The kinetics of acylation and deacylation of penicillin acylase from *Escherichia coli* ATCC 11105: evidence for lowered pK_a values of groups near the catalytic centre

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Penicillin G acylase catalysed the hydrolysis of 4-nitrophenyl acetate with a k_{cat} of 0.8 s^{-1} and a K_m of $10\text{ }\mu\text{M}$ at pH 7.5 and $20\text{ }^\circ\text{C}$. Results from stopped-flow experiments fitted a dissociation constant of 0.16 mM for the Michaelis complex, formation of an acetyl enzyme with a rate constant of 32 s^{-1} and a subsequent deacylation step with a rate constant of 0.81 s^{-1} . Non-linear Van't Hoff and Arrhenius plots for these parameters, measured at pH 7.5, may be partly explained by a conformational transition affecting catalytic groups, but a linear Arrhenius plot for the ratio of the rate constant for acylation relative to K_s was consistent with energy-compensation between the binding of the substrate and catalysis of the formation of the transition state. At

$20\text{ }^\circ\text{C}$, the pH-dependence of k_{cat} was similar to that of k_{cat}/K_m , indicating that formation of the acyl-enzyme did not affect the pK_a values (6.5 and 9.0) of an acidic and basic group in the active enzyme. The heats of ionization deduced from values of pK_a for k_{cat} , which measures the rate of deacylation, are consistent with α -amino and guanidinium groups whose pK_a values are decreased in a non-polar environment. It is proposed that, for catalytic activity, the α -amino group of the catalytic Ser^{B1} and the guanidinium group of Arg^{B263} are required in neutral and protonated states respectively.

Key words: pre-steady-state kinetics, stopped-flow spectrophotometry.

INTRODUCTION

Penicillin G acylase from *Escherichia coli* ATCC 11105, in common with several other acylases acting on penicillin or cephalosporin substrates, catalyses the hydrolysis of benzylpenicillin to release 6-aminopenicillanic acid and phenylacetic acid. The enzyme has long been of industrial interest in the synthesis of semi-synthetic β -lactam antibiotics but the catalytic pathway is still incompletely understood.

During post-translational membrane-translocation and protein assembly [1], there is excision of a 54-residue endopeptide and the mature enzyme contains an N-terminal A-chain, (M_r 23800) and a B-chain (M_r 62200) in which Ser²⁹⁰ occupies position B1 [2]. Chemical and structural evidence implicate this serine residue as a catalytic nucleophile. The inhibitor PMSF [3] which has a structural resemblance to a specific substrate, uniquely modified the hydroxyl group of the Ser^{B1} residue in the enzyme from *E. coli* [4] and the corresponding group in the sequence-related enzyme from *Kluyvera citrophila* [5]. This provided the basis for chemical conversion of the modified serine residue to cysteine (Ser $\rightarrow$ Cys) yielding an enzyme with no detectable catalytic activity, results that were underlined by the expression and processing of an inactive *E. coli* Ser $\rightarrow$ Cys mutant protein [6]. Crystal structures of the native protein, the non-covalent complex with the product phenylacetic acid, and the covalent PMSF complex [7] showed the O γ atom of Ser^{B1} to be appropriately placed to form a phenylacetyl-enzyme as an intermediate in the catalytic pathway. Similar folding motifs have been found in three different hydrolases with an N-terminal nucleophile other than serine [8]. This position of a putative catalytic nucleophile residue implies an unusual potential mobility which must be overcome by interactions with nearby groups in the protein.

Although these properties suggested the likelihood of an acylation step, they did not demonstrate its significance during

catalysis. Accumulation of an acyl-enzyme during hydrolysis of a phenylacetyl substrate has not yet been demonstrated but several reactive esters of aliphatic acids have been shown to be substrates of the sequence-related penicillin acylase from *K. citrophila* [9] and the rapid pre-steady-state release of 4-nitrophenol from 4-nitrophenyl acetate provides evidence for the accumulation of an acetyl-enzyme [10]. Among several substrates tested, this was the only one for which the rate constants for both acylation and deacylation could be determined.

This substrate has therefore been used to demonstrate a similar accumulation of an acetyl-derivative of the enzyme from *E. coli* and to show that the steady state value of k_{cat} was equal to the rate constant for the deacylation step. The results provide evidence for lowered pK_a values of ionizable groups in the region of the catalytic centre.

