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ORIGINAL PAPER

Heike Laue · Alasdair M. Cook

Purification, properties and primary structure of alanine dehydrogenase involved in taurine metabolism in the anaerobe *Bilophila wadsworthia*

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Abstract Alanine dehydrogenase [L-alanine:NAD^+ oxidoreductase (deaminating), EC 1.4.1.4.] catalyses the reversible oxidative deamination of L-alanine to pyruvate and, in the anaerobic bacterium *Bilophila wadsworthia* RZATAU, it is involved in the degradation of taurine (2-aminoethanesulfonate). The enzyme regenerates the amino-group acceptor pyruvate, which is consumed during the transamination of taurine and liberates ammonia, which is one of the degradation end products. Alanine dehydrogenase seems to be induced during growth with taurine. The enzyme was purified about 24-fold to apparent homogeneity in a three-step purification. SDS-PAGE revealed a single protein band with a molecular mass of 42 kDa. The apparent molecular mass of the native enzyme was 273 kDa, as determined by gel filtration chromatography, suggesting a homo-hexameric structure. The N-terminal amino acid sequence was determined. The pH optimum was pH 9.0 for reductive amination of pyruvate and pH 9.0–11.5 for oxidative deamination of alanine. The apparent K_m values for alanine, NAD^+, pyruvate, ammonia and NADH were 1.6, 0.15, 1.1, 31 and 0.04 mM, respectively. The alanine dehydrogenase gene was sequenced. The deduced amino acid sequence corresponded to a size of 39.9 kDa and was very similar to that of the alanine dehydrogenase from *Bacillus subtilis*.

Key words Alanine dehydrogenase · *Bilophila wadsworthia* · Taurine · Anaerobic metabolism · *ald* Gene sequence

Introduction

Bilophila wadsworthia is a strictly anaerobic, gram negative rod that was first isolated in 1989 (Baron et al. 1989). The natural habitat of this bacterium seems to be the human gastrointestinal tract (Baron 1997), although strain RZATAU was isolated from a communal sewage sludge (Laue et al. 1997). *B. wadsworthia* RZATAU grows with taurine (2-aminoethanesulfonate) as an electron acceptor in combination with an external electron donor, e.g. formate (Laue et al. 1997). During growth, ammonia, acetate and sulfide are formed as end products from taurine. At least four enzymes are involved in the degradative pathway. The oxidative deamination of alanine, catalysed by alanine dehydrogenase [L-alanine:NAD^+ oxidoreductase (deaminating), EC 1.4.1.4] regenerates the pyruvate consumed during the transamination of taurine (Fig. 1).

Alanine dehydrogenase catalyses the reversible oxidative deamination of L-alanine to pyruvate. The enzyme is involved in different metabolic pathways. In *Bacillus* sp., the main function of alanine dehydrogenase seems to be the generation of pyruvate as an energy source during sporulation (Siranosian et al. 1993). When operating in the reverse direction – the reductive amination of pyruvate – the enzyme can be involved in nitrogen assimilation as in, for example, *Rhodobacter capsulatus* (Caballero et al. 1989) and in *Streptomyces aureofaciens* (Van-

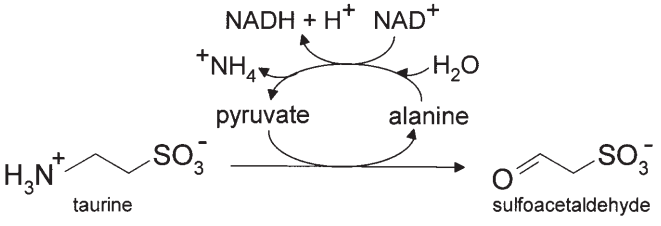

Fig. 1 Initial steps of the degradation of taurine in *Bilophila wadsworthia*. The first step is a pyruvate-dependent transamination of taurine, yielding sulfoacetaldehyde and alanine catalysed by taurine:pyruvate aminotransferase. An alanine dehydrogenase catalyses the oxidative deamination of alanine to pyruvate and ammonia. The sulfoacetaldehyde is further degraded to acetate and putatively sulfite that is finally reduced to sulfide by a dissimilatory sulfite reductase with reduction equivalents derived from the oxidative deamination of alanine and mainly from the oxidation of an external electron donor, e.g. formate (Laue et al. 1997)

