Effects of Sulfhydryl Modification Reagents on the Kinase Activity of the Epidermal Growth Factor Receptor†

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ABSTRACT: Earlier reports have indicated that epidermal growth factor (EGF) receptor autophosphorylation, thought to be a key step in receptor transmembrane signaling, can be inactivated with the relatively sulfhydryl-specific reagent *N*-ethylmaleimide (NEM); however, no Cys residue has been implicated in the catalytic mechanism of the kinase. In an effort to address the mechanism of inhibition by NEM, we have investigated effects of several sulfhydryl-modifying reagents on EGF receptor autophosphorylation and on the kinase activity of the receptor toward an exogenous peptide substrate. Kinase activity is relatively insensitive to iodoacetic acid (IAAcid) and iodoacetamide (IAAmide), though IAAmide-treated receptor displays a higher $K_{m(app)}$ with respect to ATP, relative to untreated receptor. In contrast, even low concentrations of the very specific sulfhydryl reagent *p*-chloromercuribenzoic acid (PCMB) inactivate the receptor kinase. Pretreatment of the receptor with IAAmide, but not IAAcid, provides substantial protection of the kinase from subsequent treatment with NEM and a degree of protection from subsequent treatment with PCMB. Further, inactivation by NEM, and to a lesser extent PCMB, is inhibited by coincubation of the receptor with the hydrolysis-resistant ATP analog AMP-PNP. The protective effect of IAAmide from inactivation by NEM is also lost when AMP-PNP is present during the IAAmide treatment. Pretreatment of receptor with IAAcid has no effect on subsequent modification by IAAmide. These results, taken together, suggest that NEM, PCMB, and IAAmide, but not IAAcid, modify a Cys residue of the EGF receptor kinase that is inaccessible when nucleotide is bound. Modification of this residue by a bulky reagent (NEM, PCMB) inactivates the kinase by a steric mechanism, while modification with the smaller reagent (IAAmide) results in an active enzyme with altered affinity for ATP. Further, PCMB appears to react with an additional Cys residue (or residues), also resulting in steric inactivation.

The binding of epidermal growth factor (EGF)[1] to its plasma membrane receptor initiates a chain of events leading to mitosis (Staros *et al.*, 1985; Carpenter & Cohen, 1990; Carpenter & Wahl, 1990). One early event in intracellular signaling is the activation of a protein kinase (Carpenter *et al.*, 1978) specific for tyrosyl residues (Ushiro & Cohen, 1980) that is intrinsic to the receptor (Buhrow *et al.*, 1982, 1983; Ullrich *et al.*, 1984), with resulting autophosphorylation of the EGF receptor (Buhrow *et al.*, 1982, 1983; Cohen *et al.*, 1980, 1982a) and phosphorylation of cellular substrates (Hunter & Cooper, 1981). Previous studies have demonstrated that receptor autophosphorylation is inactivated by treatment with the relatively sulfhydryl-specific reagent *N*-ethylmaleimide (NEM), and that treatment by NEM blocks the reaction of the nucleotide analog [(*p*-fluorosulfonyl)-benzoyl]-5′-adenosine (5′-FSBA) with the active site of the kinase (Buhrow *et al.*, 1982). These results taken together suggest that NEM exerts its inhibitory effect by reaction with a residue within the ATP binding site or one that is allosterically coupled to the nucleotide binding site.

Subsequent work identified Lys721, a residue highly conserved in protein kinases (Hanks *et al.*, 1988), as the site modified by 5′-FSBA (Russo *et al.*, 1985). Because of the extensive homology among protein kinases (Hanks *et al.*, 1988), cAMP-dependent protein kinase (cAPK), the first protein kinase the structure of which was solved to high resolution (Knighton *et al.*, 1991a,b; 1991b; Zheng *et al.*, 1993), has served as a model for the superfamily of protein kinases, as well as for the serine/threonine protein kinase family of which it is a member (Taylor *et al.*, 1993). In the ternary complex of cAPK with bound Mg^{2+}-ATP and inhibitor peptide, Lys72, which is homologous with Lys721 of the EGF receptor, interacts with the α- and β-phosphates of bound ATP (Zheng *et al.*, 1993). The identification of Lys721 as an important residue in ATP binding, together with the observation that maleimides can react with the ε-amino group of Lys (Brewer & Riehm, 1967; Knight & Offer, 1978), suggested the possibility that NEM exerts its affect by reacting with Lys721.