MATERIALS AND METHODS

Materials and solutions

N-(3-Carboxy-4-nitrophenyl)phenylacetamide (NIPAB) and 4-nitrophenyl esters were from Sigma-Aldrich. Acetonitrile was HPLC grade. Other reagents were of analytical grade.

Purified penicillin acylase from Boehringer Mannheim (Mannheim, Germany) was stored as a suspension in 3.2 M ammonium sulphate. The specific enzyme activity (0.4 mM NIPAB as substrate in 100 mM phosphate buffer, pH 7.5, $20\text{ }^\circ\text{C}$), determined as described by Slade et al. [4], was 16–20 kat $\cdot$ (mol of protein)^{−1}.

The 100 mM phosphate buffer, pH 7.5 contained 82.8 mM Na₂HPO₄ and 17.2 mM NaH₂PO₄; other 100 mM phosphate buffers, pH 6.6–8.0, contained a total of 100 mM phosphate (sodium salts); 50 mM Tris buffers, pH 7.2–9.0, contained the

Abbreviation used: NIPAB, *N*-(3-carboxy-4-nitrophenyl)phenylacetamide.

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required amount of HCl. Values of pH were corrected for the pH, temperature and ionic strength of the solution [11].

Substrate solutions in acetonitrile were diluted to a give a final acetonitrile concentration of 0.5% (v/v) or 5% (v/v) where this was necessary to maintain an ester in solution.

Spectrophotometric methods

A Cary model 4E spectrophotometer was used with a 10 mm light-path cuvette in a cell holder thermostatically controlled at 20 ± 0.1 °C. The protein concentration of penicillin acylase was determined from measurement of A_{280} assuming ϵ_{280} to be $1.97 \times 10^5 \text{ M}^{-1} \cdot \text{cm}^{-1}$ [12].

Kinetic methods

For the determination of K_m and k_{cat} in the steady-state, solutions (1.0 ml) contained 0.1–0.5 μM penicillin acylase and 10–120 μM 4-nitrophenyl acetate in 100 mM phosphate buffer, pH 7.5. The value of the molar absorption coefficient of the 4-nitrophenoxide anion, $1.83 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ [13], was adjusted for the effects of pH, temperature and ionic strength [11], using a value of pK_a' determined by spectrophotometric titration at each temperature. Initial velocity was determined, using linear least-squares regression, from the steady-state part of progress curves and kinetic parameters were calculated from plots of initial velocity against substrate concentration [14]. Unless otherwise stated, standard errors are shown for the indicated number of different substrate concentrations in a single experiment.

A stopped-flow spectrophotometer (Applied Photophysics model SX-17MV), with the flow-path thermostatically controlled, was used with a 10 mm light path to monitor the formation of the product 4-nitrophenol (A_{400}). Equal volumes (62.5 μl) of solutions, mixed with a dead-time of 1.8 ms, contained respectively: penicillin acylase in 100 mM phosphate buffer, pH 7.5; and 100–800 μM 4-nitrophenyl acetate, propionate, butyrate or valerate in 100 mM phosphate buffer, pH 7.5, with a concentration of acetonitrile twice that of the final mixture. The results were analysed according to relationships defining steady-state kinetic parameters (eqn. 1–5) and the burst amplitude and kinetics of approach to the steady state (eqn. 6–8) [15]:

$$k_{\text{cat}} = k_{+2}k_{+3}/(k_{+2} + k_{+3}) \quad (2)$$

$$K_m = k_{+3}(k_{-1} + k_{+2})/[(k_{+2} + k_{+3})k_{+1}] \quad (3)$$

$$K_m^{\text{acyl}} = (k_{-1} + k_{+2})/k_{+1} \quad (4)$$

$$K_s = k_{-1}/k_{+1} \quad (5)$$

$$[\text{P}_1] = At + B(1 - e^{-k't}) \quad (6)$$

$$B = [\text{E}]_0 \{k_{+2}/(k_{+2} + k_{+3})\}^2 / (1 + K_m/[\text{S}]_0)^2 \quad (7)$$

$$k' = \{(k_{+2} + k_{+3})[\text{S}]_0 + k_{+3}K_m^{\text{acyl}}\} / (K_m^{\text{acyl}} + [\text{S}]_0) \quad (8)$$

where E' , P_1 and P_2 represent the acyl-enzyme, 4-nitrophenol and the acid product respectively.

