Inactivation of penicillin acylase from *Kluyvera citrophila* by *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline: a case of time-dependent non-covalent enzyme inhibition*

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Penicillin acylase (PA) from *Kluyvera citrophila* was inhibited by *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ), a specific carboxy-group-reactive reagent. Enzyme activity progressively decreased to a residual value depending on EEDQ concentration. Neither enzymic nor non-enzymic decomposition of EEDQ is concomitant with PA inactivation. Moreover, enzyme re-activation is achieved by chromatographic removal of EEDQ, pH increase or displacement of the reagent with penicillin G. It was then concluded that PA inactivation is due to an equilibrium reaction. The kinetics of enzyme inactivation was analysed by fitting data to theoretical equations derived in accordance with this mechanism. Corrections for re-activation during the enzyme assay were a necessary introduction. The pH-dependence of the rate constant for EEDQ hydrolysis either alone or in the presence of enzyme was studied by u.v. spectroscopy. It turned out to be coincident with the pH-dependence of the forward and reverse rate constants for the inactivation process. It is suggested that previous protonation of the EEDQ molecule is required for these reactions to occur. The thermodynamic values associated with the overall reaction showed little change. Finally it is proposed that the inactivation of PA by EEDQ proceeds through a two-step reaction. The initial and rapid reversible binding is followed by a slow, time-dependent, non-covalent, reversible inactivating step. The expected behaviour in the case of enzyme modification by covalent activation of carboxy residues is also reviewed.

INTRODUCTION

Penicillin acylase (PA) hydrolyses penicillin G into 6-aminopenicillanic acid (6-APA) and phenylacetic acid. The industrial interest in this enzyme comes from the production of 6-APA, the most important intermediate compound in the production of semisynthetic penicillins. Other acylamino compounds may serve as substrates (Cole, 1969a; Plaskie et al., 1978; Margolin et al., 1980) the synthesis of amine bonds through the reverse reaction (Cole, 1969b) having been reported. Recently, other biotechnological applications have been considered, e.g. the resolution of racemic mixtures of interesting compounds such as aspartame (Fuganti and Grasselli, 1986), carnitine (Fuganti et al., 1986) and 2-furylcarbinols (Waldmann, 1989) as well as protection/deprotection reactions in sugar or amino acid chemistry (Waldmann, 1988).

Penicillin acylase from *Kluyvera citrophila* is composed of two subunits of 23 and 62 kDa that are derived from a common precursor of 93 kDa through a post-translational processing mechanism that involves the removal of signal and connecting peptides (Barbero et al., 1986). Similar subunit composition has been found in PAs from *Escherichia coli* (Schumacher et al., 1986) and *Proteus rettgeri* (Daumy et al., 1985). In fact, the comparison of the nucleotide sequences of *K. citrophila* A.T.C.C. 21285 and *E. coli* A.T.C.C. 11105 PA genes revealed 80% sequence similarity, suggesting a common ancestral origin (Barbero et al., 1986).

The elucidation of the chemical mechanism of enzyme catalysis in PA would provide valuable information not only for improving the commercial exploitation of this enzyme, but also for a better understanding of its physiological role. It is known that the enzymic hydrolysis of penicillin G proceeds through an acyl-enzyme-based mechanism (Konecny et al., 1983). Moreover, phenylmethane sulphonyl fluoride, a compound which resembles the acid moiety of penicillin G, completely abolishes enzymic activity by covalent binding in a molar ratio of 1:1 (Siewinsky et al., 1984). These results suggest the participation of a serine residue to be directly responsible for the attack to the hydrolysable amide bond (Daumy et al., 1985). However, as seen with other aminohydrolases, an increase in the nucleophilicity of serine is required for this reaction to occur.

Chemical modification is one of the most useful tools for the identification of functional groups in proteins. The participation of a histidine residue in the chemical mechanism of PA was shown fairly recently (Martín, 1990). Whether essential carboxy groups are also involved in the reaction also needs investigation.

EEDQ has been described as a specific carboxy activator for the synthesis of amide bonds, as a reagent able to inactivate proteins by covalent-bond formation (Belleau and Malek, 1968; Belleau et al., 1968), and as a slow binding inhibitor (Morrison and Walsh, 1988). The PA active site is highly hydrophobic, providing a very selective accessibility to any molecule. The reagent to be used has to be hydrophobic, EEDQ being so. Indeed, it has been shown that the binding of penicillins to PA is a function of the acid moiety of the substrate. Slight differences from phenylacetic acid usually lead to a drastic decrease in enzymic activity (Mahajan, 1984). Furthermore, an induced-fit-like mechanism has been recently proposed for PA (Martín et al., 1990).

An inactivation mechanism which proceeds through a re-

Abbreviations used: PA, penicillin acylase (penicillin amidohydrolase, penicillin amidase, EC 3.5.1.11); EEDQ, *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline; 6-APA, 6-aminopenicillanic acid.

* This manuscript is dedicated to the memory of Professor Roberto Arche.

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versible reaction at equilibrium is suggested by the results obtained from the study of the inactivation of PA by EEDQ. Remarkable information can be derived from the analysis of this reaction, although it has not been possible to confirm the involvement of a carboxy group in the chemical mechanism of catalysis.

MATERIALS AND METHODS

Penicillin G and 6-APA were supplied by Antibióticos-Farma S. A (Madrid, Spain). *p*-Dimethylaminobenzaldehyde was from Sigma (St. Louis, MO, U.S.A.). Mes and Hepes were purchased from Serva (Heidelberg, Germany). *N*-Ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) was from Fluka (Basel, Switzerland). Sephadex G-25 was from Pharmacia (Uppsala, Sweden). Other analytical grade reagents were from Merck (Darmstadt, Germany).

Protein purification

PA from *Kluyvera citrophila* was purified as previously described (Barbero et al., 1986). Enzyme activity was determined by colorimetric titration using 6-APA derivatives (Balasingham et al., 1972). The different purification fractions and the purity of the final fraction were checked on SDS/15%-(w/v)-polyacrylamide gels as described by Laemmli (1970). Gels were stained with Coomassie Blue, and protein concentration was determined by the method of Bradford (1976).