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[1] Abbreviations: AMP-PNP, 5′-adenylyl imidodiphosphate; cAPK, cAMP-dependent protein kinase; cpm, counts per minute; DMF, *N*,*N*-dimethyl formamide; EGF, epidermal growth factor; 5′-FSBA, [(*p*-fluorosulfonyl)benzoyl-5′-adenosine; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; IAAcid, iodoacetic acid; IAAmide, iodoacetamide; mEGF, murine EGF; $NaDodSO_4$, sodium dodecyl sulfate; NEM, *N*-ethylmaleimide; PCMB, *p*-chloromercuribenzoic acid; TCA, trichloroacetic acid.

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In contrast to the results obtained with NEM, EGF receptor autophosphorylation has been observed not to be inhibited by treatment with another relatively sulfhydryl-specific reagent, iodoacetamide (IAAmide) (Palaszewski *et al.*, 1990; Clark & Konstantopoulos, 1993). An interaction between the site modified by NEM and that modified by IAAmide has been suggested by the observation that treatment with IAAmide results in protection against subsequent treatment with NEM (Palaszewski *et al.*, 1990; Clark & Konstantopoulos, 1993).

We investigate here the effects of NEM and other sulfhydryl-specific reagents on receptor autophosphorylation and on the phosphorylation of an exogenous peptide substrate. The results of our chemical modifications show that reaction with Lys721 is *not* the mechanism of inactivation by NEM; rather these results imply the existence of two and perhaps more Cys residues in the EGF receptor kinase which lead to kinase inactivation when appropriately modified. At least one of these groups is protected from reaction by the hydrolysis-resistant ATP analog AMP-PNP. We suggest that relatively bulky reagents inactivate the kinase by a steric inhibition of nucleotide binding, either by directly occluding the nucleotide binding site or by locking the kinase in an inactive conformation. In contrast, reaction of a relatively small reagent at the modification-sensitive Cys residue results in an active kinase but one with altered affinity for ATP and with decreased susceptibility toward the larger, inactivating sulfhydryl-specific reagents.

EXPERIMENTAL PROCEDURES

Materials. Wild-type mEGF was prepared as described previously (Savage & Cohen, 1972). Shed membrane vesicles from A431 cells, which overexpress the EGF receptor, were prepared by a modification (Rousseau *et al.*, 1995) of the method of Cohen *et al.* (1982b). NEM, PCMB, IAAcid, IAAmide, and DMF were from Aldrich. [γ-^{32}P]-ATP (3×10^3 Ci/mmol) was from DuPont−New England Nuclear. Acrylamide, *N*,*N*′-methylenebis(acrylamide), and other reagents used in the preparation of $NaDodSO_4$ gels were from Bio-Rad. Angiotensin II and all other reagents were purchased from Sigma and were reagent grade or better. Water was purified by filtration using the Milli-Q system (Millipore).

EGF Receptor Modification by Sulfhydryl-Modifying Reagents. In preparation for autophosphorylation assays, A431 membrane vesicles (2 μL/reaction) were incubated on ice with various concentrations of sulfhydryl-modifying reagents in 100 mM HEPES, pH 7.4, with 2.5% DMF in a final reaction volume of 40 μL, unless otherwise noted below. After 10 min, ice-cold 20 mM HEPES, pH 7.4 (400 μL), was added, vesicles were pelleted by centrifugation at 14000*g* for 5 min at 4 °C, and supernatants were discarded. After being washed with an additional 400 μL of 20 mM HEPES, pH 7.4, vesicles were resuspended in 40 μL of 20 mM HEPES, pH 7.4. The suspension was incubated on ice for 10 min with the addition of 10 μL of 5 μM mEGF (final concentration 1 μM) to stimulate receptor kinase activity for subsequent assay.

In experiments involving preincubations with AMP-PNP, $MgCl_2$, and $MnCl_2$, vesicles were incubated in ice-cold solutions containing the concentrations of these reagents indicated in the figure legends for 10 min before the addition of sulfhydryl-modifying reagents as described above.

