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THE STEREOSPECIFICITY OF THE HYDROGEN TRANSFER FROM (*R*)-CARNITINE TO NAD, CATALYZED BY (*R*)-CARNITINE DEHYDROGENASE FROM *PSEUDOMONAS AERUGINOSA*

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SUMMARY

The transfer of hydrogen from the C_3 position of (*R*)-carnitine to NAD, catalyzed by (*R*)-carnitine dehydrogenase from *Pseudomonas aeruginosa* was investigated. This transfer was shown to be a direct one involving the B-position at C_4 of the pyridine ring of the nucleotide. In confirmation of this finding, the transfer of hydrogen from (*R*)-carnitine to [4-^{3}H]NAD resulted in NADH with the label confined to the A-position. These experiments show that (*R*)-carnitine dehydrogenase should be classified as a B-enzyme.

INTRODUCTION

Pyridine nucleotide-linked dehydrogenases promote a direct and stereospecific transfer of hydrogen between the substrate and the C_4 position of the nicotinamide ring of the nucleotide [1]. Examination of the steric course of reactions catalyzed by a large number of NAD-linked dehydrogenases has revealed that they promote hydrogen transfer specifically to and from either one or the other side of the C_4 prochiral position of the nicotinamide ring [1]. The enzymes fall into two classes: those which catalyze the reversible addition of hydrogen to the same side of the nicotinamide ring as does alcohol dehydrogenase (EC 1.1.1.1) designated as having A-stereospecificity [1–3] and those which catalyze transfer of hydrogen at the opposite side, designated as having B-stereospecificity [1, 4]. A direct transfer of hydrogen implies that the hydrogen atoms of the surrounding media are not involved in the reaction [1–4].

Recently Brendel et al. [5] described a new enzymatic assay for (*R*)-carnitine based on an exchange reaction between (*R*)-[3-^{3}H]carnitine and water catalyzed by the coupled action of (*R*)-carnitine dehydrogenase [(*R*)-carnitine:NAD oxidoreductase EC 1.1.1.108] from *Pseudomonas aeruginosa* and α-liponamide dehydrogenase or diaphorase (EC 1.6.4.3) from pig heart. In order to obtain more detailed information about the parameters involved in the exchange reaction, the stereospecificity of the hydrogen transfer catalyzed by (*R*)-carnitine dehydrogenase was investigated. One of

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the drawbacks of working with (*R*)-carnitine dehydrogenase of *Pseudomonas aeruginosa* is its extreme sensitivity to all ketone-trapping agents [6]. For that reason the displacement of the equilibrium of the reaction in the direction of 3-dehydrocarnitine with hydrazine [7], semicarbazide, amino-oxyacetic acid, etc. could not be accomplished.

The equilibrium constant for this reaction [6]

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

shows an extreme shift of the equilibrium to the side of the reduced substrate, as is found with many NAD-linked dehydrogenases.

In order to overcome this difficulty, the equilibrium of the reaction was displaced using high concentrations of labelled carnitine and NAD at a pH which was as high as was compatible with good activity of the enzyme [6]. (*R*)-Carnitine dehydrogenase, as shown by Aurich and co-workers [6–8] has an absolute requirement for the (*R*)-enantiomorph. Thus, only tritium from (*R*)-carnitine would be transferred to NAD, when a racemate (*RS*)-[3-^{3}H]carnitine is employed in this reaction as is apparent from maximal exchanges into water of 50% in cases in which the concomitant presence of diaphorase allows equilibration with the solvent (unpublished results).

MATERIALS AND METHODS

(*R*)-Carnitine was a gift of Otsuka Pharmaceutical Co., Osaka, Japan. NAD, (*S*)-lactate dehydrogenase from rabbit muscle (EC 1.1.1.27), (*S*)-malate dehydrogenase from pig heart (EC 1.1.1.37) and (*S*)-glutamate dehydrogenase from bovine liver (EC 1.4.1.3) were obtained from Sigma Chemical Co., and NADase (EC 3.2.2.5) isolated from *Neurospora crassa* was purchased from Worthington. NaB^3H_4 with a specific radioactivity of 12 mCi/μmole was obtained from New England Nuclear and [4-^{3}H]NAD was procured from the Radiochemical Center. Radioactivity was determined by liquid scintillation counting using 10–15 ml of a solution containing 5 g Omnifluor (New England Nuclear) in 1 l of a mixture of 2:1 (v/v) toluene–Triton X-100 (Rohm and Haas) in a Beckman LS250 Scintillation Counter. (*R*)-Carnitine dehydrogenase was purified by an $(NH_4)_2SO_4$ fractionation and Sephadex G-200 gel filtration after isolation from *P. aeruginosa* ATCC 7700 grown in a minimal media containing (*RS*)-carnitine as sole carbon source after the procedure of Aurich et al. [6, 7], and had a specific activity of 6 units/mg.

