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EPIDERMAL GROWTH FACTOR RECEPTOR TYROSINE KINASE

INVESTIGATION OF CATALYTIC MECHANISM, STRUCTURE-BASED SEARCHING AND DISCOVERY OF A POTENT INHIBITOR

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Abstract—Inhibition of tyrosine kinases is a possible approach for the treatment of cancer. We have investigated the catalytic mechanism of the epidermal growth factor receptor tyrosine kinase (EGF-RTK) in order to obtain information for use in structure-based searching for inhibitors. Initial rate studies imply that EGF-RTK forms a ternary complex together with ATP and peptide substrate. Investigation of pH and temperature dependence suggests that the kinase reaction requires the ionised form of a carboxylate ($pK = 6.3$) and the protonated form of another group ($pK = 9.1$). These characteristics are consistent with a mechanism where the carboxylate of Asp^{813}($pK = 6.3$) facilitates deprotonation of the tyrosyl hydroxyl of the peptide substrate, activating it as a nucleophile to attack the γ-phosphorus of ATP which interacts with a protonated enzyme side-chain ($pK = 9.1$), possibly the guanidinium group of Arg^{817}. This proposed catalytic mechanism was used to define a query when searching for inhibitors in a database of predicted three-dimensional structures. The procedure involved searching for compounds that mimic the ATP γ-phosphate, tyrosyl hydroxyl and the tyrosyl aromatic ring, all of which seem to interact strongly with the enzyme during catalysis. This search allowed identification of inhibitors of EGF-RTK which were used to define queries for two-dimensional searching of a larger database, leading to the discovery of 4-(3-chloroanilino)quinazoline (CAQ) which is a potent inhibitor ($K_i = 16$ nM) of the enzyme. The compound is believed to be the first representative from a new structural class of anilinoquinazoline tyrosine kinase inhibitors. It follows competitive kinetics with respect to ATP and noncompetitive kinetics when the peptide is varied, implying that it functions as an analogue of ATP. CAQ is a novel and potent lead in the search for tyrosine kinase inhibitors as potential agents for the treatment of cancer.

Key words: cancer; enzyme kinetics; pH-dependence; temperature-dependence; structure-based searching; anilinoquinazoline

Protein-tyrosine kinases (EC 2.7.1.112) play a key role in the regulation of cell division and differentiation [1–4]. Elevated tyrosine kinase activity is associated with a range of human neoplasms, so that inhibition of these enzymes represents a potential approach for the treatment of some types of cancer. Tyrosine kinases are involved in signal transduction. Binding of a specific growth factor to the extracellular domain of the enzyme leads to aggregation, autophosphorylation and activation of the intracellular catalytic domain. Different members of the tyrosine kinase family exhibit specificity for various phosphate acceptors. Conserved SH2 or SH3 regions are often located within regulatory regions of tyrosine kinases or their intracellular substrates. These regions associate strongly with specific peptide sequences containing phosphorylated tyrosine residues (such as autophosphorylation sites in tyrosine kinases) and modulate protein–protein interactions which are involved in signal transduction.

There is strong sequence homology within the tyrosine kinase family and a weaker similarity with protein serine kinases [5, 6]. Knowledge derived from the well-studied protein serine kinases [7, 8] has assisted investigation of the tyrosine kinases.

We now describe studies on the mechanism of EGF-RTK†, which suggest that certain substrate groups interact strongly with the enzyme during catalysis. Application of these theories in structure-based searching led to the discovery of a novel compound which is a potent tyrosine kinase inhibitor. We report kinetic characteristics of this compound.

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† Abbreviations: CAQ, 4-(3-chloroanilino) quinazoline; ΔH_{ion}, enthalpy of ionization; EGF-RTK, epidermal growth factor-receptor tyrosine kinase; K_i, inhibition constant; K_a, K_m for ATP; K_{pep}, K_m for peptide; V, rate at saturating substrate concentration.

MATERIALS AND METHODS

Materials

Reagents were purchased from Sigma Chemical Co. (Poole, U.K.), BDH (Lutterworth, U.K.), Amersham International (Amersham, U.K.) and CRB (Northwich, U.K.). The peptide substrate

(sequence R-R-L-I-E-D-A-E-Y-A-A-R-G) is one that has been used previously to study EGF-RTK [9]. The synthesis and chemical characterisation of CAQ have been described [10].

Methods

Preparation of EGF-RTK. Extracts containing the enzyme were prepared from A431 cells (derived from human vulval carcinoma) and human placenta. The amino-acid sequence of EGF-RTK from these sources is identical, but the cancer cells contain additional aberrant forms [11].