H. Laue (✉) · A. M. Cook
Fachbereich Biologie, Universität Konstanz,
Universitätsstrasse 10, 78457 Konstanz, Germany
e-mail: Heike.Laue@uni-konstanz.de,
Tel.: +49-7531-884385, Fax: +49-7531-882966

curova et al. 1988). Alanine dehydrogenases involved in anaerobic catabolic processes have been investigated, for example, from *Desulfotomaculum ruminis* and two marine *Desulfovibrio* strains, where the enzyme is involved in the degradation of L-alanine as an energy source (Stams and Hansen 1986).

Here, we report the purification of the alanine dehydrogenase from the anaerobic bacterium *B. wadsworthia* RZATAU, where it is involved in taurine catabolism.

Materials and methods

Organism and cultivation

Bilophila wadsworthia RZATAU (DSM 11045) was routinely grown in batch culture (0.1 l or 10 l) in an anoxic freshwater mineral salts medium containing 12 mM taurine and 80 mM formate (Laue et al. 1997). Cells were also grown with 12 mM taurine plus 25 mM pyruvate, 12 mM cysteate (2-amino-3-sulfopropionate) plus 80 mM formate, 12 mM isethionate (2-hydroxyethanesulfonate) plus 80 mM formate, 10 mM thiosulfate plus 20 mM DL-lactate, or 30 mM pyruvate.

Preparation of crude extract

Cells from 10-l cultures were harvested at the end of exponential growth in a Pellicon filtration system (Millipore), centrifuged (13,000×*g*, 40 min, 4 °C) and washed twice in 50 mM potassium phosphate buffer (pH 7.5). The cell pellets could be stored for several months at –20 °C without loss of activity. Frozen cells were thawed, suspended to 0.5 g (wet weight) ml^{-1} in 50 mM potassium phosphate buffer (pH 7.5). The bacteria were disrupted by four passages through a chilled French pressure cell (140 MPa) followed by centrifugation to remove intact cells and debris (30,000×*g*, 20 min, 4 °C). The pellet was discarded and residual membranes were removed by ultracentrifugation (160,000×*g*, 40 min, 4 °C). Nucleic acids were removed by precipitation with 2% (v/v) streptomycin sulfate, as described elsewhere (Junker et al. 1994). The supernatant fluid was referred to as crude extract.

MOPS buffer (50 mM MOPS-KOH, pH 6.5) was used instead of potassium phosphate buffer in the experiment with cell-free extracts from cells grown with different substrates.

Determination of enzyme activity

The reaction mixture for the oxidative deamination consisted of 0.1 M CHAPS buffer, pH 10.0, 50 mM L-alanine, 1.5 mM NAD^+ and enzyme in a final volume of 1 ml. The assay system for the reductive amination contained 0.1 M Tris-HCl, pH 9.0, 20 mM sodium pyruvate, 0.1 M NH_4Cl, 0.1 mM NADH and enzyme in a final volume of 1 ml. The reaction mixture was incubated at 25 °C, except for the determination of kinetic parameters and identification of products (35 °C). The reaction was started by the addition of enzyme and was followed by measuring the initial change in absorption at 340 nm with Uvicon 922 spectrophotometer. A quantity of enzyme was used that provided a linear change in absorption for at least 1 min. Most of the experiments (unless otherwise indicated) were done with alanine dehydrogenase obtained from Mono Q-chromatography. For the identification of products, the reaction mixture contained 0.1 M Tris-HCl, pH 8.0, 10 mM alanine, 4 mM NAD^+ and enzyme in a final volume of 1 ml. Samples were taken to measure alanine, pyruvate and ammonia concentrations.

Purification of alanine dehydrogenase

All chromatographic steps were performed at room temperature with a Pharmacia FPLC system (DEAE-Sepharose) or with Beckman HPLC apparatus (Mono Q, Superose 12). Absorption of column effluents was monitored at 280 nm. After each step, fractions containing significant alanine dehydrogenase activity were combined and concentrated by membrane filtration (10-kDa cut-off; Intersep, Witten, Germany) in a stirring cell (Amicon).

