

The isotope effect which accompanies dehydrogenase reactions, utilizing substrates labeled with ^{3}H, often yields insight into mechanistic details of the reaction [4–6]. A method frequently employed to evaluate isotope effects is to measure the specific radioactivity of the accumulated product at a small initial conversion, and to compare it with the specific radioactivity of the starting material [4–7]. Stevens and Attree [7] showed that under these conditions a simple relationship exists between the rate constant of the reaction of the unlabeled compound (k) to that of the labeled compound (k^*), the fraction of overall conversion of the reaction (f) and the ratio of the molar specific radioactivities of product and starting material (r).

$$k/k^* = \frac{\ln(1-f)}{\ln(1-fr)} . \qquad (1)$$

When the conversion becomes small or $f \to 0$ the following equation holds true:

$$\lim_{f \to 0} \ln(1+|f|) \cong |f|$$

It then becomes clear that under these circumstances

$$k/k^* = 1/r \qquad (2)$$

or the isotope effect becoming equal to the ratio of the specific radioactivities of starting material and product [7–9].

For purposes of convenience, kinetic isotope effects have been classified as primary and secondary [4–9]. Primary isotope effects result when bonds involving the isotopic atoms are cleaved during the rate limiting step of the overall reaction with values for ^{2}H and ^{3}H substituted compounds usually above 2; and secondary isotope effects arising whenever these bonds are little affected during the rate limiting step with values for ^{2}H and ^{3}H substituted compounds usually close to unity [4, 5]. This classification does not take into account tunneling, which in specific cases accounts for unusually large isotope effects [4–6].

The equilibrium constant for the reaction [10]:

$$\frac{[\mathrm{NAD^+}]\,[\text{(R) carnitine}]}{[\mathrm{NADH}]\,[\mathrm{H^+}]\,[\text{3-dehydrocarnitine}]} = 7.6 \times 10^{10}$$

shows that the equilibrium is far to the side of the reduced substrate, such as is the case in many NAD-linked dehydrogenases. In addition, (R) carnitine dehydrogenase from *Pseudomonas aeruginosa* is extremely sensitive towards the usual ketone trapping agents [1, 2], *i. e.*, hydrazine, semicarbazide, amino-oxyacetic acid. For these reasons the experimental procedure reported here to bring about reaction in the direction of dehydrocarnitine employs

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(R) carnitine is equivalent to (L) carnitine in the DL notation.

Table I. Isotope effect of the oxidation of (R) [3-^{3}H] carnitine catalyzed by (R) carnitine dehydrogenase from *Pseudomonas aeruginosa*.

No.	NAD μmol/ml	Carnitine dilution used [μmol]	pH	Temperatur [°C]	Specific radioactivities [dpm/μmol] (R) Carnitine	NADH	Isotope effect
1	2.8	70 [a]	8.5	13	1.8×10^5	4.6×10^4	3.9 ± 0.5
2	2.8	140 [b]	8.3	13	1.8×10^5	5.2×10^4	3.4 ± 0.6
3	2.8	70 [a]	8.2	25	1.8×10^5	4.3×10^4	3.7 ± 0.4
4	2.8	140 [b]	7.9	25	1.8×10^5	6.0×10^4	3.0 ± 0.5
5	2.8	70 [a]	8.6	37	1.8×10^5	6.5×10^4	2.8 ± 0.3
6	2.8	140 [b]	8.1	37	1.8×10^5	6.7×10^4	2.7 ± 0.4
7	15.0	12 [c]	8.5	25	1.1×10^6	3.1×10^5	3.5 ± 0.4

70 μmol carnitine·HCl (dilution a) per ml reaction medium (see Methods section).
140 μmol carnitine·HCl (dilution b) per ml reaction medium (see Methods section).
12 μmol carnitine·HCl (dilution c) per ml reaction medium (see Methods section).

the principle of mass action. Our experimental design varies from those classically used in the determination of isotope effects of reactions with NAD-linked dehydrogenases in the direction toward the oxidized substrate [4]. Using the reported equilibrium constant for the above reaction of 7.6×10^{10} [10]. the amount of NADH produced in experiments 1 – 6 in Table I is approximately 25% of the theoretically obtainable amount; at the same time only 0.4% of the carnitine has been converted to 3-dehydrocarnitine. Under these conditions of a small fraction of overall conversion, the above consideration holds true and stated in Eqn. (2), the isotope effect should equal directly the ratio of specific radioactivities of starting material and product [7–9].

Materials and Methods

(R) carnitine hydrochloride was a gift of Otsuka-Pharmaceutical Co., Osaka, Japan. (RS) carnitine hydrochloride, α-lipoamide dehydrogenase, NAD and DEAE cellulose were purchased from Sigma Chemical Company. NaB^3H_4 was from New England Nuclear. All other chemicals were of analytical grade.