The enzyme was extracted from A431 cells as follows. The cells were grown to confluence in a Nunc "cell factory" (6000 cm^2) using Dulbecco's Modified Eagle's Medium containing 5% (v/v) fetal calf serum and 1 $\mu g/cm^3$ insulin. After washing with 10 mM sodium phosphate, pH 7.4, containing 120 mM NaCl and 2.7 mM KCl (PNK buffer), cells were detached with 400 cm^3 of PNK containing 0.05% (w/v) EDTA (30 min at 37°) and collected by centrifugation (300 *g* for 10 min). The pellet was resuspended in 20 cm^3 of 20 mM borate/NaOH, pH 10.1, containing 0.2 mM EDTA and 4 mM benzamidine (BEB buffer). All subsequent processing was at 0–4°. Cells were homogenized by passage 10 times through a 21 gauge needle and then centrifuged at 400 *g* for 10 min. The supernatant was collected and the pellet was further homogenized with 10 cm^3 of BEB. This material was then centrifuged at 400 *g* for 10 min and the two supernatants were pooled prior to centrifugation at 25,000 *g* for 30 min. The pellet was retained and 5 cm^3 of 30 mM HEPES/NaOH, pH 7.4 containing 10 mM benzamidine was added. An equal volume of the same buffer containing 2% (v/v) Triton X-100 was added prior to gentle mixing for 60 min. After centrifuging at 100,000 *g* for 60 min, the supernatant contained solubilized enzyme (protein concentration 2–10 mg/cm^3) which was stored as 50 μL aliquots in liquid nitrogen.

Human placental EGF-RTK was isolated as follows. A placenta was cut into small pieces and rinsed with ice-cold PNK. The tissue was homogenized in 500 cm^3 of 0.25 M sucrose containing 25 mM benzamidine, 5 mM EDTA, 0.1 mM phenylmethanesulphonyl fluoride and NaOH to pH 7.5. The supernatant was collected following centrifugation at 600 *g* for 5 min. The pellet was homogenized again with a further 300 cm^3 of the buffer. Following centrifugation, the two supernatants were pooled and solid NaCl was added to 0.1 M, and 0.2 M $MgCl_2$ added to 0.2 mM. This extract was centrifuged at 8000 *g* for 30 min and then the supernatant was retained after filtering through one layer of nylon gauze. Following centrifugation at 40,000 *g* for 40 min, the pellets were resuspended in 40 mM HEPES/NaOH, pH 7.4 containing 10 mM benzamidine (HB buffer) and then spun again at 40,000 *g* for 40 min. The pellet was resuspended in 25 cm^3 HB and frozen in liquid nitrogen. After thawing, the preparation was mixed with an equal volume of HB containing 4% (v/v) Triton X-100. The mixture was incubated on ice for 60 min and then centrifuged at 100,000 *g* for 60 min. The supernatant was stored in the same way as the extract from A431 cells.

Tyrosine kinase assay. The standard assay was modified from that of Pike [12] and was performed in 42 mM HEPES/NaOH, pH 7.4, containing 20 μM ATP, 0.52 mM peptide, 100 μM sodium orthovanadate, 0.05% (v/v) Triton X-100, 5.5% (v/v) glycerol, 1 mM dithiothreitol, 0.5 mM $MnCl_2$, 5 mM $MgCl_2$, 1% (v/v) dimethylsulphoxide and saturating (300 nM) epidermal growth factor. ATP and peptide concentrations were varied where stated.

The following solutions were used: "Enzyme solution" was 75 mM HEPES/NaOH, pH 7.4, containing 250 μM sodium orthovanadate (to inhibit tyrosine phosphatases and ATPases), 0.05% (v/v) Triton X-100, 6.25% (v/v) glycerol, 2.5 mM dithiothreitol, 1.25 mM $MnCl_2$, 12.5 mM $MgCl_2$ and EGF-RTK preparation (typically 5% by volume). "EGF/inhibitor solution" comprised 20 mM HEPES/NaOH, pH 7.4, 5.5% (v/v) dimethylsulphoxide (containing inhibitor where indicated), 0.05% (v/v) Triton X-100, 5% (v/v) glycerol and 2 μM epidermal growth factor. "ATP/peptide" solution was 20 mM HEPES/NaOH, pH 7.4, containing 50 μM [γ-^{32}P]-ATP (1.4 Ci/mmol), 1.3 mM peptide, 0.05% (v/v) Triton X-100 and 5% (v/v) glycerol.

