

Bacterial Na⁺-translocating ferredoxin: NAD⁺ oxidoreductase

Eva Biegel and Volker Müller¹

Department of Molecular Microbiology and Bioenergetics, Institute of Molecular Biosciences, Johann Wolfgang Goethe University Frankfurt/Main, 60438 Frankfurt, Germany

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The anaerobic acetogenic bacterium *Acetobacterium woodii* carries out a unique type of Na⁺-motive, anaerobic respiration with caffeate as electron acceptor, termed “caffeate respiration.” Central, and so far the only identified membrane-bound reaction in this respiration pathway, is a ferredoxin:NAD⁺ oxidoreductase (Fno) activity. Here we show that inverted membrane vesicles of *A. woodii* couple electron transfer from reduced ferredoxin to NAD⁺ with the transport of Na⁺ from the outside into the lumen of the vesicles. Na⁺ transport was electrogenic, and accumulation was inhibited by sodium ionophores but not protonophores, demonstrating a direct coupling of Fno activity to Na⁺ transport. Results from inhibitor studies are consistent with the hypothesis that Fno activity coupled to Na⁺ translocation is catalyzed by the Rnf complex, a membrane-bound, iron-sulfur and flavin-containing electron transport complex encoded by many bacterial and some archaeal genomes. Fno is a unique type of primary Na⁺ pump and represents an early evolutionary mechanism of energy conservation that expands the redox range known to support life. In addition, it explains the lifestyle of many anaerobic bacteria and gives a mechanistic explanation for the enigma of the energetic driving force for the endergonic reduction of ferredoxin with NADH plus H⁺ as reductant in a number of aerobic bacteria.

Acetobacterium | anaerobic respiration | electron transport | Na⁺ pump | Rnf

The mechanism(s) of energy conservation in a major group of strictly anaerobic bacteria, the acetate-forming, acetogenic bacteria, is still an enigma. Acetogens can grow autotrophically with hydrogen as electron donor. The electrons are channeled to the acceptor carbon dioxide that is reduced to acetate in the Wood-Ljungdahl pathway (1–3). How this pathway is coupled to energy conservation is obscure, but in the model acetogen *Acetobacterium woodii* at least one of the reactions is coupled to primary and electrogenic Na⁺ translocation across the membrane (4). It is known that the electrochemical sodium ion gradient drives the synthesis of ATP by a unique hybrid Na⁺ F₁F₀ ATP synthase, but the enzyme generating the Na⁺ gradient remains to be identified (4–6).

In recent years it turned out that *A. woodii* can use the alternative electron acceptor caffeate [3-(3,4-dihydroxyphenyl)-2-propenoic acid] in a process called caffeate respiration (7, 8). The phenylacrylate caffeate is a major component of lignin and thus makes a considerable portion of plant-derived biomass in soils (9). Caffeate respiration in *A. woodii* is coupled to ATP synthesis by a chemiosmotic mechanism with Na⁺ as coupling ion (10). The pathway of caffeate respiration with hydrogen as reductant involves a ferredoxin-reducing hydrogenase (11), a ferredoxin:NAD⁺ oxidoreductase (Fno), an electron-transferring flavoprotein (Etf) that, in a complex with a caffeoyl-CoA-dehydrogenase, reduces caffeoyl-CoA to hydrocaffeoyl-CoA with NADH as electron donor, and an activation of caffeate to caffeoyl-CoA (12, 13). In the search for the Na⁺-translocating step of the pathway only the Fno was found to be membrane bound (13).

Here we present evidence that Fno is the Na⁺-translocating enzyme of the pathway. Furthermore, results from inhibitor studies

are consistent with the hypothesis that the Fno activity is catalyzed by Rnf, a flavin- and FeS-containing membrane-bound electron transfer complex. Because the complex is widely distributed in anaerobic as well as aerobic species it is a unique general coupling site in bacteria and archaea (12, 14, 15).