Molecular graphics

Distances between atoms, the degree of exposure of amino acid side chains to solvent, and the positions of possible hydrogen bonds were calculated using QUANTA software (Molecular Simulations, San Diego, CA, U.S.A.) and a Silicon Graphics Indigo workstation.

RESULTS AND DISCUSSION

Steady-state kinetic parameters

Steady-state parameters for four 4-nitrophenyl esters are shown in Table 1. As was observed with the enzyme from *K. citrophila* [9], there was significant catalysis of hydrolysis of each of these esters and the values of k_{cat} and K_m for 4-nitrophenyl acetate were decreased and increased respectively when the concentration of acetonitrile was increased by a factor of 10. The value of k_{cat} for 4-nitrophenyl acetate in 0.5% (v/v) acetonitrile was small compared with the values, measured under similar conditions, with derivatives of phenylacetic acid, being less than 5% of the value (20 s^{-1}) with the anilide NIPAB and less than 1% of the value (140 s^{-1}) with the ester 4-nitrophenyl phenylacetate [4]. Since the enzyme shows a marked specificity for amide and ester substrates containing the phenylacetyl or a similar group, the value of K_m , which was similar to that previously reported for other amide and ester substrates [3,16], is consistent with a value considerably less than K_s , as expected if an acyl-enzyme accumulates. Values of k_{cat} and k_{cat}/K_m were less with other esters of 4-nitrophenol, consistent with less efficient acylation by these substrates. Stereochemical factors presumably account for an absence of catalytic activity with 2,4-dinitrophenyl acetate.

Pre-steady-state kinetics

There was a biphasic time-course of release of 4-nitrophenol, illustrated in Figure 1, after mixing 4-nitrophenyl acetate and penicillin acylase under conditions such that the spontaneous rate of substrate hydrolysis was less than 1% of the enzyme-catalysed steady-state rate. Both the burst amplitude and the steady-state rate of release of product showed little dependence on substrate concentration, as expected for a range of substrate concentrations more than 3-fold greater than K_m . By contrast, the rate constant for the burst phase, k' , was strongly dependent on substrate concentration in a range much greater than K_m , consistent with $K_m^{\text{acyl}} \gg K_m$. The extrapolated value of $(k_{+2} + k_{+3})$ at substrate saturation was greater than the separately determined steady-state value of k_{cat} by a factor of approximately 50. These results imply that k_{cat} is rate-limited by k_{+3} . This was confirmed by a method of calculation independent of knowledge of the enzyme concentration [10]: the dependence of B/A on [substrate] provides an estimate of $k_{+3}(k_{+2} + k_{+3})/k_{+2}$ and in those experiments where $k_{+2} \gg k_{+3}$, this parameter is almost equal to k_{+3} . This estimate was used as a constraint in the non-linear least-squares analysis to obtain a ratio k_{+2}/k_{+3} of 38 at 20 °C. The

Table 1 Steady-state kinetic parameters with esters of aliphatic acids

The initial velocity was determined for 7 or 8 samples containing 25–700 μM ester and 0.5 or 5% (v/v) acetonitrile in 100 mM phosphate buffer, pH 7.5, at 20 °C. The value of k_{cat} for the enzyme preparations used was adjusted according to the results of active-site titration with 4-nitrophenyl acetate, i.e. 0.95 mol of active sites $\cdot (\text{mol of protein})^{-1}$. Kinetic parameters were determined using 6 or 7 different concentrations of substrate.