Radioactivity was determined by scintillation counting using 10 – 15 ml of a solution containing 5 g Omnifluor (New England Nuclear) in 1000 ml of a mixture 2:1 (v/v) of toluene Triton-X-100 (Rohm and Haas) in a Beckman LS 250 liquid scintillation counter.

Preparation of (R) carnitine dehydrogenase

Pseudomonas aeruginosa ATCC 7700 was grown after adaption in a minimal medium containing 0.5% (RS) carnitine hydrochloride as the sole carbon source [2, 3, 10], 0.01% $MgSO_4$, 1.33% Na_2HPO_4, 0.4% KH_2PO_4 and 0.01% yeast extract Difco). (RS) carnitine hydrochloride was neutralized to pH 7 before addition to the medium. After 12 hours incubation at 37 °C and under slow agitation in low form flasks, the cells were harvested, washed twice with 0.05 M Tris-HCl buffer pH 9.0, resuspended in the same buffer to an optical density of 1 at 660 nm [12], and lysed by incubation for 1 hour at 37 °C with 0.03% lysozyme (Miles-Seravac) and 0.2% Na_2H_2-EDTA $\times$ 2 H_2O [11]. The supernatant was fractionally precipitated at 5 °C with $(NH_4)_2SO_4$ [10] and the 40 – 45% salt saturated fraction was collected and found to possess a specific enzyme activity of 1 U/mg protein when measured after Aurich *et al.* [10]. This fraction was resuspended in 0.05 M Tris-HCl pH 9 and dialyzed against a continuous stream of the same buffer in a hollow fiber dialyzer (Bio-Fiber 80, Bio-Rad, Calif.). The dialysate was then placed on a 2 $\times$ 40 cm Sephadex G 200 column and eluted with 0.05 M Tris-HCl pH 9. The fractions with highest activity were combined and concentrated in the same hollow fiber device. At this stage the specific activity had risen to 5 U/mg protein and when stored at – 25 °C the activity remained virtually unchanged for at least 6 weeks.

Preparation of (RS) [3-^{3}H] carnitine hydrochloride

(RS) [3-^{3}H] carnitine hydrochloride was prepared by reduction of 37 μmol (10.4 mg) of 3-dehydrocarnitine ethyl ester bromide which was synthesized according to the method of Aurich *et al.* [12]. This was dissolved in 3 ml 0.1 M $Na_4P_2O_7 \times 12$ H_2O-HCl buffer pH 9.5 and reduced by addition of first, 5 μmol of NaB^3H_4 with a specific radioactivity of 120 μCi/μmol and then, 10 min later, of 40 μmol NaB^3H_4 with a specific radioactivity of 12 mCi/μmol. Finally, the reaction was brought to completion with 1 mmol non-labeled $NaBH_4$. The completion of the

reaction was followed by measuring optical density at 280 nm ($\varepsilon_{280\,nm} = 2 \times 10^4$) of a 1/200 dilution in a Gilford spectrophotometer. The (RS) [3-^{3}H] carnitine ethylester bromide was hydrolyzed with 40% hydrobromic acid [12] and the resulting (RS) [3-^{3}H] carnitine HBr placed on a 1×50 cm Dowex 50×8, 200 – 400 mesh, cation exchanger column in the hydrogen form, washed with 100 ml water and there-after with 1 N HCl which eluted the labeled (RS) [3-^{3}H] carnitine HCl after 150 ml [13]. The combined fractions containing the labeled carnitine were concentrated *in vacuo* and the specific radioactivity measured after the assay of Marquis and Fritz [14]. The chemical yield of the (RS) [3-^{3}H] cartinitine HCl was 90% with a specific radioactivity of 3.1 Ci/mol. The purity of the (RS) [3-^{3}H] carnitine HCl was further established by high pressure ion exchange chromatography on Aminex A-5 (J. S. Hayes, M. A. Alizade and K. Brendel, in preparation). The majority of the radioactivity (96%) eluted with an authentic carnitine sample. For the following experiments the material collected after the aforementioned chromatographic procedures was utilized.

Carnitine dilutions

Carnitine hydrochloride with three different compositions and specific radioactivities in regard to the (R)- and (S)-enantiomorphs were prepared as follows:

Dilution a. 3.8×10^{-3} μmol (RS) [3-^{3}H] carnitine with a specific radioactivity of 6.8×10^9 dpm/μmol were diluted with 70 μmol non-labeled (R) carnitine. HCl to obtain (R) carnitine HCl of a specific radioactivity of 1.8×10^5 dpm/μmol containing in addition 1.9×10^{-3} μmol of the enzymatically inactive (S) carnitine with a specific radioactivity of 6.8×10^9 dpm/μmol.