Results

Fno Activity and ²²Na⁺ Transport at Inverted Membrane Vesicles of *A. woodii*. To determine ion transport, inverted membrane vesicles (IMV) were prepared as previously described (16), yielding almost exclusively inside-out vesicles. The vesicles were tightly closed and able to hold an artificial ΔpH, tested by creating and monitoring an artificial ammonium diffusion potential (17). The Fno activity at IMVs was measured as previously described for isolated membranes (13), with reduced ferredoxin [generated by reduction with titanium (III) citrate] as electron donor. The assay was carried out under strictly anaerobic conditions in 50 mM Mops buffer (pH 6.0) containing 20 mM MgSO₄, 20 mM NaCl, 8 mM dithioerythritol (DTE), and 2.25 mg/L resazurin. Indeed, IMVs catalyzed Fno activity of 20–70 mU/mg, depending on the quality of the vesicle preparation (1 U corresponding to 1 μmol NAD⁺/min), and is in the same range as the ATPase activity (16). Fno activity was dependent on titanium (III) citrate or NAD⁺ in the assay. Ferredoxin requirement depended on the washing procedure. Vesicles washed only once did not require additional ferredoxin, whereas vesicles washed three times did. These data indicate a rather tight association of the ferredoxin with the membrane.

Transport of Na⁺ was measured at IMVs of *A. woodii* using the radioisotope ²²Na⁺. Upon addition of reduced ferredoxin and NAD⁺, ²²Na⁺ was accumulated in the lumen of IMVs (Fig. 1). ²²Na⁺ transport was strictly dependent on the presence of titanium (III) citrate and NAD⁺. As expected, ²²Na⁺ transport required addition of ferredoxin only in IMVs washed three times. ²²Na⁺ translocation was observed at IMVs prepared from cells grown on fructose or on fructose plus caffeate, indicating that the Fno activity is produced under both growth conditions. When vesicles were prepared under aerobic conditions no NAD⁺ reduction was observed and subsequently transport was abolished, indicating that Fno contains oxygen-sensitive cofactors. Depending on the preparation of the IMVs, a stoichiometry of 2–35 electrons/²²Na⁺ was obtained.

Concentration Dependence of ²²Na⁺ Transport. The rate of ²²Na⁺ transport was dependent on the NaCl concentration of the buffer (Fig. 2). At 0.1 mM NaCl it was 0.24 nmol/min · mg protein but increased with increasing Na⁺ concentrations to 2.6 nmol/min · mg protein at 10 mM NaCl. The K_m value for Na⁺ was determined to be 2.5 mM, and V_{max} was 2.6 nmol/mg · min (Fig. 2).

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¹To whom correspondence should be addressed. E-mail: vmueller@bio.uni-frankfurt.de.

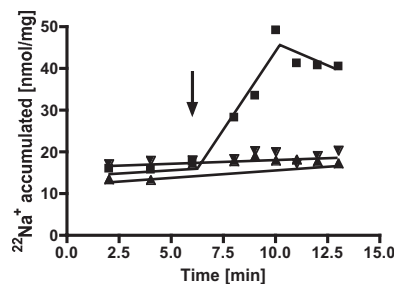


Fig. 1. Fno catalyzed $^{22}\text{Na}^+$ transport. Membrane vesicles (protein concentration 3.2 mg/mL) in 50 mM Mops buffer containing 20 mM MgSO_4 , 20 mM NaCl, 8 mM DTE, and 2.25 mg/L resazurin translocated $^{22}\text{Na}^+$ upon addition of 7.5 μg ferredoxin, 5 mM titanium citrate, and 3.5 mM NAD^+ (squares). In control experiments titanium citrate (downward-pointing triangles) or NAD^+ (upward-pointing triangles) were omitted. Arrow indicates addition of NAD^+ .

A 3.2-fold accumulation (0.48 nmol/mg) was observed at 0.1 mM NaCl, but it decreased to 2.1-fold (24 nmol/mg) at 20 mM NaCl. All subsequent experiments were performed in the presence of 20 mM NaCl.

Increasing LiCl concentrations did not have an effect on NAD^+ reduction, but with increasing LiCl concentrations $^{22}\text{Na}^+$ transport decreased, indicating that Li^+ can be transported instead of Na^+ (Fig. 3).