In experiments in which treatment of vesicles with one sulfhydryl-modifying reagent was followed by treatment with a second such reagent, vesicles were washed (as described above) after the first treatment, followed by resuspension in 30 μL of 50 mM HEPES, pH 7.4, before addition of 10 μL of a solution of the second sulfhydryl-modifying reagent in 10% DMF. In such cases, the second reaction was allowed to proceed for 5 min on ice, unless otherwise indicated, followed by a 5 min mEGF stimulation on ice prior to receptor kinase assays.

In experiments in which treatment of vesicles with one sulfhydryl-modifying reagent was followed by treatment with a second, and then third, such reagent, vesicles were washed (as described above) after the first treatment, and then resuspended in 25 μL of 50 mM HEPES, pH 7.4, before addition of 5 μL of a solution containing the second reagent in 10% DMF. The second reaction was allowed to proceed for 10 min on ice, followed by addition of 10 μL of a solution containing the third reagent. This incubation and the subsequent mEGF stimulation were each carried out for 5 min on ice. Modification reactions were followed by receptor autophosphorylation assays as described below.

In preparation for assays of angiotensin II phosphorylation, a 20-fold dilution of membrane vesicles in 25 mM HEPES, pH 7.4, was incubated with sulfhydryl-modifying reagents for 10 min on ice before vesicles were washed twice with a 25× volume (relative to the original vesicle volume) of ice-cold 20 mM HEPES, pH 7.4. Pelleted vesicles were then solubilized by dilution in a 10× volume of 50 mM HEPES, pH 7.4, 1% Triton X-100, 10% glycerol, 0.1 mM phenylmethanesulfonyl fluoride, 10 μg/mL aprotinin, and 10 μg/mL leupeptin, vortexed 1 min, and centrifuged at 14,000*g* for 10 min at 4 °C to pellet insoluble material.

EGF Receptor Autophosphorylation Assays. Following sulfhydryl modification and mEGF stimulation, phosphorylation assays were initiated by the addition of 10 μL of 6× phosphorylation mix to vesicle suspensions to yield final assay concentrations of 50 mM HEPES, pH 7.4, 5 mM $MgCl_2$, 1 mM $MnCl_2$, 10 mM Na_3VO_4, and 20 μM ATP with 0.5 μCi of [γ-^{32}P]ATP/reaction. Assays (total volume 60 μL) were quenched after 4 min by the addition of 20 μL of 4× electrophoresis sample buffer (final concentrations 62.5 mM Tris, pH 6.8, 2% $NaDodSO_4$, 10% glycerol, 50 mM dithiothreitol, and 0.001% bromphenol blue) and by heating at 100 °C for 5 min. Electrophoresis was performed by a modification of the method of Laemmli (1970), followed by autoradiography to assess the degree of EGF receptor autophosphorylation.

EGF Receptor Kinase Assays with Exogenous Substrate. Following sulfhydryl modification and receptor solubilization, 10 μL aliquots of solubilized supernatant were incubated with mEGF at a final concentration of 1.7 μM for 10 min at room temperature. Aliquots were then cooled for 10 min on ice before addition of angiotensin II and phosphorylation mix to yield assay concentrations of 50 mM HEPES, pH 7.4, 5 mM $MgCl_2$, 1 mM $MnCl_2$, 10 mM Na_3VO_4, 1.25 mM angiotensin II, and various concentrations of [γ-^{32}P]ATP (final specific activity 1250 Ci/mol) in a total assay volume of 25 μL. Kinase assays proceeded for 8 min on ice, during which time substrate phosphorylation was observed to be

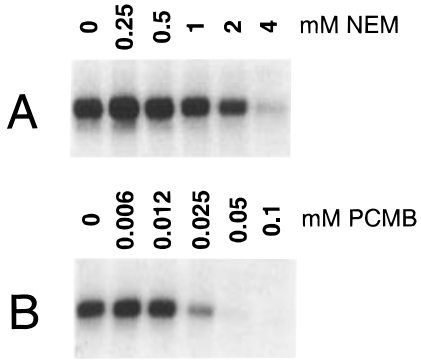

FIGURE 1: Effects of NEM and PCMB on EGF receptor autophosphorylation. Shed A431 membrane vesicles containing EGF receptor were incubated with the indicated concentrations of NEM (A) or PCMB (B) and washed, and residual receptor autophosphorylation activity was measured. The EGF receptor migrates as the most prominent band on the autoradiograph of this and subsequent figures.

linear (data not shown). Assays were quenched with 15 μL of ice-cold 13.3% trichloroacetic acid. To aid in visualizing precipitated protein, 5 μL of 5% bovine serum albumin was added to each tube. After precipitation for 10 min on ice, the samples were subjected to centrifugation at 14000*g* for 10 min at 4 °C, and 20 μL of peptide-containing supernatant was spotted onto $1^1/_4$ in. × $^5/_8$ in. Whatman phosphocellulose papers. Papers were washed 4 times in 0.80 L of 75 mM phosphoric acid (6 min/wash) before counting in 10 mL of Ecolite(+) liquid scintillation fluid using a Beckman LS7500 scintillation counter.