First, 10 μL aliquots of "enzyme solution" were dispensed into tubes on ice prior to addition of 5 μL of "EGF/inhibitor solution" and incubation for 30 min. The mixture was warmed to 25° and 10 μL "ATP/peptide" solution added before incubation for 10 min. The reaction was stopped by addition of 40 μL 5% (w/v) trichloroacetic acid and then 5 μL 1 mg/cm^3 bovine serum albumin. Following incubation on ice for 30 min, the tubes were centrifuged (10,000 *g* for 2 min). Aliquots (40 μL) of supernatant were pipetted onto 3 × 1 cm strips of Whatman p81 phosphocellulose paper and then placed in 75 mM phosphoric acid (10 cm^3/strip). The phosphoric acid was changed four times at 5 min intervals, prior to blotting the strips dry, placing in scintillation vials with 20 cm^3 water and counting.

The concentrations of the various buffers in the pH-dependence studies were calculated to give constant ionic strength ($I = 0.03$) [13].

Protein concentration. This was measured by the method of Peterson [14].

Data analysis. Except where otherwise stated, fitting was performed by unweighted nonlinear regression using "GraFit" [15]. When two substrates, or substrate and inhibitor, were varied in the same experiment, multivariate regression was used [16, 17]. Identification of the most suitable rate equation was assisted by an *F*-test [18] which was used to compare the residual sum of squares obtained after fitting the various relationships of Cleland [19]. The best rate equation was taken as that with the most fitting parameters, none of which was redundant [20, 21]. The *F*-test estimated the probability, P, that the improvement in fit associated with an extra parameter arose due to chance. Thus, this test indicated that the term was justified by the data if P was low (<1%), but not if it was high (>20%). The following additional criteria were used to help selection of the best rate equation: reasonable parameter values and SEs, and the residual differences between observed and calculated rates following a random distribution [21, 22].

RESULTS

Substrate dependence of EGF-RTK

Initial rates were measured when varying both ATP and peptide in the same experiment. Multivariate nonlinear regression was used to fit different rate equations to these data. The best fit (Fig. 1) was obtained with the "SEQEN" equation of Cleland [19]

$$v = V[\mathrm{ATP}][\mathrm{Pep}]/(K_{ia}K_{pep} + K_a[\mathrm{Pep}] + K_{pep}[\mathrm{ATP}] + [\mathrm{ATP}][\mathrm{Pep}]) \quad (1)$$

where $V = 35 \pm 3$ fmol/sec/mg protein, $K_{ia} = 15 \pm 3\,\mu$M, $K_a = 6.2 \pm 1.4\,\mu$M and $K_{pep} = 0.15 \pm 0.03$ mM. The calculated parameter values are similar to those previously reported for EGF-RTK [1, 23, 24]. A mechanism involving reversible formation of a phosphoryl enzyme intermediate would follow the "PINGPONG" rate equation [19]. This relationship can be obtained from equation (1) by setting the value of K_{ia} to infinity, and would give parallel lines on Fig. 1. Clearly, the data are not consistent with such a mechanism, and when an F-test is used to compare the "SEQEN" and "PINGPONG" equations, the estimated value of P is >>0.1%. This result is compatible with several mechanisms where the enzyme forms a ternary complex with ATP and peptide substrate (see Discussion).

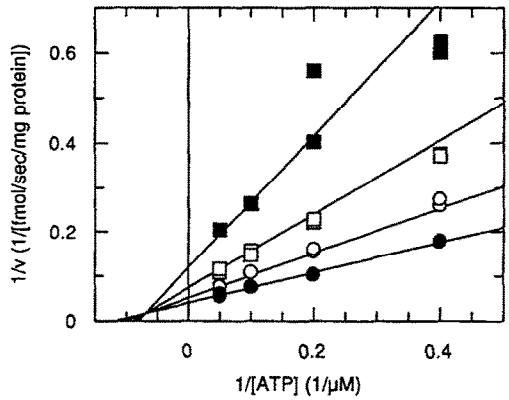

Fig. 1. Initial rate for EGF-RTK from A431 cells when ATP and peptide are varied. The concentrations of peptide substrate were as follows: ●, 0.368 mM; ○, 0.184 mM; □, 0.092 mM; ■, 0.046 mM. The best fit lines are shown. These were obtained by fitting equation (1) to the data, giving the parameter values shown in the text. Further details are given in Materials and Methods. A rate of 1 fmol/sec/mg protein corresponded to 1200 c.p.m.

