Spectroscopic Characterization of Nickel(II) Carboxypeptidase

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The electronic and ^{1}H NMR spectra of nickel-substituted carboxypeptidase have been measured and discussed in the light of the available X-ray data. The interaction with D-phenylalanine has been investigated as well as that with azide. It is proposed that D-Phe binds first at a non-metallic site, probably Arg-145, and then to the metal. The behaviour of L-Phe is more complex. Azide binds at the metal in the presence of the amino acid. These results are similar to those obtained for the parent (zinc) and cobalt-substituted enzymes. Implications for the enzymatic mechanism are discussed.

Carboxypeptidase A, CPA, is an exopeptidase containing a zinc ion bound to two imidazole groups from His-69 and -196, to a carboxylate bidentate group from Glu-72 and to solvent.[1] This enzyme catalyses the hydrolysis of the C-terminal amino acid of a polypeptide chain.[2] It is known that C-terminal products of peptide or ester hydrolysis (amino acids or carboxylates) can interact with some groups present in the active-site cavity.[3,4] The X-ray structures of several inhibitors or substrate analogues invariably show that the terminal carboxylate binds Arg-145.[5–7] Anions like N_3^-, NCO^-, NCS^- and Cl^- have little affinity for the metal ions as they prefer to bind to Arg residues inside the active cavity.[8,9] The affinity of anions for the metal increases and becomes measurable only when a carboxylate binds Arg-145.[8–11]

Nickel can substitute the zinc ion in CPA, and the derivative obtained NiCPA retains practically complete enzymatic activity.[12] The structure of NiCPA has been determined by X-ray crystallography. The nickel ion is found to be bound to the same residues as zinc in an approximately square-pyramidal arrangement.[13] Its spectral and magnetic characterization was pioneered by Gray and co-workers[14,15] before the X-ray structure was known.

We have undertaken an investigation of NiCPA as such and with the amino acids L- and D-phenylalanine and with azide in order to compare the reactivity of NiCPA, CoCPA and CPA itself. In this enzyme the residues inside the active cavity play a fundamental role which is now largely understood, whereas the role of the metal ion is still controversial. Earlier, the metal ion was believed to activate the carbonyl carbon through co-ordination with the carbonyl oxygen. Recently, the possibility that the metal ion provides a co-ordinated hydroxo group for attack at the carbonyl group has been underlined.[16] Within this framework an investigation of the reactivity of the nickel derivative is quite meaningful and may shed further light on the behaviour of the enzyme as a whole.

Experimental

Bovine carboxypeptidase A, CPA, prepared by the method of Cox *et al.*,[17] was purchased from Sigma as an aqueous crystalline suspension with toluene added. The enzyme was purified through affinity chromatography on CABS-Sepharose to remove protease contaminants.[18,19] The buffer solutions were prepared by using deionized doubly distilled water.

The zinc ion was removed from the crystalline enzyme as previously described.[20] In order to avoid contamination of apo-CPA by adventitious metal ions, plastic labware was used and all the buffers were extracted with dithizone (1,5-diphenylthiocarbazone) in CCl_4 prior to use.[21] Nickel reconstitution was accomplished by incubation of a very diluted apo-CPA solution (*ca.* 10^{-5} mol dm^{-3}) containing an excess of the metal ion (*ca.* 10^{-4} mol dm^{-3}) during 24 h at 2–4 °C.[12] At these concentration and temperature conditions the NiCPA was stable for several weeks. The enzyme concentration was determined at 278 nm by using a molar absorption coefficient of 6.4×10^4 dm^3 mol^{-1} cm^{-1}.[22] The formation of NiCPA could be monitored by electronic and ^{1}H NMR spectroscopies. The salt $NiSO_4 \cdot 6H_2O$, NaN_3, L-Phe, D-Phe and all the other chemicals were Merck analytical grade reagents. The 99.7% D_2O was obtained from Fluka.