Step 1. Crude extract was applied to a DEAE-Sepharose anion-exchange column (80 ml) equilibrated with 50 mM potassium phosphate buffer (pH 7.5). Bound protein was eluted with a 680-ml gradient over 0–1 M NaCl at a flow rate of 2.5 ml min^{-1}. Fractions of 7.5 ml were collected. Most of the alanine dehydrogenase activity did not bind to the column and the enzyme washed straight through the column. About 5% of the recovered alanine dehydrogenase activity bound to the column. It was eluted at approximately 0.2 M NaCl and discarded.

Step 2. The enzyme solution from step 1 was desalted in a Sephadex G-25 M column (Pharmacia) equilibrated with 20 mM Tris-sulfate buffer (pH 8.0). This desalted protein was applied to a Mono Q (HR 10/10) anion-exchange column equilibrated with 20 mM Tris-sulfate buffer (pH 8.0). Bound protein was eluted from the column with an 80-ml gradient over 0–0.1 M Na_2SO_4 followed by a 60-ml gradient over 0.1–1 M Na_2SO_4. The flow rate was 2 ml min^{-1} and fractions of 5 ml were collected. Alanine dehydrogenase activity eluted at about 70 mM Na_2SO_4.

Step 3. Protein from step 2 was loaded onto a gel filtration column (Superose 12, HR 10/30) equilibrated with 50 mM Tris-sulfate buffer (pH 7.5) containing 150 mM Na_2SO_4. The flow rate was 0.4 ml min^{-1} and 0.5 ml fractions were collected. Active fractions were concentrated by centrifugal ultrafiltration (10-kDa cut-off, Intersep). The molecular masses of the proteins used to calibrate the column were ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), albumin (67 kDa) and ovalbumin (43 kDa).

The enzyme was routinely stored at –20 °C. Under these conditions, the alanine dehydrogenase activity remained constant for at least 1 month. Repeated freezing and thawing led to significant loss of activity.

Gel electrophoresis and N-terminal sequence analysis

Proteins were separated by polyacrylamide (12%) gel electrophoresis in the presence of SDS according to the method of Laemmli (1970) and subsequently stained with colloidal Coomassie Brilliant Blue G-250 (Neuhoff et al. 1988). The homogeneous protein in an SDS polyacrylamide gel was blotted onto a polyvinylidene difluoride membrane. The blotted protein band was stained with the dye, cut out and subjected to Edman degradation in an Applied Biosystems 477A gas-phase system.

DNA sequencing and analysis

Degenerate primer pairs were deduced from the N-terminal sequence (MRVGIPTE) of the alanine dehydrogenase purified from *B. wadsworthia* RZATAU: 5′ ATG CGC GTB GGC ATC CCS ACC GAA 3′ and from the conserved putative pyruvate binding region (KVKEP) of other alanine dehydrogenases (Delforge et al. 1997): 5′ SGG TTC CTT SAC CTT 3′. PCR was done with washed and five-fold concentrated cells of *B. wadsworthia* RZATAU or total DNA (Ausubel et al. 1987) using the MBI *Taq* polymerase system (MBI Fermentas) but with a modified reaction buffer (2.25 mM $MgCl_2$, 50 mM Tris-HCl, 14 mM $(NH_4)_2SO_4$, pH 9.2, 10% dimethyl sulfoxide). The nucleotide sequence of the 230-bp PCR product was determined by cycle sequencing, using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit and an ABI 377 DNA sequencer (GATC GmbH), and its deduced amino acid sequence showed similarity to the sequences of other alanine dehydrogenases.

Adaptor-ligated PCR was applied to obtain the complete sequence of the *ald* gene. Total DNA from *B. wadsworthia* was digested and ligated to an adaptor of known sequence (Universal Genome Walker Kit, Clontech). Nested PCR was done with the Advantage Genomic Polymerase Mix (Clontech) and primer deduced from gene specific sequences in combination with adaptor

primer. Adaptor-ligated PCR with one primer derived from the 230-bp sequence gave a 350-bp product. The nucleotide sequence encoded the N-terminal amino acids and included the start codon and 166 nucleotides upstream. No other specific PCR product was obtained.