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

37 μmoles dehydrocarnitine ethyl ester bromide ($\varepsilon_{280\ nm} = 2 \cdot 10^4$) synthesized after Aurich et al. [9], dissolved in 2.9 ml 0.1 M $K_4P_2O_7 \cdot 3\ H_2O$–HCl buffer at pH 9.5, was reduced to (*RS*)-[3-^{3}H]carnitine ethyl ester bromide with successive additions, in 20-min intervals, of first 1.5 μmoles non-labelled $NaBH_4$, second, 24 μmoles NaB^3H_4 with a specific radioactivity of 12 mCi/μmole, and finally, 1 mmole of non-labelled $NaBH_4$ to bring the reaction to completion. The extent of the reduction was followed optically at 280 nm at a 1:200 dilution. Thereafter, 2 ml 40% HBr were added, and the mixture incubated for 24 h at 20 °C, concentrated in vacuo twice

with 2 ml H_2O, and finally poured on a 1 cm × 50 cm Dowex 50 X-8, 200–400 mesh, cation-exchange column in its hydrogen form. After washing with water, labelled carnitine was eluted with 150 ml 1 M HCl [10]. The yield from the above reduction was 93% (*RS*)-[3-^{3}H]carnitine hydrochloride with a specific radioactivity of 3.1 mCi/μmole as determined by the assay of Marquis and Fritz [11]. The position of the label was determined as previously described, and it was shown that over 99% of the label was confined to the C_3 position of (*RS*)-carnitine [5].

Isolation of NADH

NADH was isolated from the incubation mixture by the procedure of Silverstein [12] using a DEAE-cellulose column (1 cm × 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 2–3 ml of water, and the tritium content in aliquots of this solution (pH 7, 1 M NH_4HCO_3) was quantitatively transferred to either (*S*)-malate by adding 1.8 μmoles oxaloacetate and 30 units of (*S*)-malate dehydrogenase [13] or to (*S*)-lactate by adding 1.8 μmoles of pyruvate and 10 units of (*S*)-lactate dehydrogenase [14], or finally to (*S*)-glutamate by adding 1.3 μmoles α-ketoglutarate and 4 units of (*S*)-glutamate dehydrogenase [15]. The (*S*)-lactate formed was incubated for 30 min with 0.2 units of diaphorase-free NADase and diluted with 200 μmoles non-labelled (*S*)-lactate; the (*S*)-malate formed was incubated with the same amount of NADase and diluted with 2.9 μmoles of non-labelled (*S*)-malate, and finally, (*S*)-glutamate produced was diluted with 2.2 mmoles non-labelled (*S*)-glutamate. (*S*)-lactate and (*S*)-malate were isolated through a 1 cm × 20 cm Dowex 1 X-8 anion-exchange column (200–400 mesh) in the formate form with a formic acid gradient after the procedure of Busch et al. [16]. Thereafter, the eluates were concentrated, and the specific radioactivity was measured [13, 14]. (*S*)-glutamic acid was recrystallized to constant specific radioactivity from water (3 times) [15].

RESULTS AND DISCUSSION

Because it did not seem possible to use any known trapping agent in displacing the equilibrium of the reaction, we attempted to make use of the mass action principle employing high concentrations of the (*R*)-carnitine and NAD to shift the equilibrium and produce NADH in amounts sufficient to be analyzed. Due to the kinetic isotope effect of the reaction, the specific activity of the isolated NADH was significantly lower than that of the starting (*RS*)-[3-^{3}H]carnitine (unpublished results). The results in Table I show that ^{3}H transferred from carnitine to NADH, was incorporated into (*S*)-glutamate by (*S*)-glutamate dehydrogenase [15] previously shown by Levy and Vennesland [4] to be a B-enzyme. No significant incorporation of ^{3}H, however, was found to be incorporated into (*S*)-lactate or (*S*)-malate, catalyzed by their respective NAD-linked dehydrogenases. These latter enzymes are known to be A-enzymes [1–3].

In order to further prove that (*R*)-carnitine dehydrogenase is a B-enzyme, and at the same time increase the specific radioactivity of the isolated NADH, an alternate experimental setting was devised in which the equilibrium was displaced with non-

TABLE I

STEREOSPECIFICITY OF THE HYDROGEN TRANSFER FROM (*RS*)-[3-^{3}H]CARNITINE TO NAD CATALYZED BY (*R*)-CARNITINE DEHYDROGENASE