$^{22}\text{Na}^+$ Transport Is Electrogenic and Primary. If Na^+ transport is vectorial and not accompanied by charge compensation, the build-up of an electrical field ($\Delta\Psi$, inside positive) in the IMVs should inhibit further accumulation of Na^+ . The K^+ ionophore valinomycin used at high KCl concentrations should compensate for charge separation, eliminate the $\Delta\Psi$, and thus stimulate $^{22}\text{Na}^+$ transport. Indeed, this was observed. Valinomycin (17 μM) in the presence of 150 mM KCl stimulated $^{22}\text{Na}^+$ transport by 21%, indicating that $^{22}\text{Na}^+$ transport is electrogenic and accompanied by the generation of an electrical field ($\Delta\Psi$) across the IMVs. This $\Delta\Psi$ could then be used to drive the Na^+ F_1F_0 ATP synthase present in *A. woodii*. Indeed, inhibition of the Na^+ F_1F_0 ATP synthase by 1 mM *N,N'*-dicyclohexylcarbodiimide (DCCD) (16) increased Na^+ accumulation by 47%.

The protonophore 3,5-di-*tert*-butyl-hydroxybenzylidenemalonitrile (SF 6847; 100 μM) did not abolish $^{22}\text{Na}^+$ translocation (Fig. 4), excluding the possibility that Fno activity is coupled to primary proton transport that then is used to energize secondary Na^+ transport via Na^+/H^+ antiporter. The effectiveness of the protonophore was ensured by its ability to dissipate an artificial ΔpH created by an NH_4^+ diffusion potential in IMVs of *A. woodii*. There was no accumu-

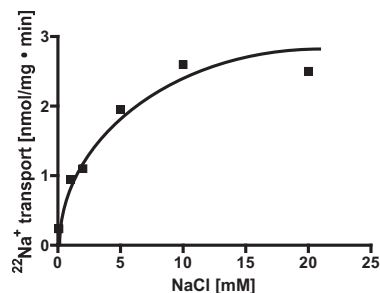


Fig. 2. Na^+ dependence of transport activity. Membrane vesicles (protein concentration 4 mg/mL) were incubated in 50 mM Mops buffer, 20 mM MgSO_4 , 8 mM DTE, 2.25 mg/L resazurin, and varying NaCl concentrations. Transport rates were calculated from the initial slopes and plotted against the Na^+ concentration.

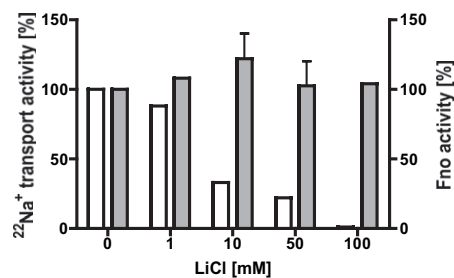


Fig. 3. Effect of LiCl on $^{22}\text{Na}^+$ transport and Fno activity. White bars represent the rate of $^{22}\text{Na}^+$ accumulation at IMVs; 100% corresponds to 0.9 nmol $^{22}\text{Na}^+/\text{mg} \cdot \text{min}$. Gray bars indicate Fno activity; 100% corresponds to 18 mU/mg.

lation of $^{22}\text{Na}^+$ in the presence of the Na^+ ionophore *N,N,N,N'*-tetracyclohexyl-1,2-phenylendioxydiacetamide (ETH 2120; 100 μM); moreover, a previously established Na^+ gradient was abolished by the Na^+ ionophore (Fig. 4), indicating the generation of a transmembrane Na^+ gradient (i.e., an uphill transport of $^{22}\text{Na}^+$ upon oxidation of reduced ferredoxin). In summary, the ionophore studies present clear evidence that the Fno activity is coupled to a primary and electrogenic $^{22}\text{Na}^+$ translocation.

Inhibition Studies on Fno Activity. So far, the data presented unequivocally demonstrate the presence of an Na^+ -translocating Fno activity but do not reveal the nature of this enzyme. Previously we had identified an operon in *A. woodii* that encodes a membrane-bound electron transfer complex with similarity to a gene cluster (*rnf*) (18) that was shown genetically to be involved in the production of reduced ferredoxin (required for nitrogen fixation) with $\text{NADH} + \text{H}^+$ as reductant in *Rhodobacter capsulatus* (19). The Rnf complexes of *A. woodii* and *Clostridium tetanomorphum* were partially purified and shown to catalyze Fno activity as well as NADH -dependent reduction of potassium ferricyanide (18, 20). Furthermore, it was shown that RnfG and RnfD of *Vibrio cholerae* contain covalently bound flavins (21). In addition, RnfC and RnfB have 4Fe4S clusters (22). To unravel whether this Rnf complex may catalyze the Na^+ -pumping Fno activity observed at IMVs, we first searched for inhibitors for the partially purified Rnf complex. Ag^+ and Cu^{2+} turned out to be very efficient inhibitors; half-maximal inhibition of NAD^+ reduction was observed at 3.75 ± 1 (SD) and 1.75 ± 0.35 μM , respectively. In addition, the inhibitors 1,10-phenanthroline ($I_{50} = 155 \pm 21$ μM), diphenyliodonium chloride ($I_{50} = 1 \pm 0.17$ μM), and diphenyleniodonium chloride