Dilution b. 3.8×10^{-3} μmol (RS) [3-^{3}H] carnitine·HCl with a specific radioactivity of 6.8×10^9 dpm/μmol were diluted with 140 μmol non-labeled (RS) carnitine HCl to obtain (RS) [3-^{3}H] carnitine HCl with a specific radioactivity of 1.8×10^5 dpm/μmol.

Dilution c. 3.8×10^{-3} μmol (RS) [3-^{3}H] carnitine ·HCl were diluted with 12 μmol non-labeled (R) carnitine·HCl to obtain a material with a specific radioactivity of 1.1×10^6 dpm/μmol and containing, in addition, 1.9×10^{-3} μmol of the enzymatically inactive (S) carnitine HCl with a specific radioactivity of 2.5×10^9 dpm/μmol.

General incubation conditions

The incubation conditions for the experiments described in Table I, for each ml of the reaction mixture, were as follows. The indicated amounts of NAD and carnitine were adjusted to pH 8 before addition to 200 μmol Tris-HCl buffer of pH 9.0 per ml reaction solution. Prior to addition of 0.05 U/ml of (R) carnitine dehydrogenase (specific activity 6 U/mg protein), the final pH of the reaction medium was adjusted to the values given in Table I. After 10 min when ΔOD_{340} was about 1.5, the NADH was isolated as described below. At this stage, 0.1 ml of the original incubate as a control was passed through a mixed bed ion exchanger consisting of equal parts of Dowex 50×8 hydrogen form, and Dowex 1×8 formate form, the components of which had been freed of adhering oxidants by prior treatment with Na_2SO_3 solution. The columns were washed with 2 ml water, and the radioactivity of the eluate which was found to be mainly in H_2O was utilized to correct the specific radioactivity of the isolated NADH [1]. This correction, probably due to a small coexisting diaphorase activity, never accounted for more than 7% of the original specific radioactivity. After completion of the reaction, the NADH was isolated as described below.

Isolation of NADH

NADH was isolated from the incubation mixtures essentially by the procedure of Silverstein [15] using a DEAE-cellulose column (1×5 cm) in the bicarbonate form, washing with 50 ml of H_2O (which eluted carnitine), 100 ml of 3.5 mM NH_4HCO_3 (which eluted the NAD), and finally, 10 ml of 0.2 M NH_4HCO_3 (which elutes the NADH). The purity of the last fraction was checked by measuring the 260/340 nm absorption ratio. If the ratio was higher than 3, the purification procedure was repeated. The isolated NADH was concentrated *in vacuo* at 40 °C, taken up in 2 to 3 ml of water, and the tritium content in aliquots of this solution was measured; NADH concentration was determined enzymatically after addition of Na-pyruvate and lactate dehydrogenase [16]. Transfer of counts from NADH into (S) glutamate was quantitative in case of (R) carnitine derived NADH which is consistent with the known stereochemistry of (R) carnitine dehydrogenase [17].

Results and Discussion

As shown in Table I, the NAD dependent oxidation of (R) carnitine catalyzed by (R) carnitine dehydrogenase of *P. aeruginosa* is accompanied by a kinetic isotope effect of approximately 3, suggestive of the cleavage of the C-H bond at C_3 in (R) carni-

tine as the rate limiting step in the overall reaction [1–5]. Even though the changes obtained by raising the temperature seem to indicate a slight but significant decrease of the overvall kinetic isotope effect, we would not like to attach any mechanistic implications to this finding at this point. The measurement of the isotope effect of the enzymatic oxidation of (R) carnitine as described in Table I differs from standard procedures. The equilibrium of the reaction between (R) carnitine and NAD on one side and 3-dehydrocarnitine and NADH on the other side cannot be displaced to the side of the oxidized substrate 3-hydrocarnitine with ketone trapping agents because these agents inactivate the enzyme [2, 3]. Instead of following the classical approach the equilibrium was displaced by mass action utilizing high concentrations of (R) carnitine and NAD at a pH of about 8. In the experiments described in Table I, a ratio of (R) carnitine to NAD of 180 (1# to 6) or 196 (#7) was chosen. The equilibrium constant for the reaction catalyzed by (R) carnitine dehydrogenase is 7.6×10^{10} which is far on the side of the reduced substrate [10]. The observed OD_{340} of about 1.5 shows that only about 20% of the maximally predictable amounts of NADH were formed; at the same time this amount of NADH represents only 0.3% oxidation of the total (R) carnitine to dehydrocarnitine. Under these conditions of small initial conversion, the Stevens and Attree equation holds true [7] and the IE of the reaction is given directly by the ratio of the specific radioactivities of the produced (S)[4-^{3}H]NADH to the original (R) [3-^{3}H] carnitine.

The presented values found for the IE of the oxidation of (R) carnitine catalyzed by (R) CDH at different pH values and temperatures indicate that the rate limiting step of this reaction is the cleavage of the C-H bond at C_3.

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