BIOORGANIC CHEMISTRY 6, 89–93 (1977)

Stereochemistry of Octopine and of Its Isomers and Their Enzymatic Properties

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Received October 13, 1976

The four isomers of octopine were prepared from pyruvic acid and L- or D-arginine and from α-keto δ-guanidinovaleric acid and L- or D-alanine by reduction with sodium cyanoborohydride. The absolute configuration of D-octopine, the natural occurring isomer being *S*(L) at the arginine center, and *R*(D) at the alanine center, was confirmed enzymatically. D-Octopine is the only isomer oxidized by NAD^+ in the presence of octopine dehydrogenase from *Pecten maximus* L. The isomer with configuration *S*(L) at the alanine center is found to be a competitive inhibitor. Isomers with *R*(D) configuration at the arginine center show no detectable effect on the enzymatic reaction.

INTRODUCTION

The stereochemistry of D-octopine has been established by synthesis from optically active α-bromopropionic acid and L-arginine (*1*). However, the kinetic experiments were run under conditions where the α-lactone might be the intermediate (*2*), leading to an overall retention instead of the expected inversion, and, on the other hand, the validity of the application of partial rotation has not been demonstrated for amino acids such as octopine. We felt that a more definite determination of the absolute configuration at the alanine center was necessary. The configuration of D-octopine at the arginine center has been demonstrated to be *S* by synthesis from L-arginine and pyruvic acid via enzymatic or catalytical reduction (*3*).

In order to prove the absolute stereochemistry, we took advantage of a new reagent, sodium cyanoborohydride, which selectively reduces the immonium salts formed from ketones and amines (*4*). α-Keto δ-guanidino valeric acid was prepared from L-arginine by oxidative desamination (*5*, *6*).

EXPERIMENTAL

Synthesis of the Four Octopine Isomers from Arginine and Pyruvic Acid

A solution of L- or D-arginine (5.0 g) and sodium pyruvate (20 g) in a 0.1 *M* phosphate buffer of pH 7.0 (250 ml) was treated with purified sodium cyanoborohydride (3.6 g) (*4*). The reaction was followed by chromatography on a cellulose plate (migration solvent, pyridine:2-pentanol:water (80:40:70); development, Sakaguchi reagent (*8*)).

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ISSN 0045-2068

After 24 hr, the excess reducing agent was destroyed by addition of 50 ml of concentrated HCl, and the solution was applied to a Dowex 50W × 8 column (H^+ form, 1.5 × 30 cm, eluted with water (*11*) and with 1 *N* ammonium hydroxide solution).

TABLE 1

YIELDS AND OPTICAL ROTATIONS OF THE OCTOPINE ISOMERS PREPARED FROM PYRUVIC ACID AND ARGININE

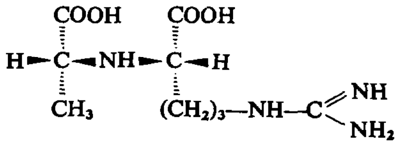
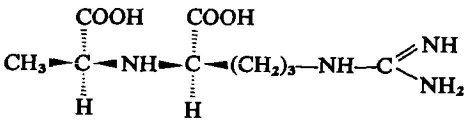
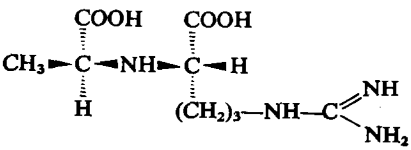
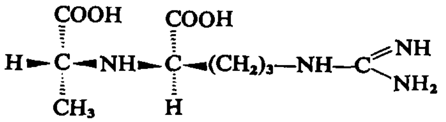
Isomer	Yield[a] (%)	$[\alpha]_D^{20}$	$[\alpha]_D^{24\,b}$
		(H₂O, 2%)	
D-Octopine	37	+20.0	+20.6
L-Octopine	38	−20.3	−20.8
L-Alloöctopine	34	+24.6	+26.8
D-Alloöctopine	28	−24.7	−26.6

[a] Calculated from arginine.