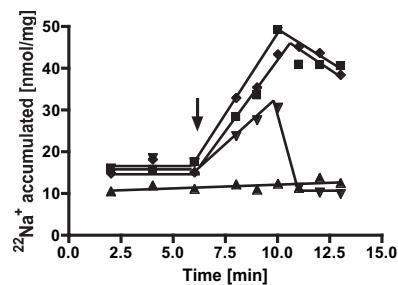


Fig. 4. $^{22}\text{Na}^+$ transport is a primary event. Membrane vesicles (protein concentration 3.2 mg/mL) in 50 mM Mops buffer (pH 6.0) containing 20 mM MgSO_4 , 20 mM NaCl, 8 mM DTE, and 2.25 mg/L resazurin showed $^{22}\text{Na}^+$ transport upon addition of 7.5 μg ferredoxin, 5 mM titanium citrate, and 3.5 mM NAD^+ (squares). ETH 2120 (100 μM) was added 6 min before (upward-pointing triangles) or 4 min after (downward-pointing triangles) addition of NAD^+ . SF 6847 (100 μM) (diamonds) was added 6 min before addition of NAD^+ . Arrow indicates addition of NAD^+ .

Table 1. Inhibition of Fno activity

Inhibitor	I ₅₀ (Fno activity) – partially purified Fno (μM)	I ₅₀ (Fno activity) – IMVs (μM)	Complete inhibition of ²² Na ⁺ transport (μM)
AgNO ₃	3.75 ± 1	5.4 ± 0.49	100
CuSO ₄	1.75 ± 0.35	2 ± 0.07	80
1,10-Phenanthroline	155 ± 21	84 ± 19	500
Diphenyliodonium chloride	1 ± 0.17	4.1 ± 4	40
Diphenyleniiodonium chloride	1 ± 0.035	2.5 ± 0.7	40

Inhibition of NAD⁺ reduction and ²²Na⁺ transport at partially purified protein and IMVs. Errors are shown as SD.

(I₅₀ = 1 ± 0.035 μM) inhibited Fno activity. 1,10-Phenanthroline is an iron chelating agent used to inhibit electron transport reactions (23, 24). Diphenyliodonium chloride and diphenyleniiodonium chloride are used as flavin inhibitors (25, 26). The inhibitors inhibited not only Fno activity of the partially purified Rnf complex but also Fno activity catalyzed by IMVs. At the same time as Fno activity, ²²Na⁺ transport catalyzed by the IMVs was inhibited by the different inhibitors of Rnf: AgNO₃ inhibited transport completely at 100 μM, CuSO₄ at 80 μM, 1,10-phenanthroline at 500 μM, and diphenyliodonium chloride and diphenyleniiodonium chloride at 40 μM (Table 1).

Discussion

In summary, this work demonstrates a unique primary sodium ion pump that couples electron transfer from reduced ferredoxin to NAD⁺ with electrogenic movement of Na⁺ out of the cell. The energy stored in the electrochemical Na⁺ gradient may then drive ATP synthesis via the well-known Na⁺ F₁F₀ ATP synthase present in *A. woodii* (6, 16). This Na⁺ pump is the only coupling site in caffeate respiration and thus explains how caffeate respiration is coupled to ATP synthesis with Na⁺ as coupling ion (Fig. 5).