Near-infrared and visible absorption spectra were recorded on a UV/VIS-NIR Perkin-Elmer Lambda 9 spectrophotometer, using microcells with an optical path length of 10 mm. Samples for spectrophotometric measurements were prepared in D_2O {1 mol dm^{-3} NaCl, 50×10^{-3} mol dm^{-3} hepes [*N'*-(2-hydroxyethyl)piperazine-*N*-ethanesulfonic acid], pD 7} and the NiCPA concentrations were approximately 1.0×10^{-3} mol dm^{-3}. The spectra were registered using as reference a solution of the native enzyme under the same conditions. Room-temperature circular dichroism (CD) spectra were recorded on a JASCO 500D spectropolarimeter using 10 mm cells. The pH of all the solutions were measured on a Crison digit-501 pH meter provided with an Ingold combined microelectrode.

The samples for NMR measurements were concentrated to $(1.5–2) \times 10^{-3}$ mol dm^{-3} protein by ultrafiltration at 2–4 °C using a Centricon microconcentrator (Amicon) with a molecular weight cut-off of 10 000. For long NMR experiments several samples with the same NiCPA concentration were used owing to the reduced stability of this metal derivative. The ^{1}H NMR spectra were recorded on a Bruker AC-200 MHz spectrometer at 20 °C (1 mol dm^{-3} NaCl, 50×10^{-3} mol dm^{-3} hepes, pH 7) using the modified driven equilibrium Fourier-transform (MODEFT) pulse sequence[23] to suppress signals from water and bulk diamagnetic protein. Spectra typically consisted of *ca.* 16 000 scans with 8K data points and a spectral width of 50 kHz. Chemical shifts were measured from the signals of water or HDO and referenced to $SiMe_4$ assumed at −4.8 ppm from the water signal. A 20 Hz line-broadening function was applied to improve the signal-to-noise ratio. The spin–lattice relaxation times (^{1}H) T_1 of the isotropically shifted signals were deter-

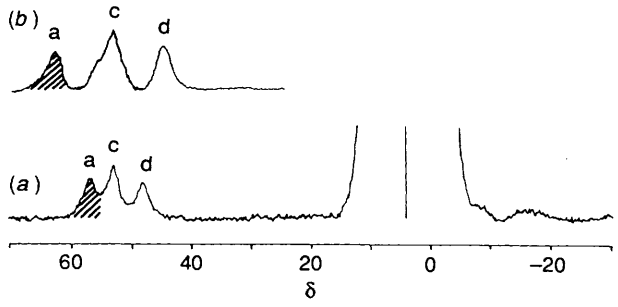
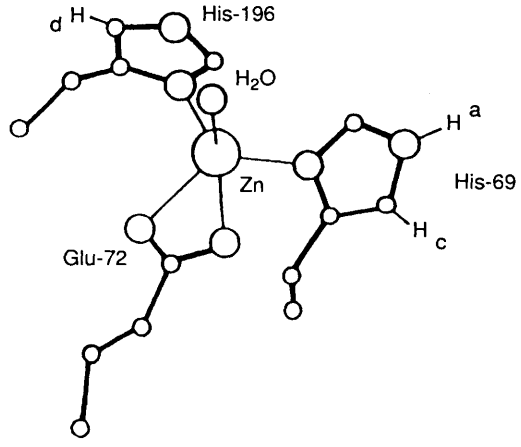


Fig. 1 The 200 MHz ^{1}H NMR spectra of 1.5 × 10^{-3} mol dm^{-3} NiCPA (*a*) and 1.5 × 10^{-3} mol dm^{-3} CoCPA (*b*) at 293 K and pH 7 (50 × 10^{-3} mol dm^{-3} hepes, 1 mol dm^{-3} NaCl)



Scheme 1

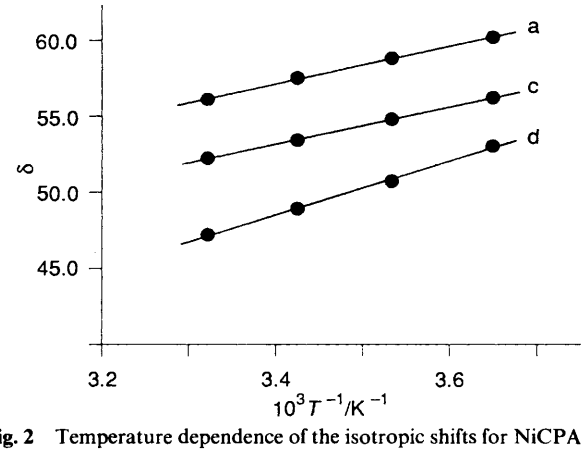


Fig. 2 Temperature dependence of the isotropic shifts for NiCPA

mined by measuring the intensity of the signals I_τ as a function of the time (τ) between subsequent pulses of the MODEFT sequence. The data were fitted using equation [23] (1) with a non-

$$I_\tau = I_0(1 - 2e^{-\tau/T_1} + e^{-2\tau/T_1}) \quad (1)$$

linear two-parameter best fitting program to obtain I_0 and T_1 values. The estimated error is about 5%.