Enzyme-activity assay

The assay mixture contained 30 μg of enzyme and 1.3 μmol of penicillin G in a final volume of 0.4 ml of 0.8 M phosphate buffer, pH 7.8. The mixture was incubated at 37 °C for 5 min in a shaking water bath. The enzyme reaction was quenched and 6-APA release was quantified by the addition of 0.86 ml of 0.5% (w/v) *p*-dimethylaminobenzaldehyde/methanol solution by the method of Balasingham et al. (1972).

Preparation of buffers

Enzyme-inactivation and EEDQ-hydrolysis experiments were performed in buffer mixtures composed of 50 mM Mes/50 mM Hepes. The lack of interference of buffers in the enzyme assay was checked. The adequate pH for each buffer was achieved by the addition of NaOH. The ionic strength of all buffers was adjusted to 100 mM NaCl by using a computer program similar to that proposed by Ellis and Morrison (1982). All pH measurements were made with a Radiometer 26 pH-meter calibrated with Beckmann standard buffers at the given temperature to a maximum error of $\pm$0.03 unit.

Kinetics of inactivation by EEDQ

EEDQ in methanol at the corresponding concentration was added to a 60 μg/ml enzyme solution in 50 mM Mes/50 mM Hepes buffer with continuous stirring. The ratio of methanol to buffer was always below 0.01 (v/v). The mixture was incubated in a shaking water bath, and enzyme activity was assayed in 0.2 ml aliquots at different times.

Re-activation experiments

Initial inactivation with 1 mM EEDQ at pH 5.5 was performed as described above. When the inactivation process had reached equilibrium, 0.3 ml aliquots of the incubation mixture were withdrawn and loaded on to a gel-filtration mini-column (Sephadex G-25; 1 cm x 5 cm). The column was spun for 5 min at 4000 rev./min. in a table centrifuge at room temperature. The column had previously been equilibrated with 50 mM Mes/50 mM Hepes (pH 5.5) buffer and spun dry in the centrifuge. The eluent, about 0.3 ml, was collected. Aliquots were withdrawn, and enzyme activity was assayed in 0.1 ml samples. Control experiments showed that dilution or loss of PA in the centrifugal-chromatography step was negligible, the efficiency of EEDQ removal, as monitored by absorbance at 312 nm, being above 90%.

The re-activation processes by pH change and displacement with penicillin G were assayed as follows: 1.7 ml of PA solution at inactivation equilibrium (1 mM EEDQ, pH 5.5) were added to 1.7 ml of 13 mM penicillin G in either 0.8 M phosphate buffer, pH 7.8, or 50 mM Mes/50 mM Hepes buffer, pH 5.5. The mixtures were incubated at 37 °C, and 0.4 ml aliquots were withdrawn at different times to monitor 6-APA-release kinetics.

Kinetics of EEDQ decomposition

The time-dependent decomposition of aqueous EEDQ solutions was studied by scanning samples at different times in a DU-70 Beckman spectrophotometer. Quartz semimicro-cuvettes (1 cm pathlength) were used.

Analysis of data

Fitting of experimental data to non-linear theoretical equations was performed by the non-linear-regression facility of the statistical software package BMDP (Dixon, 1981). Parameters were expressed with their standard deviations. Parameters for linear equations were calculated by least-squares linear-regression analysis.

RESULTS

Time–progress curves for PA inactivation at pH 5.5 by different concentrations of EEDQ are shown in Figure 1. The activity decreased to a residual value that depended on EEDQ concentration. Different mechanisms of enzyme inactivation have

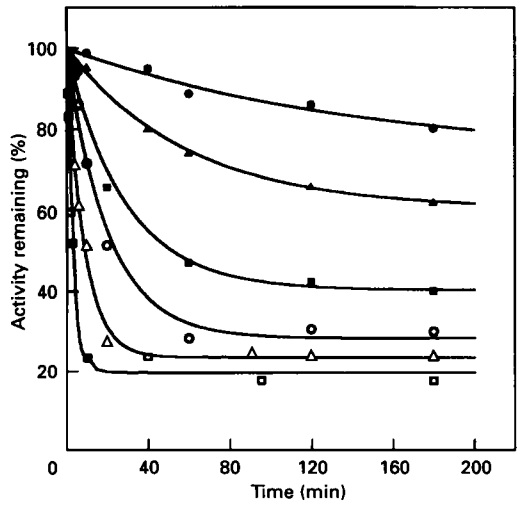


Figure 1 Time course of inactivation of PA (75 μg/ml) at different EEDQ concentrations

Reactions were performed at 20 °C in 50 mM Mes/50 mM Hepes buffer, pH 5.5. Millimolar EEDQ concentrations were 0.04 (●), 0.1 (▲), 0.2 (■), 0.4 (○), 1.0 (△) and 6.6 (□). Aliquots were withdrawn at different time intervals for enzymic assay. Data were fitted to theoretical kinetic curves based on an equilibrium mechanism.