Ester	[Acetonitrile] (%, v/v)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\text{M}^{-1} \cdot \text{s}^{-1}$)
4-Nitrophenyl acetate	0.5	0.80 ± 0.10	10 ± 6	$(8.4 \pm 4.2) \times 10^4$
4-Nitrophenyl acetate	5	0.36 ± 0.03	65 ± 18	$(5.5 \pm 1.2) \times 10^3$
4-Nitrophenyl propionate	5	0.39 ± 0.03	318 ± 52	$(1.2 \pm 0.1) \times 10^3$
4-Nitrophenyl butyrate	5	0.15 ± 0.01	99 ± 20	$(1.5 \pm 0.2) \times 10^3$
4-Nitrophenyl valerate	5	0.27 ± 0.12	551 ± 380	$(4.9 \pm 1.3) \times 10^2$

Figure 1 Progress curves for the approach to the steady state during the penicillin acylase-catalysed hydrolysis of 4-nitrophenyl acetate

The release of 4-nitrophenol was monitored (A_{400}) at 20 °C in the presence of 1.66 μM enzyme and, for traces (1)–(7), 31, 46, 84, 213, 304, 395 and 456 μM 4-nitrophenyl acetate respectively. Solutions contained 100 mM phosphate buffer, pH 7.5. Under these conditions the value of ϵ_{400} for 4-nitrophenol was $1.31 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$.

values of $(k_{+2} + k_{+3})/K_m^{\text{acyl}}$ at 5, 20 and 43 °C, obtained by fitting the data to eqn. (8), were 0.10 ± 0.01 , 0.20 ± 0.01 and $0.51 \pm 0.07 \text{ s}^{-1} \cdot \mu\text{M}^{-1}$ respectively. These values reflect relatively inefficient catalysis which, together with the evidently large value of K_m^{acyl} and the assumption of a diffusion-limited value for k_{+1} , implies that $k_{+2} \ll k_{-1}$ and that K_m^{acyl} is therefore closely similar to K_s . Kinetic parameters, measured at 20 °C and derived from progress curves recorded at 9 different substrate concentrations, gave values for k_{+2} , k_{+3} and K_s of $31.7 \pm 4.7 \text{ s}^{-1}$, $0.81 \pm 0.03 \text{ s}^{-1}$ and $156 \pm 20 \mu\text{M}$ respectively. Values of steady-state parameters, calculated from these results using eqn. (2) and (3), were $k_{\text{cat}} = 0.79 \pm 0.03 \text{ s}^{-1}$ and $K_m = 3.9 \pm 0.6 \mu\text{M}$, values which are in reasonable agreement with those determined by steady-state measurements after manual mixing (Table 1). Eqn.(7) was used to estimate the catalytic-centre concentration of enzyme, assuming the value of k_{+2}/k_{+3} to be large: a weighted plot [17] of $1/\sqrt{B}$ against $1/[\text{substrate}]$ yielded a value of 0.78 ± 0.23 of catalytic centres relative to total protein for the data shown in Figure 1. The mean value ($\pm$ S.E.M.) for three independent experiments was 0.90 ± 0.07 . Use of this fraction to correct the apparent k_{cat} determined in a separate steady-state experiment gave a value of 0.83 s^{-1} , in good agreement with the value calculated from the pre-steady-state parameters. This procedure therefore provides a more direct measure of the content of catalytic centres than titration of the decrease in catalytic activity after site-directed covalent chemical modification by PMSF [18].

Arrhenius plots of k_{cat} , k_{+2} , and k_{+3} and the Van't Hoff plot of K_s , determined at pH 7.5 for the temperature range 3–43 °C, deviated from a straight line with a decreased slope at temperatures greater than 20 °C equivalent to decreased values of ΔH of 40.1 ± 2.3 , 44 ± 5.6 , 43.2 ± 9.0 and $33.3 \pm 4.3 \text{ kJ} \cdot \text{mol}^{-1}$ respectively. As expected from the similarity in these values, the Arrhenius plot of k_{+2}/K_s shown in Figure 2 was linear, with an apparent value of ΔH of $33.6 \pm 1.0 \text{ kJ} \cdot \text{mol}^{-1}$. These results are consistent with energy compensation between binding and the rate of formation of the acetyl-enzyme [19] and with common features in the catalytic processes determining k_{+2} and k_{+3} .