pH and temperature dependence of the EGF-RTK reaction

All components of the assay were stable in the pH range 5–10. A bell-shaped profile was seen when initial rate was plotted against pH (Fig. 2), implying that activity requires deprotonation of one group and protonation of another. Measured rates were analysed by fitting of the relationship

$$\log v = \log \{c/[1 + 10^{(pK - pH)} + 10^{(pH - pK')}]\} \quad (2)$$

which was modified from the "BELL" equation of Cleland [19] where c was the pH-independent maximum rate. The best fit values are $c = 2.5 \pm 0.2$ fmol/sec/mg protein, with pK values of 6.3 ± 0.1 and 9.1 ± 0.1. The measured pK values are not necessarily in precise agreement with those of specific groups because they could be influenced by pH-dependent changes in several rate or binding constants [25]. A similar pH-dependence with pK values around 6.2 and 8.5 is seen for the serine specific enzyme, cAMP-dependent protein kinase [26].

Several different types of side-chain could be responsible for the observed pH-dependence because pK values may be perturbed by the local environment [25]. This uncertainty can sometimes be resolved by measuring the enthalpy of ionization (ΔH_{ion}) which has different values according to the type of side-chain. This parameter can be estimated by measuring the pK value at various temperatures and fitting the relationship

$$pK = (\Delta H_{ion}/2.3R)1/T) - (\Delta S_{ion}/2.3R) \quad (3)$$

where ΔS_{ion} is the entropy of ionization, R is the gas constant and T is the absolute temperature [27]. The temperature stability of EGF-RTK only allowed such characterization of the group with a pK = 6.3

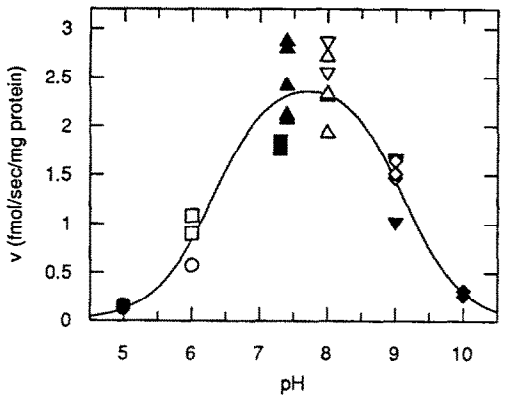

Fig. 2. pH dependence of EGF-RTK from human placenta. Assays were performed as described in Materials and Methods. The protein concentration was adjusted to give around 10,000 d.p.m. per assay. The best fit line is shown. It was obtained by fitting equation (2) to the data, giving the parameter values which are shown in the text. The concentrations of peptide and ATP were, respectively, 3.5 mM and 20 μM. The following buffers were used: ●, 37 mM succinic acid/HCl, pH 5.0; ○, 125 mM 2-(*N*-morphilino)ethanesulphonic acid/HCl, pH 6.0; □, 129 mM His/HCl, pH 6.0; ■, 230 mM imidazole/NaOH, pH 7.3; ▲, 150 mM HEPES/NaOH, pH 7.4; △, 124 mM Tris/HCl, pH 8.0; ▽, 178 mM triethanolamine hydrochloride/NaOH, pH 8.0; ▼, 158 mM diethanolamine/NaOH, pH 9.0; ◇, 135 mM 3-[(1,1-dimethyl-2-hydroxyethyl)-amino]-2-hydroxypropanesulphonic acid/NaOH, pH 9.0; ◆, 125 mM 3-(cyclohexylamino)-1-propanesulphonic acid/HCl, pH 10.0.

at 25°, where $\Delta H_{ion} = -0.2 \pm 1.4$ kcal/mol and $\Delta S_{ion} = -29 \pm 5$ cal/K/mol (Fig. 3). For a carboxyl group, the value of ΔH_{ion} is typically in the range −1.5–1.5 kcal/mol, whereas none of the other groups commonly found in proteins normally has a value below 6 kcal/mol [27]. The temperature dependence of EGR-RTK, therefore, provides evidence for the importance of a carboxylate group.