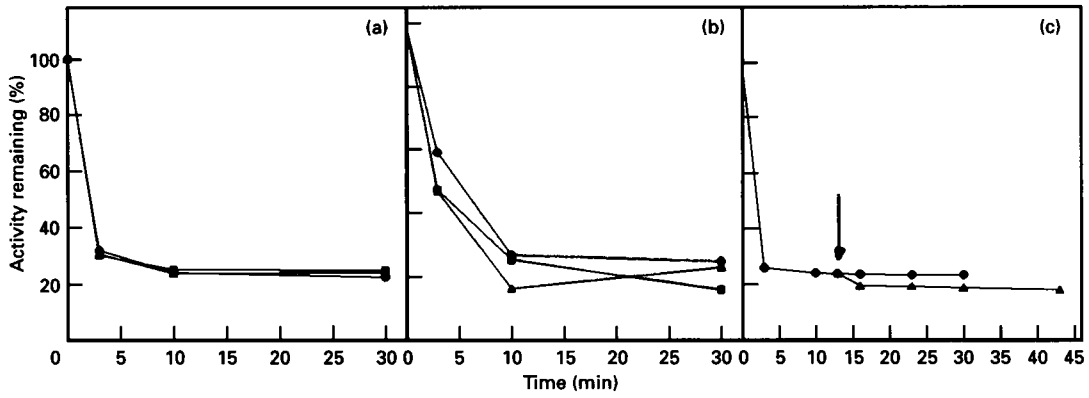


Figure 2 Effect of EEDQ hydrolysis on PA inactivation

(**a**) Enzymic hydrolysis of EEDQ. Aliquots from EEDQ (2 mM) plus PA (60 μg/ml) solution were withdrawn at 3 (●), 10 (■) and 30 (▲) min for enzymic assay. At the same time intervals 0.35 ml portions of the solution were also taken and mixed with 0.35 ml of an enzyme solution (120 μg/ml) for a new cycle of inactivation. The three inactivation cycles are depicted after correction for PA activity. (**b**) Non-enzymic hydrolysis. The experimental procedure was as described above, but without enzyme in the initial EEDQ solution. (**c**) Reinactivation cycle. Fresh EEDQ to achieve 2 mM concentration was added into an EEDQ (2 mM) plus PA (60 μg/ml) solution that had reached the residual remaining activity (arrow). The time course of inactivation for initial (●) and final (▲) solutions are performed as above.

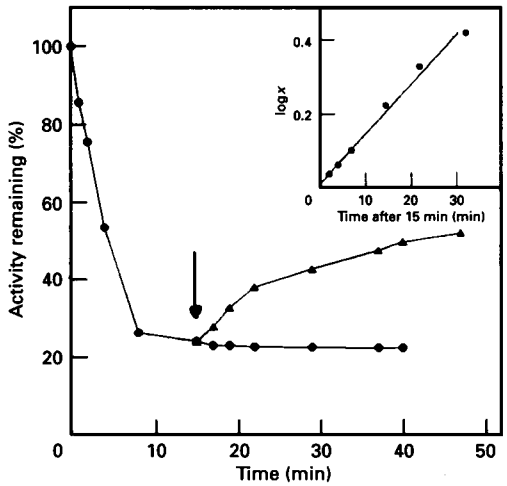


Figure 3 Re-activation of penicillin acylase by EEDQ removal

PA was inactivated by 1 mM EEDQ in 50 mM Mes/50 mM Hepes buffer, pH 5.5, at 20 °C (●). Once the residual activity was reached, aliquots were loaded on to centrifugal-chromatography columns (Sephadex G-25; 1 cm × 5 cm) to remove EEDQ (arrow). The time course of PA activity recovery (▲) was subsequently monitored by enzyme assay with penicillin G. The inset shows a semi-logarithmic plot of activity versus time after 15 min (arrow); log x is $\log[(A_{eq.}-A_0)/(A_{eq.}-A_t)]$.

been described that provide non-first-order kinetics: hydrolysis of the reagent (Pérez-Gil *et al.*, 1989); turnover on mechanism-based inhibitors (Waley, 1985); partially active modified enzyme species (Ray and Koshland, 1961); and modification reactions at equilibrium (Rakitzis, 1989).

To test the enzymic and/or non-enzymic hydrolysis of EEDQ, reaction mixtures that had reached the residual activity were mixed with a fresh solution of active enzyme for different times of preincubation. Time–progress curves (Figure 2a) were independent of the preincubation time and identical with those obtained with 'fresh' EEDQ inactivation (Figure 1). The same results were obtained when EEDQ was preincubated in the absence of enzyme (Figure 2b). Moreover, the residual PA activity value was hardly perturbed when fresh EEDQ was added (Figure 2c). These experiments indicate that neither enzymic nor non-enzymic decomposition of EEDQ takes place during PA inactivation.

If modification of enzyme were to lead to partially active forms, we would expect that extrapolation of the final slope of the inactivation kinetics could give an intercept on the ordinate axis independent of the concentration of inhibitor (Ray and Koshland, 1961). However, PA inactivation by EEDQ did not follow this behaviour.

The existence of an inactivation equilibrium implies that enzyme modification should be reversible. Re-activation of PA was achieved by removal of EEDQ and by displacement with penicillin G. A sample of PA with a residual activity of 30% due to treatment with 1 mM EEDQ was subjected to gel filtration. EEDQ concentration decreased to 70 μM after this step. Enzyme activity gradually recovered up to 70% (Figure 3) in a first-order reaction (Figure 3, inset). Forward and reverse rate constants are then comparable, and both of them should be taken into account in the formal kinetic analysis of the inactivation mechanism.

A progressive increase of enzyme activity was also observed on the addition of penicillin G (Figure 4). Release of 6-APA by a solution of inactive acylase in equilibrium with 1 mM EEDQ, pH 5.5, was measured at pH 5.5 and 7.8. Both curves became steeper with time, and the slope approached control values in the absence of EEDQ. By rearranging eqn. (5) in the Appendix, the following equation was derived:

$$\frac{[P]}{V_c} = t - \frac{[a]_0}{[A]_T} \cdot \frac{(1-e^{-kt})}{k}$$

where $V_c = k_{cat.} \cdot [A]_T$ is the enzyme reaction rate in the absence of modifying agent (all terms are fully defined in the Appendix). Although V_c is different at pH 5.5 and 7.8, plots of this equation should be coincident if both re-activation rate constants were identical. The inset in Figure 4 shows that both the pH increase from 5.5 to 7.8 and the presence of penicillin G contribute to the re-activation process. Fitting the curves to eqn. (5) in the Appendix gave the following values for the re-activation kinetic constants: 0.063 ± 0.004 min^{-1} (pH 5.5) and 0.105 ± 0.005 min^{-1} (pH 7.8). Moreover, the theoretical asymptotes calculated from the fit agreed with the experimental values of the controls in the