Figure 2 Effect of temperature on kinetic parameters at pH 7.5

Values of k_{cat} were determined in the steady state after manual mixing. Values of k_{+2} , k_{+3} and K_s were determined in stopped-flow experiments. Solutions contained 1.6 μM penicillin acylase in 100 mM phosphate buffer, pH 7.5. Mean values for 6 different substrate concentrations (12–50 μM) are shown. k_{cat} (■); k_{+2} (●); k_{+3} (▲); k_{+2}/K_s (○). The unit for k_{+2}/K_s was $\text{s}^{-1} \cdot \mu\text{M}^{-1}$; that for other rate constants was s^{-1} . Error bars show S.D. for 2 experiments. The lines were fitted using weighted linear regression.

Similar effects of temperature on steady-state kinetic parameters were reported for the hydrolysis of the specific substrate benzylpenicillin by the sequence-related penicillin G acylase from *K. citrophila* [20] and these results were interpreted by assuming a linear Arrhenius temperature-dependence of k_{+2} and k_{+3} ; the non-linearity was suggested to be caused by change in the relative contributions of the individual rate constants to steady-state kinetic parameters as a function of temperature. However, this explanation does not apply to the present results. Here, part of the effect may be explained by a change in the optimum pH as a function of temperature; there may also be a rapidly established equilibrium between two states of the enzyme comparable to that proposed for chymotrypsin [21], favouring a state making less effective catalytic use of binding energy at higher temperature.

pH dependence of kinetic parameters

The pH-dependence of steady-state kinetic parameters is shown in Figure 3, where the fitted lines were calculated according to eqn. (9):

$$k_{\text{obs}} = k_{\text{lim}} / (1 + [\text{H}^+]/K_1 + K_2/[\text{H}^+]) \quad (9)$$

The values of $\text{p}K_1$ and $\text{p}K_2$ for k_{cat} were 6.5 and 9.0 respectively and the corresponding $\text{p}K_a$ values from the plot of k_{cat}/K_m were 6.1 and 8.6 respectively, with values of S. E. M. ≤ 0.2 . These results are similar to the effects of pH observed with this enzyme [3] and that from *K. citrophila* [22] acting on substrates containing the phenylacetyl group. Interpreting the pH dependence of k_{cat} and k_{cat}/K_m in terms of acidic and basic groups in the catalytic centre of the acyl-enzyme and the free enzyme respectively, these results imply little change in the states of ionization of catalytic-centre groups on formation of the acetyl-enzyme. Within the range of pH 7.1–8.5, there was a stoichiometric pre-steady-state burst similar to that observed at pH 7.5, implying that $k_{+2} \gg k_{+3}$ and that k_{cat} was rate-limited by k_{+3} . Together with the observation that there was no significant pH-dependence of K_m ,

Figure 3 pH-dependence of steady-state kinetic parameters at 20 °C

For determination of k_{cat}/K_m the pseudo-first-order kinetics of hydrolysis of 4-nitrophenyl acetate were followed using 0.74 μM 4-nitrophenyl acetate and 20 nM penicillin acylase. The lines were fitted to eqn. (9): k_{cat}/K_m , $\circ$ (---); k_{cat} , $\square$ (—). Error bars show S.D. for 2 experiments.

Figure 4 Effect of temperature on pK_a values derived from the pH-dependence of k_{cat}

Values of k_{cat} were determined at 11–15 different pH values in the range 4–10. Error bars show the standard errors of the mean value obtained by weighted non-linear least squares regression.

with a value $10 \pm 4 \mu\text{M}$ (mean $\pm$ S.D. for experiments at pH 5.7, 6.3, 7.6 and 8.0, each using 7 substrate concentrations), and noting that $k_{\text{cat}}/K_m = k_{+2}/K_m^{\text{acyl}}$, these results imply a similar pH-dependence for k_{+2} and k_{+3} . This is consistent with the proposed involvement of the same acidic and basic groups in the formation and hydrolysis of the acyl-enzyme [7].

The apparent pK_a values obtained from the pH-dependence of k_{cat} and hence k_{+3} were affected by temperature (Figure 4) with heats of ionization for pK_1 and pK_2 of 47 ± 11 and $70 \pm 14 \text{ kJ} \cdot \text{mol}^{-1}$ respectively. Similar heats of ionization have been reported for pK_1 and pK_2 determined from the pH-dependence of

Figure 5 Possible hydrogen-bonds in the region of Arg^{B263}

Positions of non-hydrogen atoms are from the Brookhaven protein database entry 1pnk and the broken lines show possible positions of hydrogen-bonds. Labels identify B-chain residues and atoms potentially involved in hydrogen-bonding. Wat360 represents a structural water molecule which may be hydrogen-bonded to the α -amino group of Ser^{B1}, to the hydroxy group of the Ser^{B386} side chain and the $N\eta^2$ group of Arg^{B263}.

steady-state kinetic parameters in the hydrolysis of benzylpenicillin catalysed by the enzyme from *K. citrophila* [22] but those results were less clearly interpretable in terms of individual rate constants.