A possible catalytic mechanism for EGF-RTK

Studies on cAMP-dependent protein kinase suggest a mechanism involving general base catalysis by Asp^{166} and electrophilic catalysis by Lys^{168} [7, 8, 28–31]. The catalytic Asp residue is fully conserved in protein kinase sequences, being aligned with Asp^{813} in EGF-RTK [5, 6]. The carboxylate of Asp^{813}, therefore, is likely to be the group of pK = 6.3 in the reaction catalysed by EGF-RTK. Residues corresponding to Lys^{168} occur in all protein serine and threonine kinases and some protein tyrosine kinases. In the remaining kinases (including EGF-RTK), Lys^{168} is thought to be functionally replaced by an arginine residue which is displaced by two positions in the sequence [29, 32]. The current pH-dependence studies provide strong evidence in support of this hypothesis, with the guanidinium group of Arg^{817} being that with a pK = 9.1. The shift from pK = 8.5–9.1 may reflect functional substitution of a Lys side-chain by an Arg residue. When taken together with the evidence for a ternary complex (Fig. 1), these considerations are consistent with the following mechanism for EGF-RTK (Fig. 4).

1. General base catalysis by Asp^{813} (pK = 6.3) which accepts a proton from the phenolic hydroxyl of the substrate tyrosine residue.
2. Nucleophilic attack by the phenol or phenolate on the γ-phosphorus of ATP which changes from tetracoordinate to pentacoordinate, perhaps allowing stronger interaction with a protonated enzyme side-chain (pK = 9.1, possibly Arg^{817}). There is also electrophilic catalysis by a dicationic metal which chelates ATP.

A mechanism involving nucleophilic attack by the tyrosine hydroxyl oxygen on the γ-phosphorus of ATP has also been proposed for the insulin receptor tyrosine kinase. Here, substrate structure–activity relationships indicate that electron withdrawing substituents on the tyrosine aromatic ring cause decreases in rate which correlate with reduced nucleophilicity of the tyrosine hydroxyl oxygen [33, 34].

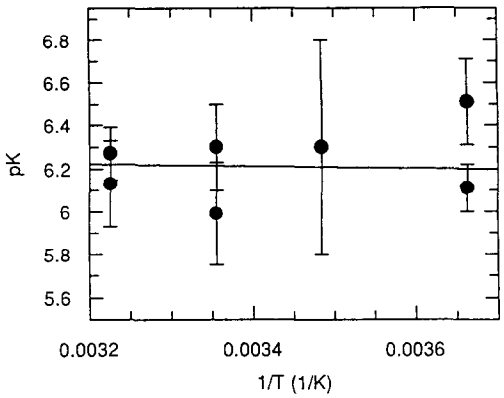

Fig. 3. Temperature-dependence of the ionization with pK = 6.3 at 25°. Experimental conditions were as described for Fig. 2. The error bars indicate the SE for each pK value. Equation (3) was fitted to the data by linear regression which was weighted according to the SEs. The best fit line is shown and the parameter values are given in the text.

It is not clear whether ionization and nucleophilic attack occur in a stepwise, or concerted, manner. The reaction is thought to follow an in-line associative mechanism because catalysis by cAMP-dependent protein kinase involves inversion of configuration for the transferred phosphate [25, 35]. Furthermore, molecular modelling of EGF-RTK is consistent with the enzyme following such a mechanism [29]. The tyrosine hydroxyl and γ-phosphate of ATP, therefore, seem to interact strongly with EGF-RTK during catalysis. We focused on groups which could mimic these interactions during structure-based searching to identify inhibitors of the enzyme.

Structure-based searching for inhibitors of EGF-RTK

The ZENECA Core Collection contains around

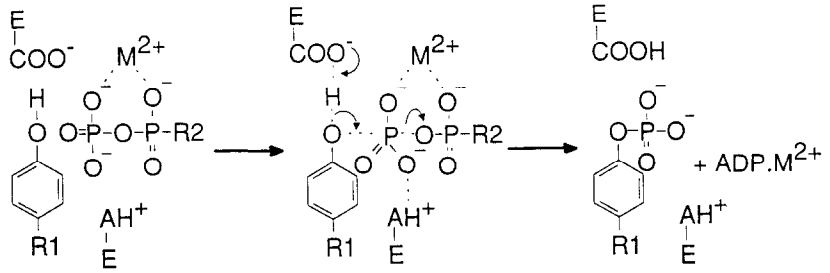

Fig. 4. A proposed catalytic mechanism for EGF-RTK. A description is given in the text. M, metal ion; R1, remainder of peptide substrate; R2, remainder of ATP substrate. Ionization of the phenol is shown as being concerted with attack on the γ-phosphate. These processes may occur in a stepwise manner. The ionising groups on the enzyme are shown to be interacting only with the reaction intermediate, but they may also interact with the substrates or products.