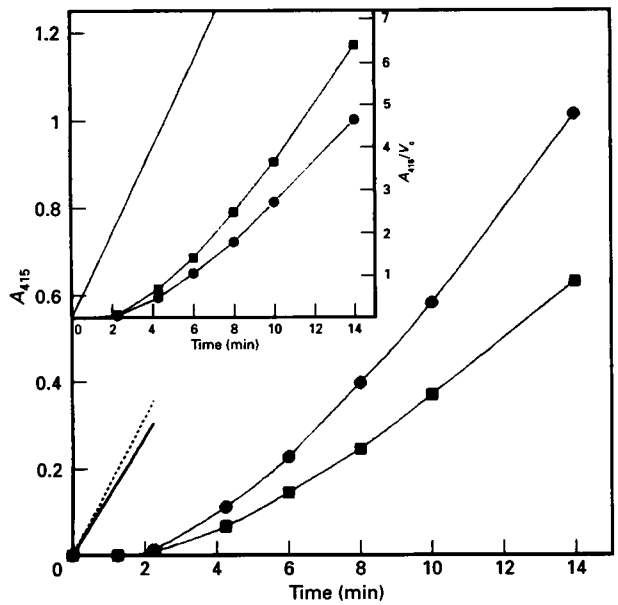


Figure 4 Re-activation of penicillin acylase by pH increase and penicillin G addition

A 1.7 ml portion of PA inactivated by 1 mM EEDQ, pH 5.5, until residual activity was added to penicillin G, either in 0.8 M phosphate buffer, pH 7.8 (●), or 50 mM Mes/50 mM Hepes, pH 5.5 (■). The resulting mixtures were incubated at 37 °C and the time course of the 6-APA release was monitored. Continuous and broken lines represent enzyme activity of controls at pH 7.8 and 5.5 respectively. The inset shows a replot of A_{415}/V_c ratio versus time, where A_{415} is the absorbance at 415 nm after titration of 6-APA release with *p*-dimethylaminobenzaldehyde and V_c is the enzyme reaction rate in the absence of EEDQ. The straight line represents the theoretical asymptote of slope 1 expected for complete re-activation.

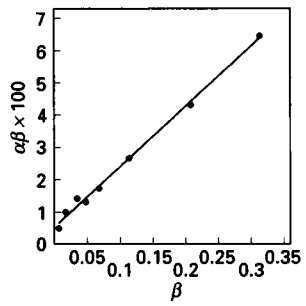


Figure 5 Determination of k_2 and C according to eqn (10) in the Appendix

α and β parameters were obtained from fitting the kinetic data of Figure 1 to eqn. (6) in the Appendix.

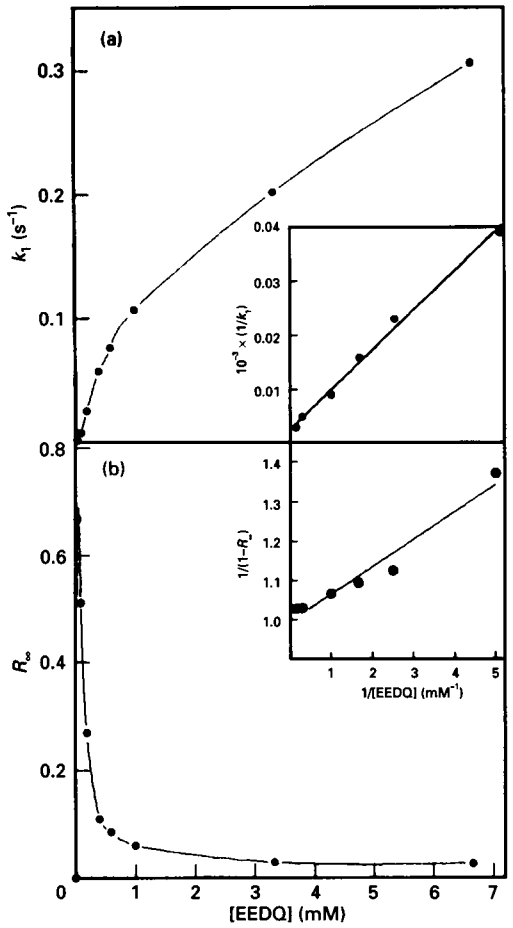


Figure 6 Dependence of (a) k_1 and (b) residual activity at equilibrium, R_∞, on EEDQ concentration

Each value of k_1 and R_∞ was estimated by fitting kinetic data of Figure 1 to eqn. (6) and subsequently using eqns. (7) and (9) in the Appendix. The insets show the (**a**) and (**b**) plots linearized according to eqns. (11) and (12) of the Appendix respectively.

absence of EEDQ. Therefore the re-activation by penicillin G leads to a 100% recovery of enzyme activity as a result of the competition between reagent and substrate for the active site of PA. The pH-dependence of re-activation suggests the presence of different states of ionization, either in the enzyme or in EEDQ, which could affect the modification reaction.

Consequently, kinetics of PA inactivation at different concentrations of EEDQ (Figure 1) were analysed according to a mechanism of enzyme modification at equilibrium. Enzyme assays had to be corrected for re-activation, as stated in the Appendix. Fitting of the experimental data to eqn. (6) in the Appendix gave the values of the α and β parameters. As predicted by eqn. (10) of the Appendix, the $\alpha \cdot \beta$-versus-β plot fitted a straight line (Figure 5). The reverse reaction rate constant, k_2, and the re-activation constant, k, were calculated from the ordinate-axis intercept. The obtained values were 7.16×10^{-3} min^{-1} for k_2 and 8.4×10^{-2} min^{-1} for k.