[b] According to (*1*).

TABLE 2

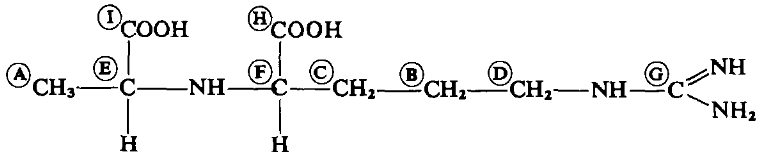
STEREOCHEMICAL FORMULAS OF OCTOPINE ISOMERS

Isomer	Configuration at the centre: Alanine	Configuration at the centre: Arginine	Configuration
D-Octopine ((+)-Octopine)	*R*(D)	*S*(L)	 $H-\overset{COOH}{\underset{CH_3}{C}}-NH-\overset{COOH}{\underset{(CH_2)_3-NH-C(=NH)NH_2}{C}}-H$
L-Octopine ((−)-Octopine)	*S*(L)	*R*(D)	 $CH_3-\overset{COOH}{\underset{H}{C}}-NH-\overset{COOH}{\underset{H}{C}}-(CH_2)_3-NH-C(=NH)NH_2$
L-Alloöctopine ((+)-Isoöctopine)	*S*(L)	*S*(L)	 $CH_3-\overset{COOH}{\underset{H}{C}}-NH-\overset{COOH}{\underset{(CH_2)_3-NH-C(=NH)NH_2}{C}}-H$
D-Alloöctopine ((−)-Isoöctopine)	*R*(D)	*R*(D)	 $H-\overset{COOH}{\underset{CH_3}{C}}-NH-\overset{COOH}{\underset{H}{C}}-(CH_2)_3-NH-C(=NH)NH_2$

The octopine-containing fractions were pooled and evaporated under reduced pressure. The diastereomers were separated by fractional crystallization from a water–ethanol (1:3 to 3:2) mixture. All crystallizations were performed at +4°C and the pH was adjusted to 6 with diluted HCl. The purity of the four isomers was checked by optical rotation and by ^{13}C-nmr spectra. Yields and physical data are reported in Tables 1 and 3.

TABLE 3

^{13}C-NMR DATA OF OCTOPINE ISOMERS[a]



$$\text{Ⓐ}CH_3-\overset{\text{Ⓘ}COOH}{\underset{H}{\text{Ⓔ}C}}-NH-\overset{\text{Ⓗ}COOH}{\underset{H}{\text{Ⓕ}C}}-\text{Ⓒ}CH_2-\text{Ⓑ}CH_2-\text{Ⓓ}CH_2-NH-\text{Ⓖ}C\begin{matrix}\nearrow NH\\ \searrow NH_2\end{matrix}$$

Isomer	Carbon								
	A	B	C	D	E	F	G	H	I
D-Octopine	−15.8	−5.8	−3.4	10.6	25.0	28.2	127.0	141.1	141.8
L-Octopine	−16.2	−6.1	−3.7	10.6	25.3	28.4	128.7	142.6	143.5
L-Allooctopine	−14.6	−5.9	−3.1	10.6	26.0	29.5	127.0	141.0	142.0
D-Allooctopine	−14.6	−5.9	−4.0	10.7	26.2	29.8	127.2	141.4	142.3

[a] For spectra, samples were dissolved in D_2O and solution adjusted to pH 1 with HCl, final concentration 0.2 *M*. Chemical shifts are in parts per million at 62.86 MHz, and are referred to a t-BuOH standard. The chemical shifts of carbon A, B, C are upfield to the standard, the others downfield.

