STEREOCHEMISTRY OF THE HYDROGEN TRANSFER TO THE COENZYME BY OCTOPINE DEHYDROGENASE

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1. Introduction

Octopine dehydrogenase extracted from muscles of *Pecten maximus* catalyzes the oxidation of D-octopine 1 to L-arginine 2 and pyruvate 3 [1, 2].

This enzyme of a molecular weight of 33 000 daltons is a monomeric one [3]. An earlier spectrophotometric study [4] has shown that the difference spectrum of the binding of the reduced coenzyme to octopine dehydrogenase exhibits a red shift of the reduced nicotinamide absorbance. According to the observations of Fisher et al. [5] this is characteristic of most B-stereospecific dehydrogenases. It seemed therefore interesting to confirm directly that octopine dehydrogenase transfers hydrogen from B side of the nicotinamide ring, using either the NADH or one of its active analogues labeled on C-4 in the pyridine ring. We chose 3-cyano PyADH [6] possessing on C-3 a group whose geometry is quite different from that of an amide.

2. Experimental

NAD^+ (98% purity), D-octopine and yeast alcohol dehydrogenase were supplied by Sigma Chemical Company. Glutamate dehydrogenase from pig liver was a gift of Dr. D. Pantaloni. Octopine dehydrogenase was prepared according to the method previously described [2]. [1–^{3}H]Ethanol, 0.54 mCi/mg, was a product of New England Nuclear. L–[2–^{3}H]glutamic acid, 10 mCi/mg, was supplied by CEA France. 3-Cyano $PyAD^+$ was prepared according to a previously elaborated procedure [6, 7].

Protein concentrations were determined spectrophotometrically using the value of $A^{280}_{1\%} = 11.4$ for octopine dehydrogenase [4].

Octopine concentration was measured colorimetrically by the method of Dumazert and Poggi [8].

The radioactivity was determined with a liquid scintillation Inter Technique SL 30 spectrophotometer.

DEAE-cellulose from Serva was repeatedly washed with 2.5 N NaOH, water, 10% potassium bicarbonate and finally with water to neutrality. Columns 50 × 2 cm were used for 1 g of nucleotides.

Dowex 1 × 2 from Fluka was washed with 3 N HCl until elimination of impurities absorbing at 260 nm. After washing with water the resin was converted to the formate form by treatment with 2 M sodium formate followed by washing with water until neutrality. Columns 40 × 2 cm were used for 1 g of nucleotides.

2.1. *Preparation of 4′ R-[4–^{3}H]NADH*

To 50 ml of 0.1 M glycine-NaOH buffer pH 9.8 containing 350 mg of NAD^+ and 2.8 ml of [1–^{3}H]-

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Abbreviations:
3-cyano $PyAD^+$: 3-cyanopyridinium adenine dinucleotide; 3-cyano PyADH: its reduced form; ADPR: ADP ribose.

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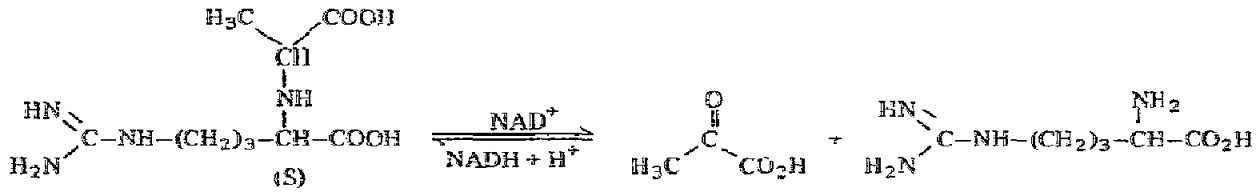

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ethanol (4.6×10^7 cpm/mmole) were added 3 mg of yeast alcohol dehydrogenase. After 1.5 hr the equilibrium was established. The solution was heated for 5 min at 60° and its volume reduced to 15 ml. This preparation was then chromatographed on DEAE-cellulose, using for elution an ammonium bicarbonate gradient from 0 to 0.4 M. The fractions containing NADH were pooled. In order to diminish the radiolysis, 4 R-[4–^{3}H]NADH was kept in solution. Yield = 200 mg (activity: 1.82×10^7 cpm/mmole).

2.2. *Preparation of 4 S-[4–^{3}H]NADH*

To 500 ml of 0.01 M phosphate buffer pH 7.6 containing 800 mg of NAD^+ and 40 mg of L-[2–^{3}H]glutamic acid (1.67×10^7 cpm/mmole) there was added 8 mg of glutamate dehydrogenase. After 5 hr the equilibrium was established. 4 S-[4–^{3}H]NADH was isolated in the same manner as 4 R-[4–^{3}H]NADH. Yield = 120 mg (activity 1.27×10^8 cpm/mmole).

