Purification and properties of carboxynorspermidine decarboxylase, a novel enzyme involved in norspermidine biosynthesis, from *Vibrio alginolyticus*

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A novel enzyme which catalyses the decarboxylation of carboxynorspermidine [$\text{H}_2\text{N}(\text{CH}_2)_3\text{NHCH}_2\text{CH}_2\text{CH}(\text{NH}_2)\text{COOH}$] to yield norspermidine [$\text{H}_2\text{N}(\text{CH}_2)_3\text{NH}(\text{CH}_2)_3\text{NH}_2$], one of the unusual polyamines, was purified to apparent homogeneity (3100-fold) from cells of *Vibrio alginolyticus*. The enzyme has an apparent M_r of 86000, with a pI of 4.25, and is a dimer composed of identical subunits with an apparent M_r of 43500. The K_m for carboxynorspermidine was 175 μM and for pyridoxal 5'-phosphate, 4.8 μM . The purified preparation had a specific activity of 24.1 μmol norspermidine produced min^{-1} (mg protein^{-1}). Carboxyspermidine, a structural homologue, was able fully to replace carboxynorspermidine as a substrate, to produce spermidine, but neither 2,3-diaminopropionic acid, 2,4-diaminobutyric acid, ornithine nor lysine showed detectable substrate activity. The optimum pH was 8.25. Dithiothreitol greatly stimulated the enzyme activity, maximum stimulation being observed at more than 5 mM-dithiothreitol.

Introduction

The aminopropyl moieties in spermidine (SPD) and spermine molecules arise from two biological pathways. One operates in a wide variety of prokaryotes and eukaryotes, and utilizes *S*-adenosyl-L-methionine, via its decarboxylated product, as an aminopropyl group donor (Williams-Ashman & Pegg, 1981). The enzymes responsible, aminopropyltransferases, have been characterized extensively in many organisms (Tabor & Tabor, 1985; Pegg, 1986). An analogous reaction has been suggested in the synthesis of newly discovered polyamines such as 2-hydroxyspermidine, norspermidine (NSPD) and norspermine (Rosano *et al.*, 1978; De Rosa *et al.*, 1978; Paulin *et al.*, 1983; Aleksijevic *et al.*, 1979; Villanueva *et al.*, 1980). The other pathway, as yet detected only in certain bacteria (Tait, 1985) and seedlings of a plant (Srivenugopal & Adiga, 1980), utilizes L-aspartic acid via its conversion to L-aspartic β -semialdehyde (ASA) as an aminopropyl-group donor. In this pathway, the Schiff base complex formed non-enzymically between ASA and

putrescine is converted into SPD through two enzyme reactions. However, the enzymes, Schiff base reductase and carboxyspermidine (C-SPD) decarboxylase, that catalyse these reactions have not yet been purified and so are less well characterized.

We have previously shown that NSPD is widely distributed in the genus *Vibrio* as a major triamine species (Yamamoto *et al.*, 1983). Our attempts to elucidate the biosynthetic pathway for this polyamine in some *Vibrio* species have indicated that L-aspartic acid (via ASA) also serves as an aminopropyl donor (Yamamoto *et al.*, 1986a). The Schiff base [$\text{H}_2\text{N}(\text{CH}_2)_3\text{N}=\text{CHCH}_2\text{CH}(\text{NH}_2)\text{COOH}$] formed between ASA and 1,3-diaminopropane (DAP) was converted into NSPD by crude cell extracts in the presence of both NADPH and pyridoxal 5'-phosphate (PLP). At present, such a mode of NSPD biogenesis is not known in any other organisms. Recently, we have purified to homogeneity a novel enzyme, L-2,4-diaminobutyric acid (DABA) decarboxylase (EC 4.1.1.-), which is responsible for the production of DAP as an amine precursor for NSPD in *V. alginolyticus* (Nakao *et al.*, 1989). In this study, a second novel enzyme, which catalyses the decarboxylation of L-carboxynorspermidine (C-NSPD) to yield NSPD, has been purified to homogeneity from *V. alginolyticus* and characterized.

Abbreviations: NSPD, norspermidine; C-NSPD, L-carboxynorspermidine; SPD, spermidine; C-SPD, L-carboxyspermidine; DABA, L-2,4-diaminobutyric acid; ASA, L-aspartic β -semialdehyde; PLP, pyridoxal 5'-phosphate; DTT, dithiothreitol; DAP, 1,3-diaminopropane.