RESULTS

To investigate the mechanism by which NEM inhibits the EGF receptor kinase, receptor present in shed membrane vesicles from A431 cells was incubated with various sulfhydryl-specific reagents, and the kinase activity of modified receptor was assayed by receptor autophosphorylation or by phosphorylation of an exogenous peptide substrate. Figure 1 shows the decrease in receptor autophosphorylation activity after incubation for 10 min on ice in the presence of increasing concentrations of either NEM (Figure 1A) or PCMB (Figure 1B). Though maleimides are relatively sulfhydryl-specific, they are also known to react with lysyl ϵ-amino groups (Brewer & Riehm, 1967; Knight & Offer, 1978); however, the high specificity of PCMB for thiol groups (Webb, 1966) argues that the reduction in activity seen with at least this reagent is attributable to modification of one or more receptor sulfhydryl groups.

We also tested the effects on autophosphorylation activity of the receptor of two other relatively sulfhydryl-specific reagents, IAAmide and IAAcid. In contrast to the sensitivity of the autophosphorylation activity to NEM and PCMB, the data in Figure 2 demonstrate the relative lack of sensitivity of receptor autophosphorylation activity to the less bulky reagents, IAAmide (Figure 2A) and IAAcid (Figure 2B).

Preincubation of receptor-containing vesicles with the hydrolysis-resistant ATP analog AMP-PNP protects the autophosphorylation activity against inactivation by NEM (Figure 3A). A smaller but significant protective effect was also observed when vesicles were incubated with AMP-PNP before treatment with PCMB (data not shown). This is consistent with modification of sulfhydryl groups by NEM and PCMB at a site or sites within the ATP binding site, or

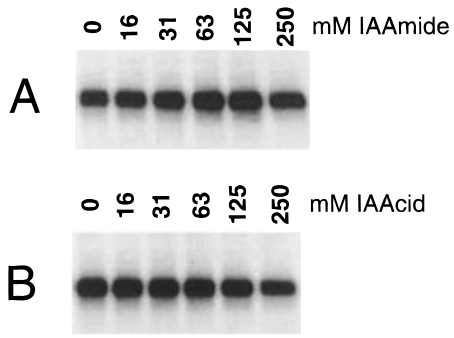

FIGURE 2: Effects of IAAmide and IAAcid on EGF receptor autophosphorylation. Vesicles were incubated with the indicated concentrations of IAAmide (A) or IAAcid (B) and washed, and the residual receptor autophosphorylation activity was measured.