1500 molecules selected as representing the diversity within the larger ZENECA Company Compound Collection (around 250,000 compounds). The program "CONCORD" [36] was used to predict a single low energy conformation for each compound in the Core Collection. Next, a model for the multisubstrate complex (Fig. 4) was built using the software, "VIKING" (a program written within ZENECA Pharmaceuticals). The "ALADDIN" software [37, 38] then was used to search the ZENECA Core Collection using a query derived from the model for the multisubstrate complex as follows.

1. The centre of the tyrosyl aromatic ring [target (1)] was represented by the centre of any six-membered ring.
2. The tyrosyl phenolic oxygen [target (2)] was represented by any oxygen, or any nitrogen except those with at least three non-hydrogens attached.
3. The non-bridging oxygens of the γ-phosphate [targets (3a–c)] were represented by any oxygen, or any nitrogen that can accept a hydrogen bond.

These targets were selected on the basis of their likely importance in enzyme–substrate interactions and the level of confidence in their positions in the multisubstrate model. Target (1) was chosen because hydrophobic interactions are known to be important in molecular recognition [25]. Targets (2) and (3) were selected because the groups have different characteristics in the putative tightly bound intermediate when compared to the ground state where substrates are bound less tightly. In order to qualify as a hit during three-dimensional searching, a molecule was required to contain atoms corresponding to targets (1) and (2) together with any two out of the three targets (3a–c). The acceptable distances between the targets are given in Table 1.

The three-dimensional search query matched 152 compounds in the Core Collection, three of which gave over 50% inhibition of EGF-RTK when present at a concentration of 50 μM under standard assay conditions. Two of the three compounds contained common substructures (basic aromatic heterocycles) which were used to define queries for subsequent two-dimensional searching of the ZENECA Company Compound Collection. CAQ (Fig. 5, IC_{50} = 40 nM under standard conditions) was discovered in these searches. This compound is not in the ZENECA Core Collection. Structure-based searching, therefore, identified a potent tyrosine kinase inhibitor without the need for synthesis of new compounds.

Mechanism of inhibition by CAQ

This has been investigated by measuring EGF-RTK activity when varying the concentrations of either inhibitor and ATP, or inhibitor and peptide. Different rate equations were fitted to initial velocity data by multivariate nonlinear regression. When ATP was varied at 0.23 mM peptide, competitive kinetics (K_i = 16 ± 1 nM) were followed [Fig. 6(A)] according to the "COMP" equation [19]:

$$v = V[\mathrm{ATP}]/\{K_a(1 + [\mathrm{I}]/K_i) + [\mathrm{ATP}]\} \quad (4)$$

where V = 5.0 ± 0.1 fmol/sec/mg protein and K_a = 13.0 ± 0.9 μM. Inclusion of an additional parameter

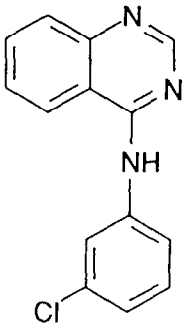

Fig. 5. Structure of CAQ.

to give mixed noncompetitive inhibition was rejected (P = 96%). When shown as Lineweaver–Burk plots [Fig. 6(A)], there is some deterioration in the quality of the fit when the rate is low because the substrate concentration is low and inhibitor concentration is high. The use of inverse rate exaggerates this effect [39]. This artefact does not occur when rate is plotted against substrate concentration, and there is an acceptable quality of fit when the data are graphed in this way. The Lineweaver–Burk plots do illustrate a tendency towards cooperative kinetics for ATP at high inhibitor concentrations (Hill coefficient 1.2 ± 0.2 at 100 nM CAQ) [see 25, 27]. This may be due to the inhibitor perturbing growth factor-induced aggregation and stimulation of EGF-RTK activity [1, 40, 41].

When peptide was varied at 20 μM ATP, inhibition followed pure noncompetitive kinetics [K_i = 32 ± 1 nM, Fig. 6(B)] according to the relationship

$$v = V[\mathrm{Pep}]/\{(K_{pep} + [\mathrm{Pep}])(1 + [\mathrm{I}]/K_i)\} \quad (5)$$

where V = 6.6 ± 0.1 fmol/sec/mg protein and K_{pep} = 0.25 ± 0.01 mM. Equation (5) was derived from the "NONCOMP" relationship of Cleland [19] by assuming that $K_{ii} = K_{is} = K_i$. (This assumption was supported by an F-test which gave a value for P = 94%.)