Methods

Bacterial strain and growth conditions. *Vibrio alginolyticus* ATCC 17749 was grown for 6–8 h as described previously (Nakao *et al.*, 1989), and the cell pellets collected were stored at -30°C until enzyme purification.

Preparation of C-NSPD and C-SPD. We first attempted to synthesize the substrate C-NSPD chemically, according to the procedure of Srivenugopal & Adiga (1980), but could only obtain very low yields. Therefore, we prepared it from DAP and ASA by the action of the *V. alginolyticus* Schiff base reductase. ASA was prepared by ozonolysis of L-allylglycine and purified (Black & Wright, 1955). The enzyme preparation employed was the $(\text{NH}_4)_2\text{SO}_4$ fraction (50–70% saturation) as described below. The incubation medium consisted of 125 mM-potassium phosphate buffer, pH 7.5, 2.2 mM-NADPH, 5 mM-dithiothreitol (DTT), 7 mM-DAP, 5 mM-ASA and 30 mg of protein in a total volume of 7 ml. Fifty test tubes, each containing the above medium, were incubated at 37°C for 2 h. The reaction was terminated by the addition of 2.4 ml 20% (w/v) HClO_4 to each tube, and the precipitate was removed by centrifugation. The combined supernatant was applied to a column (2.7×11 cm) of Amberlite CG-120, 100–200 mesh, in the H^+ form. The column was washed with 400 ml distilled water, followed by 400 ml 1.5 M-HCl, and then eluted with 400 ml 6 M-HCl. The eluate was evaporated to dryness at 60°C in a rotary evaporator under reduced pressure. The residue, dissolved in a small volume of 0.1 M-HCl, was applied to a column (2.7×11 cm) of Dowex 50W $\times$ 8, 200–400 mesh, in the H^+ form. After washing with 400 ml distilled water followed by 200 ml each of 1, 1.5 and 2 M-HCl, the column was eluted with a stepwise increase in the concentration of HCl (0.1 M per 50 ml). Three fractions, eluting with 2.7–2.9 M-HCl, were pooled and evaporated to dryness. The resulting oil was recrystallized with water/2-propanol to give approximately 220 mg of C-NSPD trihydrochloride: m.p. $231\text{--}232^{\circ}\text{C}$; $[\alpha]_{\text{D}}^{25} \pm 12^{\circ}$ (1%, w/v, in H_2O).

The $(\text{NH}_4)_2\text{SO}_4$ precipitate (50–70% saturation) from *Paracoccus denitrificans* IFO 13301 (NCIB 8944), which possesses a pathway for the production of SPD via C-SPD (Tait, 1985), was employed for the preparation of C-SPD. Cells grown for 5 h under the same conditions as those for *V. alginolyticus* were used as the enzyme source. Incubation and purification were done as described above except that DAP was substituted for putrescine. C-SPD was eluted in the 2.8 and 2.9 M-HCl fractions from a column of Dowex 50W $\times$ 8. Recrystallization of the residue with the same solvents as those for C-NSPD gave 50 mg of C-SPD trihydrochloride: m.p. $221\text{--}222^{\circ}\text{C}$; $[\alpha]_{\text{D}}^{25} \pm 5.8^{\circ}$ (1%, w/v, in H_2O).

The identity and purity of these compounds were confirmed by GC, GC-MS (Yamamoto *et al.*, 1986a) and TLC on an Avicel SF cellulose plate (Funakoshi Pharmaceutical Co., Japan) in a solvent system of ethanol/ammonium hydroxide/water (18:5:1, by vol.). No contaminating materials which provided GC peaks and reacted with ninhydrin were present in any preparation. The R_F values for C-NSPD and C-SPD were 0.40 and 0.44 respectively.