It is worth noting that k values derived from inactivation and penicillin G re-activation experiments are in good agreement. The dependence of the forward rate constant, k_1, and the residual remaining activity at equilibrium, R_∞, on EEDQ concentration is presented in Figure 6. The k_1 profile was hyperbolic, in accord with the formation of a saturable enzyme–EEDQ intermediate. The dissociation equilibrium constant, K_i, and the first-order rate constant, $k_{1(0)}$, were respectively 2.6 mM and 0.35 min^{-1}.

The intercept of the plot according to eqn. (12) of the Appendix is approx. 1 (Figure 6b, inset), as would be expected if $k_{1(0)}$ were much higher than k_2 (0.35 min^{-1} as against 7.16×10^{-3} min^{-1}). The value of k_2 (9.36×10^{-3} min^{-1} from the slopes) is in good agreement with the previously determined k_2 value.

The pH-dependence of the inactivation process was investigated in order to obtain information about the nature of the enzyme-modification reaction and the ionizable groups involved. Aqueous solutions of EEDQ decomposed to the corresponding quinoline derivative as deduced from comparison with the reference u.v. spectra of quinoline and quinolylium ion (DMS UV, 1966).

The pH-dependence of the rate constants k_h, k_1 and k_2 and the

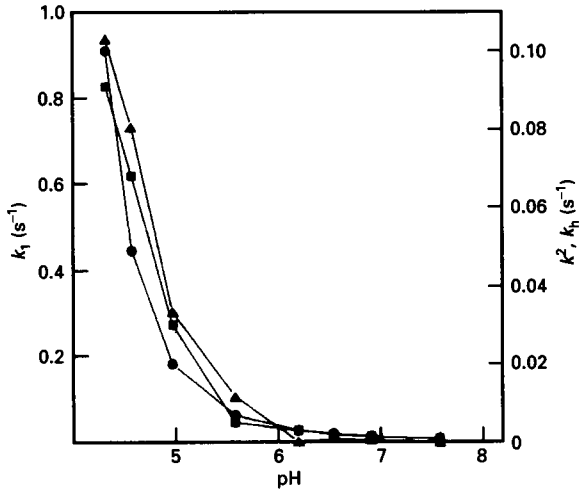


Figure 7 pH-dependence of k_h, k_1 and k_2

Hydrolysis kinetics of 0.5 mM EEDQ were examined at different pH values in 50 mM Mes/50 mM Hepes buffers at 20 °C. Inactivation kinetics were independently observed by incubation of PA (60 μg/ml) with EEDQ (0.5 mM) in the same buffer as that described above: ● k_h; ▲, k_1; ■, k_2. The EEDQ hydrolysis rate constant, k_h, was determined by fitting absorbance changes (increase at 312 nm or decrease at 262 nm) to first-order kinetics.

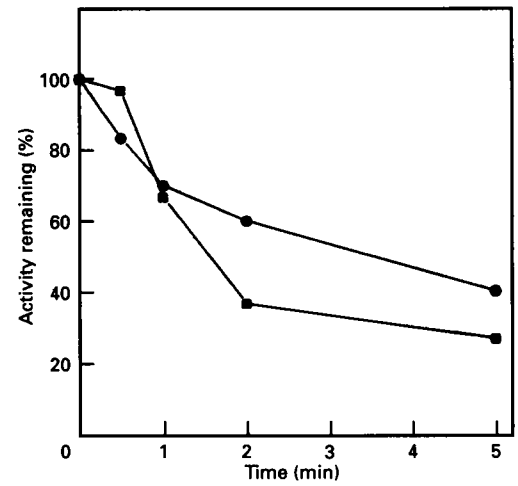
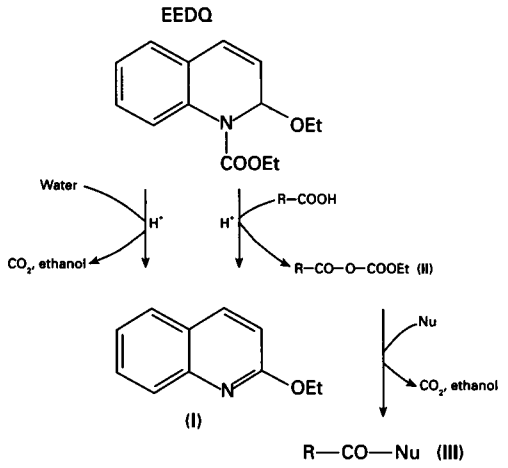


Figure 8 Comparison of PA inactivation by EEDQ and 2-ethyoxyquinoline

Hydrolysis of 0.5 mM EEDQ was performed in 50 mM Mes/50 mM Hepes buffer, pH 4.3, monitoring the reaction by spectroscopic scanning until complete. Time course of inactivation with either 2-ethoxyquinoline (●) or fresh EEDQ (■) was carried out at 0.5 mM concentration of each reagent.



Scheme 1

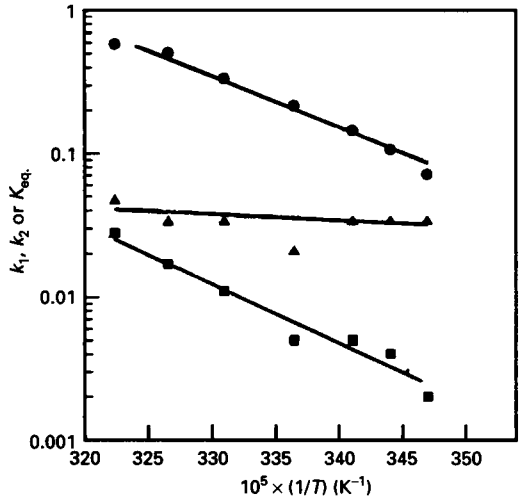


Figure 9 Arrhenius plots of k_1 and k_2 and van't Hoff plot of K_{eq}

k_1 (●) and k_2 (■) values were determined from inactivation kinetics of PA (50 μg/ml) with 0.5 mM EEDQ in 50 mM Mes/50 mM Hepes buffer, pH 5.5, at different temperatures of incubation. K_{eq} (▲) values were calculated as the k_2/k_1 ratio.

inactivation equilibrium constant $K_{eq.}(=k_2/k_1)$ are shown in Figure 7. All the rate constants showed similar profiles corresponding to an acid-catalysed mechanism in which $K_{eq.}$ is pH-independent. These results point to the same ionizable group, i.e. the nitrogen atom of EEDQ, which is essential for the three reactions. It should be pointed out that k_1 is 10-fold higher than k_h and the EEDQ hydrolysis rate constant was not increased by the presence of PA. Moreover, 2-ethoxyquinoline, the product of EEDQ hydrolysis, also inactivated PA until a residual activity similar to that reached by EEDQ (Figure 8). We therefore suggest that PA inactivation by EEDQ occurs through a reversible non-covalent reaction. This conclusion is supported by the fact that covalent modifications of carboxy groups are essentially irreversible, as is EEDQ hydrolysis (Scheme 1).