Table 1. Criteria for identification of hits during three-dimensional database searching

	Second target				
First target	1	2	3a	3b	3c
1		2.76	3.53	4.46	4.94
2			2.34	2.34	2.34
3a				2.81	2.81
3b					2.81

The search targets corresponded to the following features of the proposed reaction intermediate (Fig. 4). Target (1), the centre of the tyrosyl aromatic ring; target (2), the tyrosyl phenolic oxygen; targets (3a–c), non-bridging oxygens of the ATP γ-phosphate. A compound was classified as a hit if it contained the required targets within ± 1Å of the distances given. More details are given in the text.

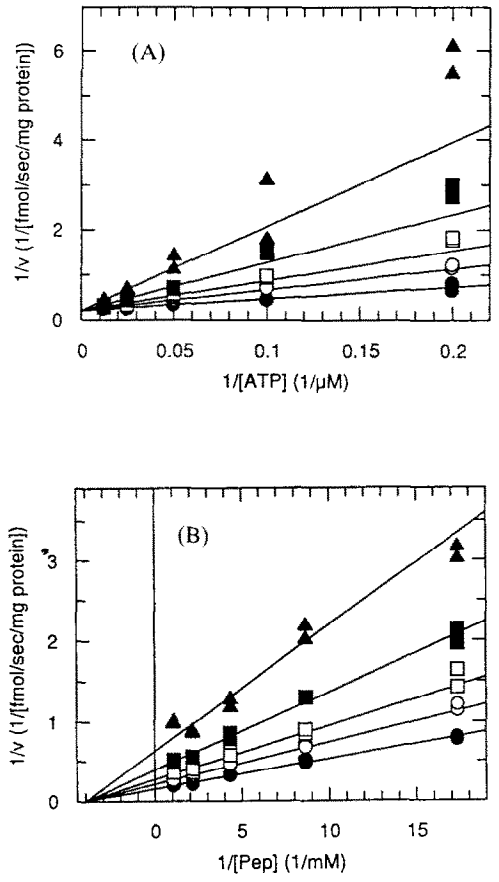

Fig. 6. Inhibition of EGF-RTK from A431 cells by CAQ. Details are given in Materials and Methods. The concentrations of CAQ were as follows: ●, 0 nM; ○, 12.5 nM; □, 25 nM; ■, 50 nM, ▲, 100 nM. The best fit lines are shown and the parameter values given in the text. (A) When ATP is varied. The best fit was obtained using equation (4). A rate of 1 fmol/sec/mg protein corresponded to 500 c.p.m. (B) When peptide is varied. Equation (5) gave the best fit. A rate of 1 fmol/sec/mg protein corresponded to 580 c.p.m.

DISCUSSION

Substrate-dependence of EGF-RTK

Identification of equation (1) as giving the best fit suggests a rapid equilibrium kinetic mechanism with formation of a ternary complex between the enzyme, ATP and peptide. Posner *et al.* [42] report that utilisation of two different peptide substrates by EGF-RTK also follows equation (1). This rate equation is obtained for three different mechanisms involving ternary complexes: where the substrates associate with the enzyme in a random order, or ATP binds first, or peptide binds first [27]. This kinetic equivalence is apparently resolved by product inhibition studies which show addition of substrates in a random order [42]. Such a mechanism is consistent with kinetics implying a ternary complex for a number of protein kinases [7, 8, 43]. Conversely, initial rate data suggesting a pingpong mechanism with only binary complexes have been reported for cAMP-dependent protein kinase [44, 45]. These proposals for a pingpong mechanism seem inconsistent with stereochemical studies which show inversion of configuration of the phosphate transferred by cAMP-dependent protein kinase, suggesting that there is no phosphoryl enzyme intermediate [35].

Mechanism of inhibition by CAQ

The proposed catalytic mechanism (Fig. 4) was used to define a query (Table 1) for structure-based searching which led to the discovery of CAQ (Fig. 5), which is a potent inhibitor of EGF-RTK. The compound is thought to be the first example from a new structural class of anilinoquinazoline tyrosine kinase inhibitors [10]. CAQ follows competitive kinetics with respect to ATP [Fig. 6(A)], indicating that it is displaced by this substrate. The observation of pure noncompetitive inhibition when the peptide is varied [Fig. 6(B)] shows that CAQ binds with similar affinity before and after association with this substrate. These results imply that either CAQ uses the ATP site, or the two ligands use different sites