During sequence analysis of the taurine:pyruvate aminotransferase (*tpa*) gene (Laue and Cook, unpublished results), a truncated ORF with high sequence similarities to alanine dehydrogenases was found. Therefore the complete *ald* gene was amplified using primer derived from the sequence upstream from the alanine dehydrogenase (*ald*) gene obtained by adaptor-ligated PCR and from the *tpa* gene. The *ald* gene was sequenced completely by cycle sequencing and primer walking. Database searches were performed with BLAST (Altschul et al. 1997).

Analytical methods

Reverse-phase chromatography with diode array detection was done as described elsewhere (Laue et al. 1996). Alanine was quantified by HPLC after derivatisation with 2,4-dinitrofluorobenzene (Denger et al. 1997). Pyruvate was analysed by a HPLC system with a refraction index detector (Galushko et al. 1999). Ammonia was measured enzymically by the specific reaction of glutamate dehydrogenase (Bergmeyer 1983). Protein concentrations were determined by the method of Bradford (1976) with bovine serum albumin as standard. Chemicals of highest commercially available purity were purchased from Fluka, Merck or Sigma, and gas mixtures (N_2/CO_2) were from Sauerstoffwerk, Friedrichshafen, Germany.

Nucleotide sequence accession number

The sequence encoding the alanine dehydrogenase from *B. wadsworthia* RZATAU has been deposited in GenBank (accession no. AF269148).

Results

Alanine dehydrogenase as an inducible enzyme

Alanine dehydrogenase activity was significant higher in cell-free extracts from cells grown with taurine plus formate (relative activity, 100%; cf. Table 1) than in extracts from taurine-plus-pyruvate-grown cells (19%). Lower activities were detected in cells grown with pyruvate as single substrate (6%), thiosulfate plus lactate (4%), cysteate plus formate (1%) and isethionate plus formate (1%). SDS-PAGE analysis indicated a significantly larger, 42 kDa band, in the extract of taurine-plus-formate-grown cells than in extracts of taurine-plus-pyruvate-, thiosulfate-plus-lactate-, or pyruvate-grown cells. Extracts of cysteate-plus-formate-, or isethionate-plus-formate-grown cells showed no prominent 42-kDa band (data not shown). The enzyme from cells grown with taurine plus formate was chosen for purification.

Table 1 Purification of alanine dehydrogenase from *Bilophila wadsworthia* RZATAU

Purification step	Protein (mg)	Activity (μkat)	Specific activity (kat kg^{-1})	Yield (%)	Purification (*x*-fold)
Crude extract	499	14.1	2.83	100	1
DEAE-Sepharose	36.6	4.8	21.0	34	7.4
Mono Q	6.2	1.6	52	11	18
Superose 12	0.4	0.14	68	1	24

Purification of alanine dehydrogenase

Alanine dehydrogenase was purified 24-fold by a three-step purification involving two different anion exchange columns – DEAE-Sepharose and Mono Q – and gel filtration chromatography (Table 1). After Mono Q chromatography, the enzyme was already about 95% pure (Fig. 2) and this material was used for most experiments. After gel filtration, SDS-PAGE analysis showed one single, homogenous protein band (Fig. 2). The final yield was very low (1%).

Molecular properties

The molecular mass of the denatured protein determined by SDS-PAGE was 42 kDa (Fig. 2). The protein eluted from gel filtration columns under non-denaturing conditions with an apparent molecular mass of 273 kDa. N-terminal sequencing of the purified protein yielded a unique sequence. It therefore appears that the alanine dehydrogenase is composed of six identical subunits.

The ultraviolet/ visible spectrum of the purified alanine dehydrogenase obtained from gel filtration chromatography showed no absorption maxima except at 280 nm (data not shown). Thus the enzyme seems to have no prosthetic groups.