Results

Spectral Studies of NiCPA.—The ^{1}H NMR spectrum of an aqueous solution of NiCPA, at pH 7, displays three resolved paramagnetically shifted signals at δ 57.5 (a), 53.4 (c) and 48.9 (d), as previously described [24] [Fig. 1(*a*)]. These signals are assigned to the NH(ε 2) and HC(δ 2) protons of the two co-ordinated histidines, His-69 and -196 (Scheme 1). When the spectrum of NiCPA is recorded in D_2O the signal at δ 57.5

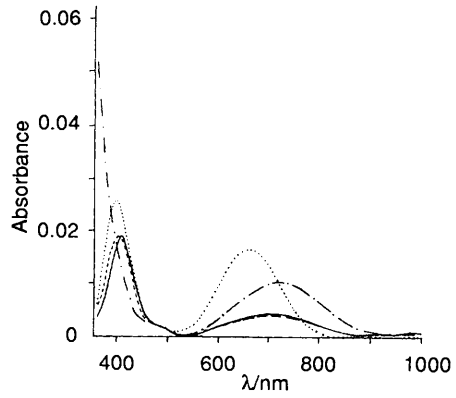


Fig. 3 Electronic spectra in the visible region of NiCPA alone (——) and the adducts NiCPA(D-Phe) (– – –), NiCPA(D-Phe)$_2$ (· · · ·) and NiCPA(D-Phe)(N_3^-) (— · —). Solution conditions are 0.8 × 10^{-3} mol dm^{-3} NiCPA, 295 K and pH 7 (50 × 10^{-3} mol dm^{-3} hepes, 1 mol dm^{-3} NaCl)

disappears completely indicating the presence of one exchangeable NH proton. This spectrum exhibits the same pattern as does the ^{1}H NMR spectrum of CoCPA [Fig. 1(*b*)]. We can assign the signals c and d to the non-exchangeable δ protons and the signal a to a NH residue.[8] The second His NH is not observed probably because it is in fast exchange with bulk water. Of the two NH groups, that of the His-196 is expected to exchange rapidly because it is weakly hydrogen bonded to a water molecule, while that of His-69 is hydrogen bonded to Asp-142.[1] The only remarkable difference between the ^{1}H NMR spectra of NiCPA and CoCPA is the larger spread of the signals in the latter. It is possible that larger magnetic anisotropy, and therefore larger dipolar shifts, are operative in the latter derivative.

We have observed that the stability of NiCPA solutions is very dependent on the temperature and enzyme concentration. In the usual range of concentrations of the ^{1}H NMR studies [*ca.* (1–2) × 10^{-3} mol dm^{-3}] nickel-substituted CPA is not stable, and the intensity of the proton signals decreases to about one half after 4 h. Despite this drawback, variable-temperature ^{1}H NMR spectra of NiCPA were recorded between 5 and 30 °C. In Fig. 2 the observed isotropic shifts of the *meta*-like protons are plotted *vs.* T^{-1}. The chemical shifts of the three proton signals decrease with increasing temperature and extrapolate to values within or close to the diamagnetic region. These results indicate that the shifts of the NiCPA derivative are largely contact in origin.

We have also recorded the ^{1}H NMR spectra of NiCPA as a function of pH. In the range pH 6–9 no change is observed in the positions of the NH(ε 2) and HC(δ 2) signals. At pH >9 the intensity of signals c and d starts to decrease and a new set of isotropically shifted signals appears under slow-exchange conditions. However, an analysis of the ^{1}H NMR spectra was not performed because the signals are very broad and the enzyme undergoes denaturation.