Changes in the thermodynamic parameters of the forward and reverse reactions were calculated from the temperature-dependence of k_1 and k_2 at 1 mM EEDQ, pH 5.6 (Figure 9). The activation energy, E, and the pre-exponential factor, A, were calculated from the Arrhenius plots. From these values, the activation enthalpy, $\Delta H\ddagger$, entropy, $\Delta S\ddagger$, and free energy, $\Delta G\ddagger$, were calculated as follows:

$$\Delta H\ddagger = E - RT$$

$$\Delta S\ddagger = R \cdot \ln\left(\frac{Ah}{kT}\right)$$

$$\Delta G\ddagger = \Delta H\ddagger - T \cdot \Delta S\ddagger$$

where k is the Boltzmann constant and h is the Planck constant. The values at 25 °C were as follows: $\Delta H\ddagger_1 = 69.0$ kJ·mol^{-1}; $\Delta S\ddagger_1$, -52.2 J·mol^{-1}·K^{-1}; $\Delta G\ddagger_1$, 84.4 kJ·mol^{-1}; $\Delta H\ddagger_2 =$ 77.0 kJ·mol^{-1}; $\Delta S\ddagger_2$, -53.1 J·mol^{-1}·K^{-1}; $\Delta G\ddagger_2$, 92.8 kJ·mol^{-1}.

It is remarkable that the initial and final states in the mechanism are not significantly different, although the energy barriers are

high. However, the complex-formation from two free molecules should produce an entropy decrease (Fersht, 1985). These results will be discussed below in comparison with the formation of the PA–penicillin G complex (Martín et al., 1990).

DISCUSSION

EEDQ has been reported to be a carboxy activator in amide-bond chemical synthesis (Belleau and Malek, 1968) and irreversibly inactivates proteins by covalent modification of carboxy residues (Belleau et al., 1968, 1969; Pougeois et al., 1978; Pougeois, 1983; Harb *et al.* 1986). Yet the mechanism of the reaction is unclear. Transient formation of a mixed carbonic anhydride seems to be involved (Belleau and Malek, 1968; Belleau et al., 1969). Although the ethoxy substituent at position 2 markedly affects EEDQ reactivity, other non-substituted *N*-carbethoxyquinoline derivatives also abolish protein activity in aqueous solutions (Belleau et al., 1968). This evidence points to the carbamate moiety as that responsible for EEDQ reactivity, which is dependent on general acid catalysis (see the Results section and Pougeois et al., 1978; Harb et al., 1986). The carbamate function should therefore be activated by protonation and subsequently eliminated to yield 2-ethoxyquinoline (I). Ethanol and carbonic anhydride are also released in hydrolysis of mixed carbonic anhydride (**II**) in carboxy activation (Scheme 1). The release of a quinoline derivative from EEDQ hydrolysis has been confirmed in the present study. The carboxy-activated intermediate (**II**) can be cleaved by a nucleophile to give ethanol, carbonic anhydride and the corresponding carboxyl derivative (**III**). It should be noted that the carboxy function will regenerate if the nucleophile is water. In this sense, covalent modification of proteins by EEDQ should be interpreted on the basis of cross-link formation between the activated carboxy and a sterically accessible nucleophilic residue from the protein, as other authors have also proposed (Belleau et al., 1969). In addition to EEDQ reactivity, the nucleophilic attack on the activated carboxy also depends on pH. Thereby the pK value of the nucleophile might be reflected in the modification rate constant, k_{in}. The proton-catalysed EEDQ hydrolysis is a second-order reaction (Figure 7). If the deprotonated form of nucleophile is the only reactive species, and assuming second-order for carboxy activation, the following pH-dependence of k_{in} is obtained:

$$k_{in} = k_0 \cdot K \cdot \frac{[H^+]}{K + [H^-]}$$

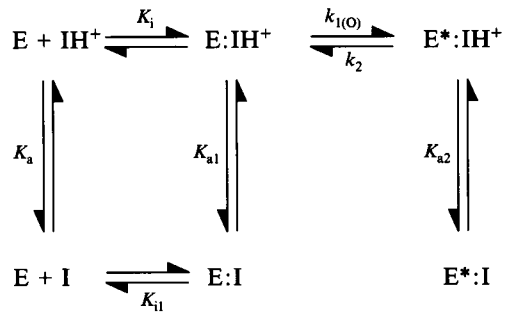
where K is the acid–base equilibrium constant of nucleophile and k_0 is the real second-order rate constant. This equation corresponds to sigmoidal titration curves with a maximum plateauing at low pH, as has been reported for 5′-nucleotidase (Harb *et al.*, 1986) and F_1-ATPase (Pougeois et al., 1978) inactivation by EEDQ. The observed pK value was attributed to the covalently modified carboxy group. The unusually high pK value for these carboxy groups in the enzyme was rationalized on the basis of a hydrophobic environment. However, this conclusion should be reviewed in terms of the equation above.