2.3. *Preparation of 4 R-[4–^{3}H]3-cyano PyADH*

The same method as for the preparation of 4 R-[4–^{3}H]NADH was used except that the incubation was performed at pH 8.5 in presence of [1–^{3}H]-ethanol, 6×10^7 cpm/mmole. The purity of the resulting 4 R-[4–^{3}H] 3-cyano PyADH was estimated by the ratio A_{260}/A_{323}. Actually due to the fact that the remaining 3-cyano $PyAD^+$ is degraded to ADPR during the chromatography on DEAE-cellulose, the 3-cyano PyADH obtained was contaminated with ADPR. The ratio of the absorptions at 260 nm and 323 nm was 3 for 3-cyano PyADH and 3.5 for the elution product. Consequently the latter contains only 85% of 4 R-[4–^{3}H]3-cyano PyADH (yield 75%; activity 1.15×10^7 cpm/mmole).

2.4. *Oxidation of 4 R- and 4 S-[4–^{3}H]NADH by octopine dehydrogenase*

To 25 ml of a solution adjusted to pH 6.6 with 0.1 M KH_2PO_4 and containing 40.3 μmoles of 4 R-[4–^{3}H]NADH, 33.4 μmoles of L-arginine and 33.4 μmoles of sodium pyruvate were added 50 μg of octopine dehydrogenase (in five increments). After 30 min the reaction stopped. The pH was brought to 4.5 by addition of 2 N acetic acid and the mixture was chromatographed on Dowex 1 × 2 formate form. Octopine was eluted by water and NAD^+ by 0.2 formic acid. The absence of arginine in isolated octopine was verified by thin-layer chromatography on cellulose using as solvent: pyridine–isoamyl alcohol–water: 80–40–70. The R_f were: octopine 0.29, L-arginine 0.08.

The same procedure was used for the oxidation of 4 S-[4–^{3}H]NADH.

2.5. *Oxidation of 4 R-[4–^{3}H]3-cyano PyADH*

This experiment was performed in the same way as for the oxidation of 4 R-[4–^{3}H]NADH except that a 40-fold excess of L-arginine and sodium pyruvate over the coenzyme and a 6-fold quantity of enzyme were employed. Only 52% of 3-cyano PyADH were oxidized. The large excess of L-arginine made difficult the estimation of the specific radioactivity of octopine.

3. Results and discussion

The results of the studies of the stereospecificity of hydrogen transfer catalyzed by octopine dehydrogenase are summarized in the table. As can be seen there is no transfer of tritium from 4 R-[4–^{3}H]NADH to octopine and essentially all the radioactivity (96%) remains in NAD^+.

On the contrary, in the presence of 4 S-[4–^{3}H]-NADH only 3.5% of the initial radioactivity is detected in NAD^+ and 99% is transferred to octopine.

It is therefore obvious that octopine dehydrogenase transfers the hydrogen from the side of NADH to the substrate without exchange with protons of the me-

Table 1
Stereospecificity of hydrogen transfer from coenzyme to octopine.

Source of label	Radioactivity (cpm per mmole)*			(%)	
	Reduced coenzyme	Oxidized coenzyme	Octopine	Not transferred	Transferred
4 R-[4-^{3}H]NADH	1.82×10^7	1.75×10^7	0.16×10^7	96	8
4 S-[4-^{3}H]NADH	1.24×10^8	0.04×10^8	1.22×10^8	3.5	99
4 R-[4-^{3}H]cyano PyADH	1.15×10^7	1.05×10^7		91	

* Estimated error ± 4%.

dium and thus belongs to the B class of dehydrogenases.

4 R-[4-^{3}H]3-cyano PyADH does not transfer its tritium atom to octopine since 91% of radioactivity remains in 3-cyano PyAD$^+$. The stereochemical behaviour of this analogue is the same as that of NAD$^+$ towards octopine dehydrogenase as well as towards some other dehydrogenases [9].

Octopine dehydrogenase can be compared to a certain extent to glutamate dehydrogenase. The dehydrogenation performed by both enzymes occurs at a —C—N— system while in most other dehydrogenases a —C—O— system is involved [10].

Earlier spectrophotometric [4, 5, 11, 12] and spectropolarimetric [13, 14] studies have led us to an assumption that some analogies might exist between these two enzymes at their coenzyme binding sites. The B stereospecificity of octopine dehydrogenase gives now an additional argument in favour of this hypothesis.

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