The electronic spectra (Fig. 3) show three main absorptions at < 10 000, 14 600 and 24 250 cm^{-1}. They had been assigned to an essentially six-co-ordinated complex mostly on the basis of the low molar absorption.[25] The *Dq* value was found to be lower than expected for two nitrogen donors. The CD spectra (Fig. 4) show absorptions at 12 800, 14 600, 21 000 and 25 000 cm^{-1}; it is possible that the band at 21 000 cm^{-1} is one component, together with that at 25 000 cm^{-1}, of the F ⟶ P transition. Such splitting would be consistent with the five-co-ordinated square-pyramidal structure found through X-ray investigation which was described as octahedral minus one ligand.[13] Also the bands at 12 800 and 14 600 cm^{-1} are indicative of a symmetry lower than cubic.

Interaction of NiCPA *with* D-Phe.—The ^{1}H NMR spectrum

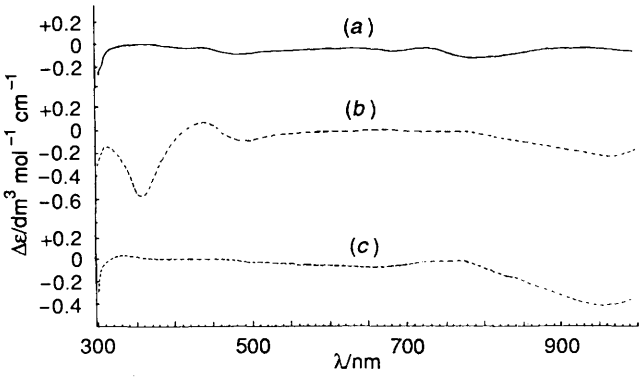


Fig. 4 Room-temperature CD spectra of 0.5 × 10^{-3} mol dm^{-3} NiCPA and its adducts: NiCPA (*a*), NiCPA(D-Phe)(N_3^-) (*b*) and NiCPA-(D-Phe)$_2$ (*c*)

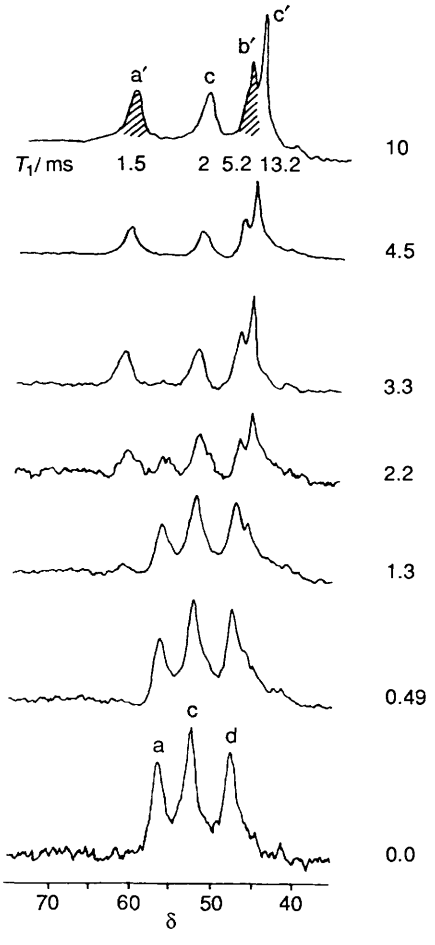


Fig. 5 Proton NMR titration of 1.7 × 10^{-3} mol dm^{-3} NiCPA with increasing concentration (mmol dm^{-3}) of D-Phe at 294 K and pH 7

of NiCPA is, seemingly, not sensitive to the addition of D-Phe up to concentrations as large as 1.0 × 10^{-3} mol dm^{-3}. For higher D-Phe concentrations a marked change occurs in the ^{1}H NMR spectrum. The variation is essentially complete at 5 × 10^{-3} mol dm^{-3} D-Phe. In particular, whereas signal c remains at the same position, a and d of NiCPA disappear progressively and new signals appear at δ 62.5 (a'), 48.4 (b'), 46.9 (c') and 17.5 (e) (Fig. 5). When the spectrum of the adduct is recorded in D_2O signals a' and b' disappear completely. An affinity constant of about 10^3 dm^3 mol^{-1} was estimated from the variation of the relative intensities of the proton signals a' and c as a function of the D-Phe concentration. Carbon-13 T_2 measurements on 10^{-3} mol dm^{-3} D-Phe ^{13}C-enriched at the