Enzyme assay. The production of NSPD was assayed in the standard mixture (1.0 ml), containing 100 μmol Tris/HCl buffer, pH 8.25, 1.4 μmol C-NSPD, 0.1 μmol PLP, 5 μmol DTT and enzyme protein (0.1–100 μg), incubated at 37°C for 30 min. After termination of the reaction with 0.4 ml of 20% (w/v) HClO_4 , an internal standard (1,10-diaminooctane) was added to the mixture, which was then centrifuged at 1500 g for 10 min. The supernatant was treated for derivatization, followed by GC analysis with nitrogen-selective detection, as previously described (Yamamoto *et al.*, 1986a). The reproducibility of the method used was assessed by calculating the recoveries of known amounts of NSPD (50 and 100 nmol) added to the

assay mixtures denatured by the prior addition of HClO_4 . The recovery for NSPD was $97 \pm 6\%$ ($n=10$). Also, under standard assay conditions, enzyme activity was a linear function of both incubation time (5–120 min) and the amount of enzyme protein present. One unit of activity (U) was defined as the amount of enzyme that catalysed the formation of 1 μmol NSPD min^{-1} under standard assay conditions.

Enzyme purification. Unless otherwise noted, all procedures were done at $2\text{--}4^{\circ}\text{C}$ and all dialyses were done against 100 vols of the specified buffers for 12 h. A crude estimate of the protein content of column fractions was made by measuring absorbance at 280 nm. For accurate measurement of the protein content of pooled column fractions and final enzyme preparation the Lowry method or that of Bradford (1976) was used depending upon the amount of material available.

(i) The frozen cells (200 g) were thawed and suspended in 800 ml 20 mM-Tris/HCl, pH 7.5, containing 1 mM-DTT and 0.1 mM-EDTA (buffer A). Samples consisting of 200 ml of cell suspension were placed in 500 ml beakers kept in a salt-ice bath and sonicated with an ultrasonic homogenizer. Cell debris was removed by centrifugation at 40000 g for 30 min, and a small portion of the combined supernatant dialysed against buffer A to determine the activity.

(ii) Solid $(\text{NH}_4)_2\text{SO}_4$ was added slowly, with stirring, to the combined supernatant, to give a final concentration of 50% saturation. The solution was centrifuged at 35000 g for 20 min and the pellet discarded. Additional $(\text{NH}_4)_2\text{SO}_4$ was added to the supernatant to obtain a final concentration of 70% saturation, and the new precipitate was centrifuged as above. The pellet was dissolved in buffer A and dialysed against two changes of the same buffer.

(iii) The dialysed $(\text{NH}_4)_2\text{SO}_4$ fraction (60 ml) was divided into three 20 ml portions, each of which was applied to a DEAE-Sephacrose CL-6B column (2.2×25 cm) equilibrated with buffer A. The column was washed with 300 ml of buffer A containing 0.2 M-NaCl and then eluted with a 500 ml linear gradient of 0.2–0.4 M-NaCl in the same buffer at a flow rate of 15 ml h^{-1} . Active fractions eluting between 0.32 and 0.33 M-NaCl were pooled (25 ml $\times$ 3).

(iv) The pooled fraction of step (iii) was treated with solid $(\text{NH}_4)_2\text{SO}_4$ to bring it to 80% saturation, and the precipitate was collected and dissolved in a small amount of buffer A, containing 0.4 M-NaCl, 40 μM -PLP and 0.02% NaN_3 . The solution was applied to a Sephacryl S-200 HR column (2.1×88 cm) equilibrated with the same buffer and the column was eluted with the same buffer at a flow rate of 10 ml h^{-1} . Active fractions (tube nos 31–33) were pooled (15 ml).

(v) After dialysis against 20 mM-sodium phosphate buffer, pH 5.8, containing 1 mM-DTT and 0.1 mM-EDTA, the step (iv) fraction was applied to a DEAE-Sephacrose CL-6B column (1.3×20 cm) equilibrated with the same buffer. The column was washed with 80 ml of the above buffer, additionally containing 0.25 M-NaCl, and then eluted with a 200 ml linear gradient of 0.25–0.75 M-NaCl in the same buffer at a flow rate of 10 ml h^{-1} . Active fractions eluting between 0.42 and 0.45 M-NaCl were pooled (15 ml).

(vi) The active fraction was dialysed against 10 mM-potassium phosphate buffer, pH 7.5, containing 1 mM-DTT and 0.1 mM-EDTA, and the dialysed solution was applied to a hydroxyapatite column (1.65×10.5 cm) equilibrated with the same buffer. After washing with 120 ml of the same buffer, the column was eluted with a 120 ml linear gradient of 10–50 mM-potassium phosphate buffer, pH 7.5, at a flow rate of 10 ml h^{-1} . Active fractions eluting between 39 and 42 mM-potassium phosphate were pooled (12 ml).