The free energy change of the Fno reaction is -19 kJ/mol, assuming a redox potential of ferredoxin of -420 mV and NAD⁺ of -320 mV. This would allow for the translocation of 1 mol Na⁺ across the membrane, assuming an electrochemical ion potential of -200 mV at the cytoplasmic membrane of *A. woodii*. Thus, during caffeate respiration 3 mol of ferredoxin have to be

reduced to get the three ions required for synthesis of 1 mol ATP. However, it is conceivable that additional ferredoxin is reduced in the course of the caffeyl-CoA dehydrogenase reaction by electron bifurcation (see below) (27, 28).

What is the nature of the sodium-motive Fno in *A. woodii*? A membrane-bound protein complex with Fno activity was partially purified from *A. woodii* and shown to be encoded by the *rnf* operon (18). The partially purified Rnf complex is inhibited by AgNO₃ and CuSO₄, the flavin-directed inhibitors diphenyliodonium chloride and diphenyleniiodonium chloride, and 1,10 phenanthroline, which is described as an iron chelating agent. At the same time, these inhibitors inhibited ferredoxin-driven Na⁺ translocation in IMVs of *A. woodii*. This is consistent with the hypothesis that the Rnf complex catalyzes the observed Na⁺ translocation coupled to Fno.

Rnf is a proposed membrane-bound electron transport complex containing six subunits with flavins and iron-sulfur centers as electron carriers. It was originally described in mutants of *R. capsulatus* (*Rhodobacter nitrogen fixation*), but the genes are present in the genomes of many aerobic and anaerobic bacteria. NAD⁺ was regarded as the most electronegative electron donor in classic bioenergetics, but in recent years it turned out that ferredoxin is an electron carrier widely used by anaerobes (28, 29). Ferredoxin is used as primary electron acceptor by hydrogenases, the pyruvate:ferredoxin oxidoreductase, some pyruvate:formate lyases, formate dehydrogenases, and CO dehydrogenases (11, 30–33). In addition, some anaerobic bacteria couple

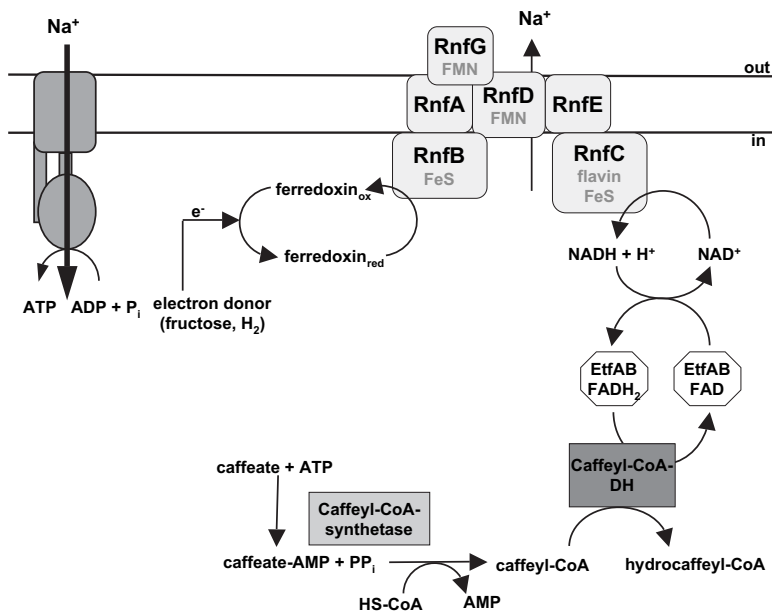


Fig. 5. Model of caffeate respiration in *A. woodii*. Flow of electrons from electron donors (fructose or hydrogen) to acceptor caffeate is shown. For explanations see text.

the exergonic reduction of fermentation intermediates to the endergonic reduction of ferredoxin with NADH as a reductant in a process called “electron bifurcation” (27, 28). The reduced ferredoxin is then assumed to be oxidized by Rnf. In anaerobes it may often be the only way to generate an ion gradient for synthesis of ATP by a chemiosmotic mechanism. In aerobes, it may function to drive the endergonic reduction of ferredoxin or iron (sulfur) centers in other proteins. The use of Na⁺ as coupling ion for *A. woodii* Fno/Rnf was expected because it is known that *A. woodii* bases its bioenergetics on a sodium ion current across the cytoplasmic membrane (10, 15, 34). The same may be true in other anaerobes or aerobes. However, most organisms that harbor Rnf have no documented Na⁺ bioenergetics, and therefore it is likely that these species use H⁺ as coupling ion for Rnf (19).