(*R*)-Carnitine with a specific radioactivity of $1.1 \cdot 10^6$ dpm/μmole was prepared by the dilution of 0.024 μmoles (*RS*)-[3-^{3}H]carnitine with a specific radioactivity of 3.1 mCi/μmole, with 143 μmoles non-labelled (*RS*)-carnitine, and was mixed with 120 mg NAD (pH 8), 3 mmoles Tris–HCl buffer (pH 9.0), and water, to a volume of 11.2 ml and at a final pH of 8.7 at 25 °C. The reaction was started with 0.5 units (*R*)-carnitine dehydrogenase with a specific activity of 6 units/mg, and, after 60 min when $\Delta A_{340\,nm}$ was less than 0.02 per min, the NADH was isolated. 0.1 ml of the original incubate was passed through a mixed-bed ion exchanger (equal parts of Dowex 50 X-8 hydrogen form, and Dowex 1 X-8 formate form), washed with 2 ml water, and the radioactivity counted. The radioactivity which appeared in the eluate was subtracted from the control done the same way, but without enzymes, and was utilized to correct the specific radioactivity of the isolated NADH. This correction accounted for less than 7% of the original specific activity. The NADH produced represented a conversion of only 0.5% of the total amount of (*R*)-carnitine present. ^{3}H in NADH was then transferred to the various substrates as described in the text. Specific activities of these substrates were corrected for dilution.

Expt No.	Specific radioactivity in dpm/μmole			
	NADH	(*S*)-lactate	(*S*)-malate	(*S*)-glutamate
1	$3.1 \cdot 10^5$	$2.8 \cdot 10^3$	$2.1 \cdot 10^3$	$2.9 \cdot 10^5$
2	$2.9 \cdot 10^5$	$2.0 \cdot 10^3$	$1.8 \cdot 10^3$	$3.0 \cdot 10^5$
3	$3.6 \cdot 10^5$	—	—	$3.5 \cdot 10^5$

labelled (*R*)-carnitine and [4-^{3}H]NAD. In the reduction of the labelled NAD by non-labelled (*R*)-carnitine catalyzed by (*R*)-carnitine dehydrogenase the chirality of the C_4 position of the nicotinamide ring of the nucleotide would now be the opposite of what it had been before. Thus, if direct transfer of ^{3}H from labelled carnitine to NAD takes place at the B-position of C_4 of NADH, the ^{3}H should now be located in the A-position.

Table II shows that the ^{3}H content of NADH obtained under the second set of experimental conditions is now transferred to (*S*)-malate by (*S*)-malate dehydro-

TABLE II

STEREOSPECIFICITY OF THE HYDROGEN TRANSFER FROM NON-LABELLED (*R*)-CARNITINE TO [4-^{3}H]NAD CATALYZED BY (*R*)-CARNITINE DEHYDROGENASE

[4-^{3}H]NAD with a specific radioactivity of $1.6 \cdot 10^7$ dpm/μmole was prepared by dilution of 0.25 μmoles [4-^{3}H]NAD with a specific radioactivity of 50 Ci/mole, with 1.5 μmoles non-labelled NAD, and incubated with 143 μmoles non-labelled (*R*)-carnitine (neutralized to pH 8), and 200 μmoles Tris–HCl buffer (pH 9.0), in a total volume of 1.1 ml and at a final pH of 8.6. The reaction was started with 0.2 units of (*R*)-carnitine dehydrogenase, and 60 min later the NADH was isolated. The amounts of NADH generated correspond to a 10% reduction of the original NAD. ^{3}H in NADH was then transferred to the various substrates as described in the text. Controls and corrections for ^3H_2O and dilutions were done as described in Table I.

Expt No.	Specific radioactivity in dpm/μmole		
	NADH	(*S*)-malate	(*S*)-glutamate
1	$1.4 \cdot 10^7$	$1.4 \cdot 10^7$	$2.0 \cdot 10^5$
2	$1.3 \cdot 10^7$	$1.4 \cdot 10^7$	$1.9 \cdot 10^5$

genase, an A-enzyme; whereas, almost no incorporation takes place into (*S*)-glutamate catalyzed by (*S*)-glutamate dehydrogenase, a B-enzyme [4].

Both experiments confirm the observation that (*R*)-carnitine dehydrogenase and (*S*)-glutamate dehydrogenase are enzymes of the same stereospecificity regarding the hydrogen transfer to NAD, and therefore, it can be concluded that (*R*)-carnitine dehydrogenase from *P. aeruginosa* should be classified as a B-enzyme. (*R*)-Carnitine dehydrogenase is not present in the *P. aeruginosa* cells in the absence of (*R*)-carnitine and is induced only in the presence of (*R*)-carnitine [17]. A similar situation occurs with *Pseudomonas testosteroni* in the presence of steroids which induce the formation of 3α-, 3β- and 17β-steroid dehydrogenases, all of which are B-enzymes [18, 19]. This observation, although limited to a few examples, might suggest that inducible dehydrogenases in the *Pseudomonas* genera may always be B-enzymes.

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