(vii) Chromatofocusing was done according to the supplier's instructions (*Technical Handbook: Chromatofocusing with Polybuffer and PBE*, Pharmacia, Uppsala). A PBE column (0.95×14.5 cm) was equilibrated with 25 mM-piperazine/HCl buffer, pH 5.5, containing 1 mM-DTT. The original Polybuffer 74 was diluted 10-fold by vol. with 1 mM-DTT solution, adjusted to pH 4.0 with 0.1 M-HCl and degassed.

The step (vi) enzyme solution which had been dialysed against the same buffer was applied to the column, followed by elution with 180 ml of Polybuffer 74, pH 4.0. However, C-NSPD decarboxylase activity was not observed in any fraction, although the pH of fractions monitored at the same time dropped linearly to pH 4. The column was then eluted with 20 mM-Tris/HCl buffer, pH 7.5, containing 1 M-NaCl and 1 mM-DTT; 3 ml fractions were collected. Activity was recovered in fraction 6, which was dialysed against buffer A containing 0.02% NaN₃ and stored at 4 °C after concentration to about 0.25 ml by use of a Centricon 30 (Amicon).

Electrophoretic analyses. PAGE under non-denaturing conditions was done on a 7.5% (w/v) polyacrylamide slab gel as described by Williams & Reisfeld (1964) except that DTT was added to buffers for preparation of gels to give a final concentration of 1 mM. SDS-PAGE was done by using a 10% (w/v) polyacrylamide slab gel according to Laemmli (1970). Before loading, the enzyme sample was heated for 3 min with 2% (w/v) SDS and 5% (w/v) 2-mercaptoethanol in a boiling-water bath. Isoelectric focusing was done on a 5% (w/v) polyacrylamide gel plate containing 2% (w/v) Ampholine pH 3.5–9.5 using a horizontal isoelectric focusing apparatus (Bio-Rad, model 1415). The samples were run at an initial setting of 4 W for 2 h, at 1500 V for 3 h and then at 1700 V for 30 min. Protein bands were visualized by staining with Coomassie Brilliant Blue R-250. For monitoring the distribution of enzyme activity after native PAGE, the gels were sliced horizontally into 5 mm sections, each of which was macerated in buffer A overnight at 4 °C to elute the enzyme.

Determination of M_r by gel filtration and SDS-PAGE. The M_r of the native enzyme was determined, as described by Andrews (1965), by using a Sephacryl S-200 HR column (2.1 × 88 cm) calibrated with protein standards. The M_r of the subunit was determined from the relative mobility on SDS-PAGE compared to the standards.

Chemicals. Diamines and polyamines were purchased from Aldrich; DEAE-Sepharose CL-6B, Sephacryl S-200 HR, PBE 94, Polybuffer 74 and Ampholine were from Pharmacia LKB; DTT, PLP, L-allylglycine, standard protein kit for SDS-PAGE (SDS-6H) and standard proteins for gel filtration were from Sigma; and NADPH and hydroxyapatite were from Oriental Yeast (Japan) and Wako (Japan), respectively.

Results

Preliminary experiments with a crude cell extract containing the whole enzyme system for NSPD biosynthesis showed that no NSPD was formed unless incubation was first done in the presence of NADPH.

This at least indicates that decarboxylation of the Schiff base complex is not a first-step reaction. The reaction sequence has thus been shown to be:

Purification of C-NSPD decarboxylase

The steps followed in the purification of C-NSPD decarboxylase from *V. alginolyticus* ATCC 17749 are shown in Table 1. C-NSPD decarboxylase appeared to be a completely cytoplasmic enzyme, found in the S40 cell-free extract. The first DEAE-Sepharose CL-6B chromatography led to a 9% increase in the total enzyme activity of the crude extract, with a large increase in purification. The increase in the total activity suggests the possible presence of an enzyme inhibitor(s) in the crude extract that was removed. Hydroxyapatite chromatography also brought about a large increase in purification with a good recovery, but some minor contaminating proteins were still detected by PAGE. Chromatofocusing was very convenient for removing these proteins, since the enzyme was recovered by eluting the PBE column with 1 M-NaCl after extensive washing with Polybuffer 74. This agreed well with the fact that the enzyme had a relatively low pI (see below).