Our work shows experimentally that electron transfer from reduced ferredoxin to NAD⁺ as acceptor drives the generation of a transmembrane ion gradient. It should be mentioned in this connection that other anaerobes, such as *Methanosarcina* or *Pyrococcus* species, have related systems that use Ech hydrogenase or Eha hydrogenase to use the redox difference between ferredoxin and H⁺/H₂ to establish a transmembrane ion gradient (35–37).

Materials and Methods

Measurement of Fno Activity. The measurement of Fno activity was conducted as previously described (13). The buffer used was 50 mM Mops (pH 6.0) containing in addition 20 mM MgSO₄, 20 mM NaCl, 8 mM DTE, and 2.25 mg/L resazurin (to monitor redox state).

Growth of Cells and Preparation of Vesicles. *A. woodii* (DSM 1030) was grown under anaerobic conditions using 20 mM fructose or 20 mM fructose and 5 mM caffeate as electron acceptor as previously described (16, 38). The preparation of vesicles was done under strictly anaerobic conditions in an anaerobic chamber (Coy Laboratory Products) as previously described (16). For preparation of vesicles the growth medium was supplemented with 420 mM sucrose and 8.1 mM MgSO₄. Five liters of medium were inoculated (with 200 mL culture), and the optical density was followed at 600 nm. At OD₆₀₀ of 0.7–0.9, 70 μg penicillin G/mg were added to the medium to induce protoplast formation. During further incubation *A. woodii* formed protoplasts as monitored by microscopic observations. After 20 h the culture

consisted almost entirely of spherical forms that were highly sensitive to low osmolarity. These protoplasts were harvested anaerobically by centrifugation (6,250 × g, 20 min, 4 °C) and washed in cold vesicle buffer [50 mM Tris/HCl (pH 8.0) containing 25 mM MgSO₄, 420 mM sucrose, 8 mM DTE, and 2.25 mg/L resazurin, flushed with N₂ for 20 min]. After washing, the protoplasts were resuspended in a total volume of 300 mL vesicle buffer with lysozyme (1 mg/mL) and incubated for 30 min at room temperature. The protoplasts were centrifuged (6,250 × g, 20 min, 4 °C) and resuspended in 10–20 mL vesicle buffer. The protoplasts were passed through a French pressure cell at 41 MPa and centrifuged three times (4,500 × g, 35 min, 4 °C). The resulting supernatant (= crude vesicles) was centrifuged further by ultracentrifugation at 120,000 × g, 40 min, 4 °C. The pellet was washed in vesicle buffer and centrifuged again. The resulting pellet was solved in the same buffer in a volume of 3–5 mL.

Protein concentrations were determined by the method of Bradford (39).

Measurement of ²²Na⁺-Translocation. The experiments were performed under anaerobic conditions in 50 mM Mops buffer (pH 6.0) containing 20 mM MgSO₄, 8 mM DTE, and 2.25 mg/L resazurin (to monitor redox state) at 30 °C in a shaking water bath as previously described (16). In 3.5-mL glass vials buffer, supplements (17 μM valinomycin, 1 mM DCCD, NaCl as indicated, 150 mM KCl, and 7.5 μg ferredoxin) and ²²NaCl (final activity 0.5 μCi/mL) were combined and incubated for 120 min to ensure equilibration of ²²Na⁺ before the reaction was started. Titanium citrate (5 mM), 100 μM ETH 2120 or 100 μM SF 6847 were added just before taking of samples started. After 6 min 3.5 mM NAD⁺ was added. When the effect of DCCD on transport was tested, sodium was omitted from the assay.

LiCl. The sodium concentration in all experiments was 1 mM, and LiCl was increased from none to 100 mM. ²²Na⁺ transport and Fno activity were measured in presence of different LiCl concentrations.

Inhibition Experiments. AgNO₃, CuSO₄, 1,10-phenanthroline, diphenyliodonium chloride, and diphenyleniodonium chloride were preincubated with IMVs for 10 min before starting the NAD⁺ reduction or transport. For experiments with AgNO₃ and CuSO₄, DTE was omitted from all buffers.

Partial Purification. The protein was enriched as previously described (18).

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