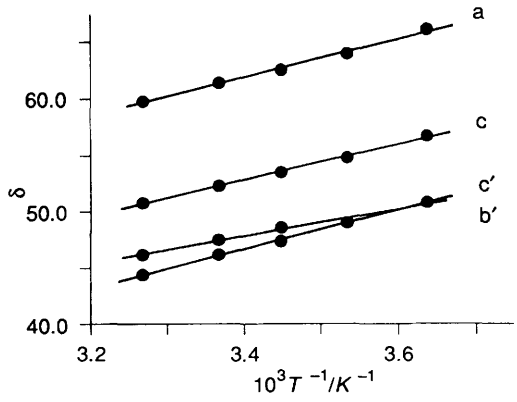


Fig. 6 Temperature dependence of the isotropic shifts for 2.7 × 10^{-3} mol dm^{-3} NiCPA(D-Phe)$_2$ at pH 7 (50 × 10^{-3} mol dm^{-3} hepes, 1 mol dm^{-3} NaCl)

carboxylate in the presence of 1 × 10^{-3} mol dm^{-3} NiCPA show a sizeable T_2 decrease with respect to the native enzyme (ΔT_2^{-1} = 79.2 s^{-1}) indicating that D-Phe interacts with the protein in the proximity of the paramagnetic metal ion. This is similar to what is observed with CoCPA although the different values of the affinity constants for the metal derivatives prevented a deeper analysis of the ^{13}C relaxation data.

Analogously to the cobalt enzyme, this spectral behaviour can be explained by assuming the successive formation of two complexes, NiCPA(D-Phe) and NiCPA(D-Phe)$_2$. The first D-Phe molecule does not affect the nickel(II) chromophore substantially and binds at a non-metallic site. The second molecule induces marked changes in the ^{1}H NMR spectrum probably due to direct binding to a metallic site. It should be noted that occupancy of the first site in the native crystal occurs upon soaking crystals in 5 × 10^{-3} mol dm^{-3} D-Phe.[26] The structures of L-Phe and its *N*-benzoyl derivative are a relevant model for the binding of two molecules to the protein.[27]

At variance with NiCPA, NiCPA(D-Phe)$_2$ is rather stable in solution. Variable-temperature ^{1}H NMR spectra of NiCPA-(D-Phe)$_2$ were recorded between 5 and 30 °C (Fig. 6). The temperature dependence of the shifts is of the Curie type indicating that they are essentially contact in origin without detectable orbital contribution. The T_1 values of the protons of co-ordinated histidines are indicated in Fig. 5. Proton NMR data for nickel(II) proteins are scarce; recently T_1 values for Cu_2Ni_2SOD (SOD = superoxide dismutase) have been reported.[28] In this derivative the nickel ion is four-co-ordinated and the T_1 values for the NH(ε 2) and HC(δ 2) protons of co-ordinated histidines are in the range 12–18 ms. In the present NiCPA(D-Phe)$_2$ case the T_1 values for the NH(ε 2) and HC(δ 2) protons are smaller (*ca.* 2 ms). This is expected for pseudo-octahedral or square-pyramidal nickel(II) systems when the ground state is orbitally non-degenerate.

The absorption spectra of these complexes in the range 340–1000 nm are shown in Fig. 3. The spectrum of NiCPA in the presence of 1 × 10^{-3} mol dm^{-3} D-Phe displays two bands at about 25 000 (ε = 24) and 14 300 cm^{-1} (ε = 7 dm^3 mol^{-1} cm^{-1}) and is practically identical to that displayed by NiCPA. The spectrum of the 1:2 complex also presents two bands, slightly shifted to shorter wavelengths, and centred at 25 250 (ε = 32) and 14 700 cm^{-1} (ε = 20 dm^3 mol^{-1} cm^{-1}). The spectral variations upon D-Phe binding at the metal are similar to those observed and analysed[15] when β-phenylpropionate is bound to nickel(II), *i.e.* an ipsochromic shift of the bands and an increase in intensity. The spectra of the latter derivative were interpreted as due to five-co-ordination.[15] This interpretation is quite reasonable and the intensity enhancement is attributed to deviation from tetragonality.