A total purification of 3130-fold and a specific activity of 24.1 U (mg protein)⁻¹ was obtained after these purification steps.

Properties of the purified enzyme

Stability. The enzyme was stable at 4 °C and pH 7.5 in the presence of DTT, EDTA and NaN₃ for at least one month. The enzyme was greatly stabilized by the addition of both DTT and EDTA during purification. Surprisingly, the presence of PLP was not essential for maintenance of enzyme activity during the current purification process, although PLP was essential for

Table 1. *Purification of carboxynorspermidine decarboxylase from V. alginolyticus*

A typical purification from 200 g wet wt of cells is shown.

Step	Total protein (mg)	Total activity (U)	Specific activity [U (mg protein) ⁻¹]	Purification (-fold)	Recovery (%)
Crude extract	5100	39.2	0.0077	1	100
50–70% Ammonium sulphate	2972	38.3	0.013	1.7	98
1st DEAE-Sepharose CL-6B	234	42.7	0.18	23.4	109
Sephacryl S-200 HR	35.5	29.2	0.82	106.5	74
2nd DEAE-Sepharose CL-6B	19.9	20.6	1.04	135.1	53
Hydroxyapatite	1.41	18.9	13.40	1740	48
Chromatofocusing	0.384	9.25	24.10	3130	24

Fig. 1. Cofactor requirement for C-NSPD decarboxylase activity. The purified enzyme (0.1 μ g) was incubated under standard assay conditions except that PLP (a) or DTT (b) was added at the indicated concentrations. In (b), the purified enzyme had been dialysed for 4 h against two changes of 200 vols 20 mM-Tris/HCl buffer, pH 7.5, containing 0.1 mM-EDTA, to remove DTT. The relative activity is expressed as a percentage of the maximum activity (73.3 and 70.0 nmol NSPD formed in 30 min for a and b, respectively) observed under the experimental conditions.

enzyme activity in the assay (see below). Freezing and thawing the purified preparation resulted in almost complete loss of activity.

Purity and isoelectric point. The purified enzyme migrated as a single protein band in PAGE under non-denaturing conditions (data not shown). Analysis of a duplicate unstained gel for enzyme activity established that the protein band corresponded with the enzyme activity. The protein band with enzyme activity was electro-focused at a pH of 4.25.

M_r of enzyme and subunit. The M_r value of the enzyme was determined to be 86000 ± 1000 with a Sephacryl S-200 HR column calibrated with standard M_r markers. SDS-PAGE of the purified enzyme showed a single protein band corresponding to an M_r of 43500 ± 500 , which was unaltered when the protein was not reduced with 2-mercaptoethanol before electrophoresis (data not shown). C-NSPD decarboxylase therefore appears to be a dimer of subunits of identical size, which are not linked by disulphide bonds.

Cofactors required for optimum enzyme activity. When PLP was omitted from the standard assay mixture, the purified enzyme showed only a slight activity, less than 2% of maximum. Near-maximum stimulation was obtained at 100 μ M-PLP (Fig. 1a). Hyperbolic saturation kinetics were observed for PLP and the K_m value was calculated to be 4.8 μ M. Without addition of DTT to the assay mixture, less than 9% of the maximum activity was retained in the purified enzyme that had been dialysed for 4 h against two changes of 200 vols 20 mM-Tris/HCl buffer, pH 7.5, containing 0.1 mM-EDTA. The addition of DTT to the assay mixture, however, brought about a

remarkable stimulation of enzyme activity, maximum stimulation being observed at 5–10 mM-DTT (Fig. 1b).

Optimum temperature and pH. The optimum temperature for enzyme activity in the standard assay was 37 °C; 75% and 51% of the maximum activity remained at 30 °C and 45 °C, respectively, but the activity decreased rapidly at temperatures above 55 °C or below 20 °C. The effect of pH on enzyme activity was determined using 100 mM-potassium phosphate buffer in the range 6.25–7.25, and 100 mM-Tris/HCl buffer in the range 7.5–9.0. Maximum activity was found at pH 8.25; 57% and 72% of the maximum activity were observed at pH 7.5 and 9.0, respectively.