The sequence of the first 15 amino acids from the amino terminus of the purified alanine dehydrogenase

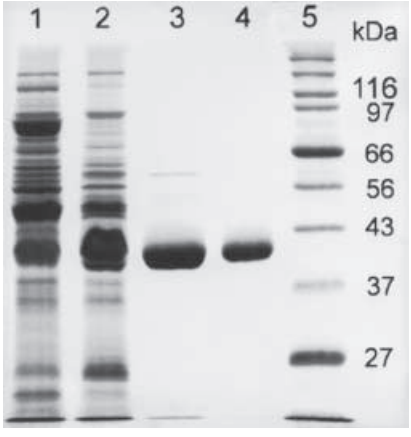

Fig. 2 Purification of alanine dehydrogenase monitored by SDS-PAGE. Protein was separated on a 12% polyacrylamide gel and subsequently stained with colloidal Coomassie Brilliant Blue. *Lane 1* Crude extract (20 μg), *lane 2* active fractions from DEAE-Sepharose chromatography (10 μg), *lane 3* active fraction from Mono Q-chromatography (10 μg), *lane 4* purified alanine dehydrogenase after gel filtration on Superose 12 (3 μg), *lane 5* molecular mass standards (New England BioLabs)

was identified as M R V G I P T E I K V Q E F R. The N-terminal amino acid sequence of the protein showed significant similarities to the N-terminal amino acid sequences of several alanine dehydrogenases.

Catalytic properties

The deamination of alanine was confirmed by HPLC analysis. Disappearance of alanine was concomitant with the formation of pyruvate and ammonia. $NADP(H)^+$ did not serve as a substrate. In addition to L-alanine the following substrates were deaminated by the alanine dehydrogenase: L-2-aminobutyrate (relative activity 10.4%), L-valine (4.8%), L-norvaline (4.6%), L-serine (4.4%), D-alanine (3.5%) and L-isoleucine (0.2%). Taurine was inert. In the amination reaction, the enzyme utilised in addition to pyruvate (100%) oxaloacetate (26.3%), 2-oxobutyrate (15.3%), β-hydroxypyruvate (9.3%) and glyoxylate (1.0%), but not 2-oxoglutarate and phenylpyruvate.

Enzyme activity was assayed as a function of pH in the forward and reverse reactions. Whereas the reductive amination of pyruvate had a sharp pH optimum at pH 9, the oxidative deamination of alanine had a broad optimum over pH 9.0–11.5, and showed higher activity in the mixed buffer system than in potassium phosphate buffer. When the enzyme was assayed at different temperatures, the maximum activity for reductive amination was measured at 55–60 °C and for oxidative deamination at 50–55 °C. But at 60 °C an inactivation of the enzyme was observed after about 1 min, while at 65 °C the enzyme was inactive after about 10 s. The activation energy was calculated by using the Arrhenius plot as 50 kJ mol^{-1} and 57 kJ mol^{-1} for amination and deamination reactions, respectively.

The K_m values for the substrates in the forward and reverse reactions were determined at the optimum pH for the appropriate reaction and at 35 °C. The apparent K_m values for alanine, NAD^+, pyruvate, ammonia and NADH were 1.6, 0.15, 1.1, 31 and 0.04 mM, respectively. The V_{max} value for the reductive amination of pyruvate (17 μkat) was 14-fold higher than the value determined for the oxidative deamination of alanine (1.2 μkat).

Sequence analysis

A 1.5-kb sequence was amplified with the PCR primers (see Materials and methods) and sequenced. An ORF of 1,134 nucleotides encoding a protein which included the N-terminal peptide of alanine dehydrogenase was found. The molecular mass of the deduced 377 amino acid protein was calculated to be 39.9 kDa, which was close to the value obtained by SDS-PAGE (42 kDa). The theoretical isoelectric point was estimated as 6.5.

The start codon is preceded by a putative ribosomal binding site (GGAGG). There are two stretches resembling the –35 and –10 regions of an *Escherichia coli* σ^{70} promotor (TTGGGA-N_{19}-TATAAA) 71 nucleotides upstream from the translational start of the *ald* gene and a putative terminator sequence $\overrightarrow{\text{CCAGTCTCATG}}\text{TCCG-}\overleftarrow{\text{CATGAGACTGG}}\text{CCAGTTTT}$; inverted repeats indicated by arrows) 19 nucleotides downstream from the stop codon. In contrast to the total G+C content of the *B. wadsworthia* DNA (39–40 mol%), the *ald* gene has a significantly higher G+C content (61.9 mol%).