The simplest interpretation of the present results involves ionizable amino-acid side-chain groups close to the catalytic centre although indirect effects cannot be wholly excluded. Crystal structures have provided a plausible candidate for a catalytic base acting in both acylation and deacylation steps [7]. The α -amino group of Ser^{B1} is sufficiently close to the $O\gamma$ atom of the same residue to accept a proton, either directly or through a bridging water molecule, so fulfilling the necessary function of increasing the nucleophilicity of Ser^{B1}, and there are no other groups close enough to act either as a general base or as a general acid. It has therefore been proposed that the α -amino group is required to be present in the neutral base form. Considering groups expected to possess pK_a values closest to pK_1 , the observed heat of ionization for pK_1 is greater than the value of zero expected for a carboxy group and is consistent with the expected values of approximately 40 and 50 $\text{kJ} \cdot \text{mol}^{-1}$ for peptide and protein α - and ϵ -amino groups respectively and approximately 30 $\text{kJ} \cdot \text{mol}^{-1}$ for imidazolium [23]. Although the value of pK_1 is somewhat less than that expected for an α -amino group, a decreased value is plausible in a protein environment that is less polar than that of the aqueous solvent. Additionally, the ionization of a carboxylic acid, lysine or histidine side chain is unlikely to account for pK_1 because the shortest distances from the $O\gamma$ atom of Ser^{B1} to aspartic acid or glutamic acid, histidine and lysine side chains are 10, 12.5 and 14 Å respectively.

Although no other basic groups are close to the $O\gamma$ atom of Ser^{B1}, the $N\eta^1$ atom of Arg^{B263} is capable of forming a hydrogen bond with the $O\delta^1$ atom of Asn^{B241} and this residue is placed to form a hydrogen-bond with the α -amino nitrogen of Ser^{B1} [7]. Figure 5 shows possible hydrogen bonds in the region of Arg^{B263}, the $N\eta^1$ atom of which is potentially hydrogen-bonded to the main-chain carbonyl oxygen atom of Leu^{B387}. The fractional exposure to solvent of Arg^{B263} is less than 0.1 and the observed value of pK_2 , which is less than the normal value of about 12 for

an exposed arginine side chain, is consistent with the side chain being in an environment of low polarity. The heat of ionization of the arginine side chain has been reported to be $60 \text{ kJ} \cdot \text{mol}^{-1}$ [24], a value similar to that found here. The side chains of eight A-chain and nineteen B-chain arginine residues occupy an abnormal environment with an average fractional exposure to solvent of less than 0.5 but only Arg^{B263} is located nearer than 9 Å from the O γ atom of Ser^{B1}. The heat of ionization of an exposed phenolic group, $25 \text{ kJ} \cdot \text{mol}^{-1}$, is smaller than that observed for pK₂ and the nearest phenolic oxygen is more than 8.5 Å from the catalytic O γ atom. It is unlikely that either pK₁ or pK₂ reflects the ionization of the Ser^{B1} hydroxy group which, in an aqueous environment, may be expected to have a value of pK_a > 14, rather greater than that for a typical solvent-exposed guanidinium group. Furthermore, if the pK_a of the hydroxy group corresponded to pK₂, only a small fraction of the enzyme molecules would contain the reactive species at the optimum pH; the greater perturbation needed for pK_a to correspond to pK₁ would represent an unfavourable Gibbs energy greater than $40 \text{ kJ} \cdot \text{mol}^{-1}$. The presence of the protonated guanidinium group of Arg^{B263} is therefore proposed to be obligatory for catalysis, in the orientation of the hydrogen-bonding network, orienting the N-terminal catalytic serine residue and contributing to a decrease in the pK_a of the α -amino group.

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