Kinetic characteristics and substrate specificity. A Lineweaver–Burk treatment of results obtained when the C-NSPD concentration was varied gave a K_m of 175 μ M with a V of 26.6 μ mol NSPD min^{-1} (mg protein) $^{-1}$. C-SPD, the alternative substrate, was decarboxylated at almost the same rate as that for C-NSPD, to yield SPD. However, neither L-2,3-diaminopropionic acid, DABA, L-ornithine nor L-lysine served as substrates for C-NSPD decarboxylase. Moreover, none of these diamino acids, at the same concentration as that of C-NSPD (1.4 mM), inhibited the formation of NSPD from C-NSPD.

Influence of cations. The effect of different cations (all 1 mM) on the activity of C-NSPD decarboxylase was examined with the purified enzyme. Fe^{3+} , Fe^{2+} and Mn^{2+} severely inhibited the enzyme activity (88%, 82% and 50%, respectively), whereas Zn^{2+} had a slightly inhibitory effect (23%), and Mg^{2+} , Ca^{2+} , Cu^{2+} and Cu^{+} had no effect. A substantial loss of activity (about 20%) was observed when the enzyme was dialysed for 12 h or

stored at 4 °C for 2 d without addition of EDTA. Na⁺ and K⁺ (up to 1 M) did not affect enzyme activity.

Discussion

The results presented herein, together with those reported earlier (Yamamoto *et al.*, 1986*a*), establish the occurrence of a newly recognized decarboxylase participating in NSPD biosynthesis of *V. alginolyticus*. We have designated this enzyme as C-NSPD decarboxylase on the basis of its apparent specificity, and probable role in catalysing the final step in NSPD biosynthesis *in vivo*. Certainly, the close correspondence between the time of appearance of this enzyme activity and the accumulation of NSPD (Yamamoto *et al.*, 1986*b*) strongly suggests a functional interrelationship. Nevertheless, it is conceivable that C-NSPD only exists transiently in this bacterium, since our attempts to demonstrate its natural occurrence have not so far been successful. This suggests that the *in vivo* formation of C-NSPD may be subject to strict regulation by inhibition by the end product, NSPD.

The purification procedures described for C-NSPD decarboxylase from *V. alginolyticus* provided a pure, apparently homogeneous protein in 24% yield. One important factor contributing to the success of the purification was the efficient preparation of the substrate, C-NSPD, by use of Schiff base reductase from this bacterium. C-NSPD decarboxylase has an M_r of 86000 and is composed of two apparently identical subunits with an M_r of 43500. As with most decarboxylases, it required PLP for enzyme activity. The K_m value for PLP was calculated at a saturating level of C-NSPD to be 4.8 µM. However, it is difficult to extrapolate this value to *in vivo* conditions, because C-NSPD was undetectable in cells. The requirement of the enzyme for DTT, to achieve maximum activity, agreed with the observation for C-SPD decarboxylase of *Lathyrus sativus* (Srivenugopal & Adiga, 1980). EDTA was very effective in stabilizing the enzyme, suggesting that it may protect the enzyme from contaminating metal cations.

Interestingly, C-SPD acted as a substrate towards C-NSPD decarboxylase, the reaction rate being comparable with that of C-NSPD. However, the rate of SPD formation was much lower (<10%) than that of NSPD formation, when the crude cell extract, having the whole enzymic system for NSPD biosynthesis, was incubated in the presence of putrescine, instead of DAP (Yamamoto *et al.*, 1986*a*). This suggests that the reductase dependent on NADPH may be more specific to the Schiff base formed between DAP and ASA than that between putrescine and ASA. In contrast, the enzyme was inactive towards DABA, which is structur-

ally similar to C-NSPD and C-SPD, indicating that the attachment of an aminopropyl or aminobutyl moiety to the terminal amino group of DABA is essential for substrate activity. On the other hand, whether or not NSPD affects the enzyme activity *in vitro* could not be clarified because the quantification of NSPD formed enzymically was hampered by the presence of large amounts of added NSPD.

Further efforts will be directed towards purification and characterization of the Schiff base reductase of *V. alginolyticus* to understand the whole biosynthetic pathway for NSPD. The regulatory mechanisms of the enzymes involved in NSPD production, and the importance of NSPD in this bacterium may then be further clarified.

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