Preparation of α-Keto δ-guanidino Valeric Acid

The procedure of Meister (*5*, *6*) was modified. Recrystallized L-arginine hydrochloride (5 g) in water (250 ml) was treated at 0°C with oxygen. The pH of the solution was constantly kept at 7.2 by addition of 0.1 *M* HCl in presence of snake venom from *Crotalus adamenteus* (200 mg, Sigma) and catalase (200 mg) from bovine liver (Sigma), which had been dialyzed at 4°C against 1 m*M* phosphate buffer (pH 7). After 4 days, no more arginine was detected. The solution was dialyzed for 2 days against 2 × 1 liter of water. The dialysate was reduced under vacuum at a temperature below 40°C to a small volume (50 ml). The product (3.8 g) crystallized during cooling from this solution, and was recrystallized several times from water. The purity was checked by paper electrophoresis (*7*). *Anal.* Calcd. for $C_6H_{11}N_3O_3$; C, 41.3; H, 6.4; N, 24.3. Found: C, 41.0; H, 7.0; N, 23.8. The ir spectra showed a carbonyl group at 1720 cm^{-1}.

Preparation of the Octopine Isomers from α-Keto δ-guanidino Valeric Acid and Alanine

A solution of L- or D-alanine (0.5 g) and α-keto δ-guanidino valeric acid (0.25 g) in 0.1 M phosphate buffer, pH 7 (12.5 ml) was treated with sodium cyanoborohydride (0.2 g). After 24 hr, the reaction was stopped by addition of 3 ml of concentrated HCl, and the solution was applied to a Dowex column as described above. The pure octopine solution then obtained was neutralized and tested for enzymatic activity.

RESULTS

The enzymatic tests with octopine dehydrogenase from the shellfish *Pecten maximus* L. (gift of Dr. A. Olomucki) were performed according to (*9*). The enzyme used had a specific activity of 320 U/mg. D-Octopine proved to be the only isomer oxidized by NAD^+ in the presence of octopine dehydrogenase: $K_m = 2 \times 10^{-3}$ *M* compared to the published value of 1.5×10^{-3} *M* (*10*).

L-Alloöctopine is a competitive inhibitor with respect to D-octopine: $K_i = 7.3 \times 10^{-4}$ *M*. L-Octopine and D-alloöctopine showed neither activity nor inhibition at a concentration of 10^{-2} *M*.

The octopine prepared from D-alanine was enzymatically active in contrast to the isomer prepared from L-alanine.

DISCUSSION

The preparation of D-octopine by the reduction with cyanoborohydride, starting either with D-alanine and α-keto δ-guanidino valeric acid or with L-arginine and pyruvic acid, proves definitely that the configuration at the alanine center is *R*(D), and at the arginine center, *S*(L). The reaction mixtures of octopine and its isomer were resolved by selective recrystallization. The separation through the picrate and flavianate described by previous workers (*1*) was completely inefficient in our hands for undetermined reasons.

The octopine dehydrogenase shows an exclusive preference for the isomer having the *S*(L) configuration at the arginine center. Neither activity nor inhibition was detected at 10^{-2} *M* for the isomers having the *R*(D) configuration, L-octopine and D-alloöctopine.

Surprisingly the center close to the active site does not show stereospecificity; both isomers are bound to the enzyme, one being a substrate, the other a competitive inhibitor to the substrate. In contrast, L-lactate is the only stereoisomer which binds to L-lactate dehydrogenase, and no inhibition has even been detected with D-lactate (*11*).

The fact that both octopine dehydrogenase and lactate dehydrogenase are involved in the pyruvate metabolism suggests (*12*) that these enzymes could be similar from an evolutionary point of view. This similarity should be reflected in the stereospecificity (*13*). However, the chirality of the NAD^+ reduction is pro-*S* for octopine dehydrogenase (*14*) and pro-*R* for lactate dehydrogenase; furthermore, the stereospecificities for the substrates are opposite. These two differences in stereospecificity suggest that in the evolution lactate dehydrogenase and octopine dehydrogenase separated quite early.

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