The results presented here indicate that the inactivation of PA from *Kluyvera citrophila* by EEDQ proceeds through a two-step reaction. An initial and rapid reversible binding is followed by a slow non-covalent inactivating reversible step. The analysis of the inactivation kinetics is consistent with the mechanism of reactions at equilibrium (Rakitzis, 1989). Several authors have studied the equilibrium between enzymes and modifying agents to produce covalently modified proteins, such as inactivation of dehydrogenases (Chen and Engel, 1975a,b; Ogawa and Fujioka, 1980) and ornithine transcarbamylases (Marshall and Cohen, 1977) by pyridoxal-5′-phosphate, and glyceraldehyde-3-phosphate dehydrogenase by butane-2,3-dione (Rakitzis, 1989). It is noteworthy that the reversion of a covalent modification is only possible in addition reactions (e.g. the formation of a Schiff base from pyridoxal and lysine residues). Substitution reactions might occasionally be reversible, provided that the second product of the reaction is present in high enough concentrations (Rakitzis, 1989). As covalent modification of proteins by EEDQ implies the irreversible decomposition of reagent, as established above, the equilibrium state would never be reached. Moreover, it has been proved that no loss of EEDQ is concomitant with PA inactivation.

PA re-activation by penicillin G displacement of EEDQ suggests that the inhibitor binds at the active site in a competitive-inhibition-like mechanism. A time-dependent reversible mechanism has also been described for the inhibition of acyl-CoA:cholesterol *O*-acyltransferase by CoA (Kinnunen et al., 1988). EEDQ non-covalent competitive inhibition has been reported for subtilisin, whose active site has a strong affinity for aromatic molecules (Belleau et al., 1969). It should be borne in mind that the PA active site is also highly hydrophobic, its structure being reorganized by interaction with the substrate according to an induced-fit mechanism (Mahajan, 1984; Martín et al., 1990). Slight differences from the phenylacetyl group in the acid moiety of the substrate usually lead to a drastic decrease in enzymic activity (Mahajan, 1984).

The observed association rate constants for non-covalent enzyme–ligand interactions are in the range of 10^6–10^8 $M^{-1} \cdot s^{-1}$, not very far from the diffusion-controlled encounter frequency. Much slower steps are often associated with protein isomerizations involving conformational changes (Fersht, 1985). Furthermore, the rate of inactivation by covalent modification may be occasionally limited by the rate of interconversion of two conformational states of the enzyme (Gore et al., 1973). Since PA is inactivated by non-covalent EEDQ binding, the unusual slow inactivation rate constant, $k_{1(0)}$, should be due to an induced conformational change in the protein that leads to an inactive enzyme. It is noteworthy that the $k_{1(0)}$ value (0.35 min^{-1} at 20 °C and pH 5.5) is not far from the first-order rate constant for pyridoxal-induced conformational transition in glutamate dehydrogenase (Gore et al., 1973). A conformational transition in PA could not be demonstrated by near-u.v. c.d., because the EEDQ signal disturbed the PA spectra.

The temperature-dependence of the overall forward and reverse rate constants was investigated in order to determine the thermodynamic magnitudes associated to the reaction from free enzyme to the inactive EEDQ–PA complex. It is well known that binding of two molecules to form only one leads to the loss of one set of rotational and translational entropies. This loss may sometimes be offset by an increase in the internal entropy due to new modes of internal rotation and vibration. This entropy loss is in the order of 188 $J \cdot mol^{-1} \cdot K^{-1}$ (55–60 kJ/mol at 25 °C) (Fersht, 1985). However, the entropy decrease for the formation of the inactive EEDQ–PA complex is only 0.8 $J \cdot mol^{-1} \cdot K^{-1}$, so some compensating entropy effect must exist. In this respect the role of hydrophobicity in ligand binding has been satisfactorily interpreted in terms of its contribution to entropy increases by the loss of surface accessible area (Chothia, 1974, 1975; Janin and Chothia, 1975). On this basis, an entropy increase of 188 $J \cdot mol^{-1} \cdot K^{-1}$ should correspond to a loss of surface area of about 5 nm^2. This area cannot only be accounted for by EEDQ, so a substantial part of enzyme surface must also be buried. The existence of a conformational transition is supported by the fact that EEDQ binding hardly modifies the hydrophobicity of the



Scheme 2

Key to symbols: E, active enzyme; E*, inactive enzyme; I, inhibitor; K_a, K_{a1} and K_{a2} acid equilibrium constants. K_i and K_{i1} enzyme–inhibitor association constants.

highly apolar PA active site. It should be pointed out that penicillin G binding produces an entropy decrease of about 113 $J \cdot mol^{-1} \cdot K^{-1}$ (Martín et al., 1990). This value is consistent with an entropy compensation due to the almost complete burying of penicillin G without modification of the accessible surface area of PA. In this sense it is proposed that the rate-limiting step in the EEDQ-induced inactivation mechanism is the reversible conformational change of PA subsequent to EEDQ binding. Moreover, the enzyme–substrate complex is enthalpically stabilized by 60.6 kJ/mol with respect to free enzyme (Martín et al., 1990), whereas the formation of an inactive EEDQ–PA complex decreases enthalpy to only 8.5 kJ/mol. Different interactions may therefore be involved in the stabilization of penicillin G or EEDQ at the PA active site.

Protonation of EEDQ is essential to induce both PA inactivation and reversion to an active enzyme, as deduced from pH-dependence of the rate constants of inactivation and hydrolysis (Figure 7). EEDQ acid–base equilibrium is greatly displaced to the deprotonated species over the assayed pH range. PA inactivation could then be interpreted as a proton-catalysed reaction between enzyme plus EEDQ and the inactive EEDQ–enzyme complex. A general mechanism can be formally proposed by including all of the possible EEDQ protonation and EEDQ–enzyme association equilibria (Scheme 2). However, binding of positively charged EEDQ should be less favourable than that of the deprotonated species, owing to the high hydrophobicity of PA active site. Thus EEDQ protonation might take place after the formation of the reversible initial complex. The instability of the active site due to the presence of this positive charge could be the driving force producing the conformational change that finally leads to inactive PA. It should be noted that the inactivation process is faster than EEDQ hydrolysis, and the equilibrium is reached with negligible EEDQ decomposition. On the basis of the mechanism depicted in Scheme 2, the re-activation by a pH increase is caused by modification of the EEDQ acid–base equilibrium. Since binding of penicillin G is much faster than the reversion of the inactive PA–EEDQ complex, the fact that the re-activation rate constant is significantly higher in the presence than in the absence of penicillin G should be interpreted as a result of the high affinity of the acylase for penicillin G, even in the EEDQ–PA complex.