Interaction of NiCPA *with* L-Phe.—The ^{1}H NMR spectrum of NiCPA shows minor variations upon addition of L-Phe up to

4.4×10^{-3} mol dm^{-3}. At higher concentrations the original spectrum decreases in intensity, new signals appear between δ 15 and 30 and eventually all of them disappear leaving only one broad signal at about δ 30. The spectral pattern is more complex than in the case of D-Phe indicating that more than two species are formed upon increasing the L-Phe concentration. It is possible that at high concentrations the ligand binds the metal, as with D-Phe. With a large excess of L-Phe, nickel(II) remains bound to the protein as demonstrated by the dialysis of NiCPA *versus* a solution of L-Phe.

The Azide Derivatives.—A water solution of 2×10^{-3} mol dm^{-3} Ni^{II}CPA and 0.1 mol dm^{-3} N_3^- shows the same ^1H NMR and electronic spectra as those of NiCPA alone. Addition of D-Phe to this solution causes major changes in the electronic (Fig. 3) and ^1H NMR spectra. The NMR spectrum (not shown) is dominated by a broad asymmetric signal with a maximum at δ *ca.* 60 which partially disappears when the ^1H NMR spectrum is recorded in D_2O. This spectral behaviour indicates that azide interacts with NiCPA, at neutral pH, only in the presence of the amino acids in a fashion similar to that displayed by CoCPA.

Discussion

Nickel(II) carboxypeptidase interacts with the amino acid D-Phe, presumably forming complexes of stoichiometry 1:1 in analogy to the cobalt and zinc derivatives.[26] The ^1H NMR spectrum of NiCPA is only slightly modified when the 1:1 complex is formed, consistent with a non-metallic binding site for the amino acid. In addition to the 1:1 complex, D-Phe gives rise to a 1:2 complex as proposed in the case of CoCPA. When binding of a second D-Phe molecule occurs the ^1H NMR spectrum of NiCPA is dramatically changed, indicating that the amino acid binds the metal ion directly, and the ligand is in slow exchange on the NMR time-scale. The amino acid L-Phe gives rise to at least three species, two of which are probably similar to those obtained with D-Phe.

Azide does not interact with the metal in NiCPA at neutral pH, as shown by the electronic spectra. Nevertheless, in the presence of the amino acids L- or D-Phe, azide does bind the metal ion in the form of a ternary complex. Again, this is similar to CoCPA.[8-11] In the case of native zinc and in the presence of L-Phe, azide has been found by X-ray investigations to interact both with Arg-145 and the metal ion.[29]

The visible and CD spectra of all the derivatives with amino acid and azide inhibitors are also similar to each other and resemble those of NiCPA. The position of the absorption bands and their relative intensity are consistent with a pseudo-tetragonal five-co-ordinated nickel(II) stereochemistry. The enhanced intensity of the spectra of some adducts probably reflects a more distorted geometry.

The temperature dependence of the isotropic shifts can provide additional information about the co-ordination geometry of the nickel ion in NiCPA and its adducts. If the ground state is orbitally non-degenerate a T^{-1} dependence of the shifts is expected, with a near-zero intercept at infinite temperature.[30] This is indeed what has been found for NiCPA and NiCPA-(D-Phe)$_2$.

The present results may be relevant to the enzymatic mechanism. They are indicative of how the terminal amino acid interacts with the protein after cleavage of the peptidic bond. The terminal amino acid probably binds like D-Phe in CoCPA·D-Phe,[26] if allowance is made for the different configuration at the asymmetric carbon. So, Glu-270 is expected to interact with the amino group of the amino acid and the Glu-270-co-ordinated water hydrogen bond is weakened. As a result, the metal ion becomes available for anion binding. Therefore M(amino acid)$_2$ (M = Co, Ni or Zn) and the ternary complex with N_3^- are instructive models for the step following cleavage of the peptidic bond. These events occur independently of the kind of metal ion, thus justifying why the enzyme is active with Zn, Co and Ni. The different metal ions have slightly different co-ordination geometries as a compromise between the protein steric requirements and the preference of the metal ion for a given stereochemistry. The monodentate *versus* bidentate behaviour of Glu-72, which is obtained by small conformational changes of the residue, and the position of the water molecule provide some flexibility around the metal ions. This may account for the small variations in the low pK_a of the activity profile,[31] although the catalytic steps appear to be essentially the same.

Acknowledgements

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