The deduced amino acid sequences showed high similarities to those from other alanine dehydrogenases. The *B. wadsworthia* enzyme was 57%, 56%, 54% and 51% identical to the enzymes from *Bacillus subtilis* (Siranosian et al. 1993), *Bacillus sphaericus* (Kuroda et al. 1990), *Mycobacterium tuberculosis* (Cole et al. 1998) and *Phormidium lapideum* (Baker et al. 1998), respectively. All of these sequences including that of *Bilophila wadsworthia* contained the conserved residues (KVKEP) of the putative pyruvate binding region (Delforge et al. 1997) and of the putative βαβ Rossmann fold responsible for nucleotide binding (Wierenga et al. 1986; Bork and Grunwald 1990).

Discussion

Alanine dehydrogenase of *B. wadsworthia* RZATAU seems to be induced by taurine. In cell-free extracts from cells grown in the absence of taurine, alanine dehydrogenase activity was significantly lower than in the presence of taurine as an electron acceptor. In the absence of taurine, probably only a basic level of alanine dehydrogenase was expressed. However, in the presence of pyruvate instead of formate as electron acceptor plus taurine, the activity was five-fold lower. We presume that the expression of alanine dehydrogenase is repressed by pyruvate, though other regulation types cannot be excluded. In the presence of pyruvate, the enzyme seems not to be necessary in large amounts – especially during the initial part of growth – to regenerate pyruvate as an amino-group acceptor. The alanine dehydrogenase from *Mycobacterium* strain HE5 seems to be regulated by different factors. A high enzyme activity was only detected in cells grown with nitrogen-containing substrates, but not with pyruvate plus ammonia, and the oxidative deamination of L-alanine was significantly stimulated by morpholine (Schuffenhauer et al. 1999).

The alanine dehydrogenase from *B. wadsworthia* RZATAU was purified about 24-fold and characterised. Based on the data of the purification protocol, it can be calculated that the enzyme represents about 4% of the soluble part of proteins in taurine-plus-formate-grown cells. The enzyme appears to consist of six identical subunits of apparently 42 kDa.

Alanine dehydrogenases have been purified from various organisms. While the quaternary structure of the enzyme from *B. wadsworthia* matches to the largest group of hexameric alanine dehydrogenases purified from *Anabaena cylindrica* (Rowell and Stewart 1975), *Bacillus* spp (e.g. Ohashima and Soda 1979), *Mycobacterium tuberculosis* (Andersen et al. 1992), *Phormidium lapideum*

(Sawa et al. 1994) and *Thermus thermophilus* (Vali et al. 1980), there are also tetrameric, monomeric and even octomeric structures (cf. Brunhuber and Blanchard 1994).

The *Bilophila* alanine dehydrogenase exhibits a pH optimum at pH 9 for the reductive amination of pyruvate and a broad optimum at pH 9–11.5 for the oxidative deamination of alanine. These data coincide with those from other alanine dehydrogenases (Brunhuber and Blanchard 1994), except for the unusually broad maximum of the deamination reaction also including relatively low pH values.

The enzyme requires NAD^+ as a cofactor that cannot be replaced by $NADP^+$. In the deamination reaction, the *B. wadsworthia* enzyme acts on L-alanine, L-2-aminobutyrate (10% activity), around 4% activity with L-valine, L-norvaline, L-serine and D-alanine, as well as acting slightly on L-isoleucine. Alanine dehydrogenases from *Bacillus subtilis* (Yoshida and Freese 1965) and *Enterobacter aerogenes* (Chowdhury et al. 1998) exhibit a similar substrate spectrum for amino acids, while the enzymes from other organisms, such as *M. tuberculosis* (Andersen et al. 1992), *P. lapideum* (Sawa et al. 1994) and *S. fradiae* (Vancura et al. 1989), are restricted exclusively to L-alanine. The substrate spectrum of alanine dehydrogenases for the reductive amination is usually broader. The *Bilophila* enzyme aminates oxaloacetate, 2-oxobutyrate, β-hydroxypyruvate and, at lower activity, glyoxylate.

The apparent K_m values of the *Bilophila* enzyme are of the same magnitude as those of alanine dehydrogenases from other species. The K_m value for L-alanine (1.6 mM) belongs to the lower K_m values, in comparison with other alanine dehydrogenases (Ohshima and Soda 1990), making the *Bilophila* enzyme apparently suitable for the direction of oxidative deamination. This is supported by the relatively high K_m value for ammonia (31 mM).

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