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APPENDIX

Kinetics of enzyme modification reactions at equilibrium: correction for re-activation in the enzyme assay

The kinetic formalism followed in this paper is based on the original model of Rakitzis (1989). The general scheme of protein modification reactions at equilibrium can be presented as the reversible interconversion of three species. If an enzyme A is in rapid equilibrium with a modifying agent M to form a reversible complex which yields an inactive enzyme a through a first-order

reaction, the global process can be described by a unique equilibrium:

$$A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} a$$

The dependence of k_1 on the concentration of modifying agent is given by:

$$k_1 = k_{1(0)}[M]/(K_i + [M])$$

where $k_{1(0)}$ is equal to the value of the protein inactivation rate constant when all of the enzyme is reversibly complexed with modifying agent and K_i is the dissociation equilibrium constant of the complex to give free protein and reagent. The time-dependence of the remaining enzyme activity, R, is given by eqn. (1):

$$R = \frac{k_2}{k_1+k_2} + \frac{k_1}{k_1+k_2} e^{-(k_1+k_2)t} \quad (1)$$

This equation is deduced assuming that (i) only the species a are inactive, since the complex can be reverted by addition of substrate and (ii) the concentration of a remains constant during the enzyme activity assay.

However, the model described by Rakitzis (1989) must be corrected if a re-activation from the species a may occur during the enzyme assay, giving an apparent value of the remaining activity, $R_{app.}$. This process can be described by the first-order reaction:

$$a \xrightarrow{k} A' \quad (2)$$

where A′ represents the active enzyme species that are directly obtained in the re-activation process, i.e. free enzyme or enzyme–substrate complex. In the more general case, the rate constant k is different from k_2. Integration of the rate law of eqn. (2) give the following re-activation kinetic equation:

$$[A'] = [a]_0(1 - e^{-kt})$$

where $[a]_0$ is the concentration of the inactive species a at zero time in the enzyme assay. The velocity of product, P, release is defined by:

$$\frac{d[P]}{dt} = \sum_i k_{cat.,i}[A_i] \quad (3)$$

where A_i represents each of the active enzyme species and $k_{cat.,i}$ is its respective catalytic constant of the enzyme reaction. If $[A]_0$ is the total concentration of active enzyme at time zero and assuming the same catalytic constant values for free enzyme, A, and the re-activated enzyme, A′, eqn. (3) is transformed into:

$$\frac{d[P]}{dt} = k_{cat.}([A]_0 + [A']) = k_{cat.}\{[A]_0 + [a]_0(1 - e^{-kt})\} \quad (4)$$

The integration of eqn. (4) yields the kinetic equation:

$$[P] = k_{cat.}[A]_T t - \frac{k_{cat.}}{k}[a]_0(1 - e^{-kt}) \quad (5)$$

where $[A]_T = [A]_0 + [a]_0$ is the total concentration of A. The real, R, and apparent, $R_{app.}$, remaining activities are respectively defined as:

$$R = 1 - \frac{[a]_0}{[A]_T}$$

$$R_{app.} = \frac{[P]}{k_{cat.}[A]_T t} = 1 - \frac{(1 - e^{-kt})}{kt}\frac{[a]_0}{[A]_T}$$

The equation for $R_{app.}$ is valid whatever the mechanism of enzyme inactivation is, and it should be used whenever reversion of modification occurs. Taking into account eqn. (1) and rearranging:

$$R_{app.} = \alpha + (1 - \alpha)e^{-\beta t} \quad (6)$$

where $\alpha = 1 - Ck_1/(k_1 + k_2)$, $\beta = k_1 + k_2$, $C = (1 - e^{-kt'})/kt'$ and t' is the time of incubation for the enzymic assay. It is worth noting that eqns. (1) and (6) are formally identical; that is, the re-activation does not introduce any mathematical deviation from the predicted equations for reactions at equilibrium. Fitting the experimental data to eqn. (6) allows one to calculate the parameters α and β and hence the rate constants k_1 and k_2 and the residual remaining activity at equilibrium, $R_\infty = k_2/(k_1 + k_2)$:

$$k_1 = \frac{\beta - \alpha \cdot \beta}{C} \quad (7)$$

$$k_2 = \frac{\alpha \cdot \beta - \beta(1 - C)}{C} \quad (8)$$

$$R_\infty = \frac{\alpha + C - 1}{C} \quad (9)$$

Parameter C can be obtained from fitting the re-activation kinetics to eqn. (5) through an additional experiment. Anyway, from eqn. (8) expression (10) is derived:

$$\alpha \cdot \beta = k_2 C + \beta(1 - C) \quad (10)$$

Since k_2 and C are independent of the concentration of modifying agent, the plot of $\alpha \cdot \beta$ versus β for inactivation experiments at different concentrations of M fits a straight line, which permits one to calculate both C and k_2 values.

The dependence of k_1 and R_∞ on the concentration of M is analysed to obtain $k_{1(0)}$ and K_i. The derived theoretical equations can be linearized to:

$$\frac{1}{k_1} = \frac{1}{k_{1(0)}} + \frac{K_i}{k_{1(0)}}\frac{1}{M} \quad (11)$$

$$\frac{1}{1 - R_\infty} = 1 + \frac{k_2}{k_{1(0)}} + \frac{K_2 K_i}{k_{1(0)}}\frac{1}{[M]} \quad (12)$$

In addition to $k_{1(0)}$ and K_i, K_2 values can also be obtained from these equations.

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