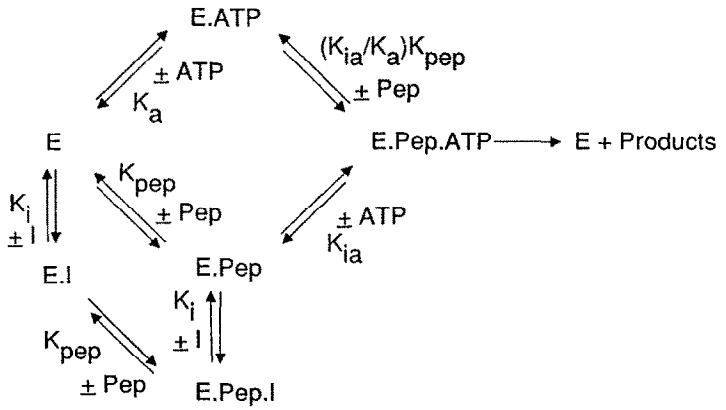

Fig. 7. A possible kinetic mechanism for EGF-RTK.

which are mutually exclusive. Both of these alternatives are consistent with the observation that CAQ and a close structural analogue of ATP, 5′-adenylyl-β,γ-imidodiphosphate, exhibit the same inhibition profile for EGF-RTK* [42]. A possible kinetic mechanism for the action of CAQ is shown in Fig. 7.

The K_i value of 16 nM calculated from equation (4) is an estimate of the dissociation constant of CAQ at zero ATP. Conversely, the K_i value of 32 nM derived from equation (5) relates to 20 μM ATP and corresponds to a dissociation constant of around 10 nM at zero ATP because CAQ is displaced by this substrate. Thus there is good agreement between the K_i value measured when ATP is varied and that calculated when peptide is varied.

Factors which may contribute to the potency of CAQ

The absolute affinity of an enzyme for a substrate or inhibitor is given by the value of the microscopic dissociation constant. Equation (1) gives two estimates of the absolute affinity for ATP, K_a and K_{ia}, which reflect association with free EGF-RTK and enzyme–peptide complex. It is not clear which dissociation constant refers to which process, but K_a is smaller in magnitude and so is an estimate of the maximum affinity for ATP. The value of K_i in equation (4) is the microscopic dissociation constant for CAQ [whereas that in equation (5) is a macroscopic constant because it varies according to the concentration of ATP]. Thus, CAQ appears to act as an ATP-analogue, but bind with a much higher affinity ($K_a/K_i = 390$). Several factors may contribute to this effect. Dissociation is favoured more for ATP than CAQ because the substrate is more polar. Unbound ATP exists predominantly as a metal chelate [46] which is more flexible than free CAQ, leading to greater entropy loss upon binding, which again disfavours formation of the enzyme–substrate complex. The greater affinity for the inhibitor could also be due to it making some interactions which are used by tightly bound intermediates, but not by ATP in the ground state.

CAQ would have been identified as a hit in 3-D database searching if it had been in the ZENECA Core Collection. It matches the search query as follows: the aromatic ring of the anilino function overlays the tyrosine aromatic ring, the anilino nitrogen corresponds to the tyrosine hydroxyl oxygen, and the quinazoline nitrogens represent oxygens of the γ-phosphate. These ideas are consistent with the observed competitive kinetics with respect to ATP. However, the noncompetitive inhibition with respect to peptide suggests, but does not prove, that CAQ does not occupy the tyrosine subsite. A definitive answer to this question requires 3-D structural data.

CAQ as a lead in the search for cancer therapeutics

The observation of competitive kinetics with respect to ATP has implications for the activity of CAQ inside cells where the concentration of ATP is typically around 2 mM. The measured K_i value of 16 nM can be used to predict an IC_{50} of 3 μM at 2 mM ATP. This estimate is close to the IC_{50} value of 1 μM which has been measured for inhibition of EGF-stimulated growth in two cell-lines: KB (human oral carcinoma) and normal rat kidney*. This may be a chance correlation because different factors influence potency in the EGF-RTK activity assay and the cell assay. However, these results do suggest a link between inhibition of EGF-RTK and inhibition of EGF-stimulated cell-growth. This idea is supported by the observation that similar concentrations of CAQ are required to inhibit EGF-dependent phosphorylation of proteins in KB cells*. In spite of apparently functioning as an ATP-analogue, CAQ exhibits selectivity in that a 100 μM concentration of the compound leads to only 6% inhibition of protein kinase C*. CAQ, therefore, is a novel, potent and selective tyrosine kinase inhibitor which is being exploited as a lead in the search for agents to treat cancer [10].

* Cook PN, Green LR, Newboult E, Curry BJ, Cartlidge SA, Woodburn JR and Ward WHJ, unpublished data.

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