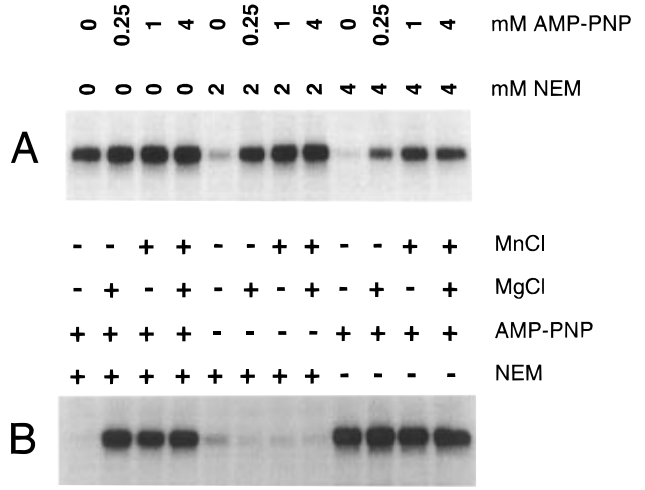

FIGURE 3: Effects of preincubation of the EGF receptor with AMP-PNP on receptor sensitivity to NEM. (A) Vesicles were preincubated with 5 mM $MgCl_2$, 1 mM $MnCl_2$, and the indicated concentrations of the hydrolysis-resistant ATP analog AMP-PNP before treatment with the indicated concentrations of NEM and assay of receptor autophosphorylation activity. (B) Preincubation of vesicles was carried out with 4 mM AMP-PNP in the absence or presence of 5 mM $MgCl_2$ and/or 1 mM $MnCl_2$ before treatment with 4 mM NEM. Lanes 1−4 demonstrate that the protective effect of AMP-PNP depends on the presence of either divalent cation. Remaining control lanes demonstrate that AMP-PNP is required for the protective effect (lanes 5−8) and that pretreatment of vesicles with these reagents has no effect on kinase activity in the absence of NEM (lanes 9−12).

at a site or sites that are allosterically coupled to the nucleotide binding site. Effective protection by AMP-PNP requires the presence of Mg^{2+} or Mn^{2+} (Figure 3B). Mg^{2+} and Mn^{2+} were effective at concentrations similar to those required for receptor autophosphorylation, suggesting that the conformation assumed by AMP-PNP in protection assays is similar to that of ATP in its role as a physiologic substrate.

When vesicles were pretreated with IAAmide, washed, and incubated with NEM, a concentration-dependent protection from inactivation was observed (Figure 4A), implying a modification of the receptor kinase by IAAmide. A smaller but significant protective effect was observed when vesicles were treated with PCMB after preincubation with IAAmide (see below). In contrast to the protective effect of IAAmide, pretreatment with IAAcid had no effect on subsequent inactivation by other reagents (Figure 4B). This lack of effect could be due either to failure of IAAcid to react with the receptor kinase at the site modified by

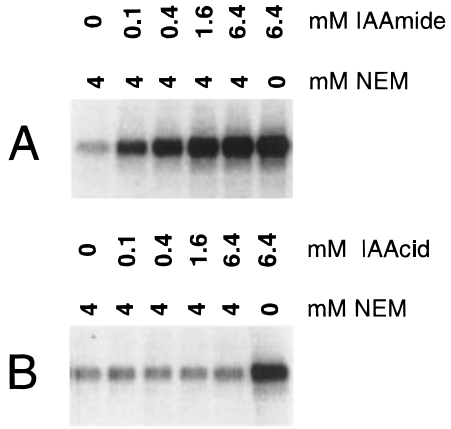

FIGURE 4: Effect of pretreatment of the EGF receptor with IAAmide or IAAcid on receptor sensitivity to NEM. Vesicles were pretreated with various concentrations of IAAmide (A) or IAAcid (B) before treatment with NEM. Results of a subsequent receptor autophosphorylation assay are depicted.

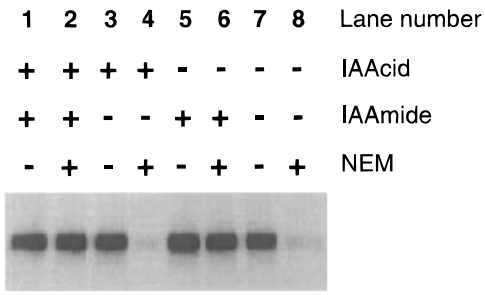

FIGURE 5: Effect of pretreatment of the EGF receptor with IAAcid on IAAmide protection of the receptor from NEM. Vesicles were pretreated with or without 12.5 mM IAAcid, and then washed before treatment with or without 12.5 mM IAAmide and subsequently with 4 mM NEM. Results of a subsequent receptor autophosphorylation assay are shown.

IAAmide or to modification of the site by IAAcid, with the charge differences in these reagents leading to different effects on the receptor kinase. Pretreatment of vesicles with IAAcid prior to treatment with IAAmide and subsequent incubation with NEM had no effect on the ability of IAAmide to protect against modification by NEM (Figure 5). These results imply that IAAcid does not react with the site modified by IAAmide.

Incubation of IAAmide-modified receptor with NEM for even long periods of time (on the order of at least 2 h) gives rise to receptor with substantial kinase activity, whereas incubation with PCMB after IAAmide modification progressively leads to an essentially complete loss of kinase activity (Figure 6). These data, together with the data from the AMP-PNP protection experiments, argue that IAAmide and NEM share a common site of modification in the kinase, whereas PCMB, while it likely reacts at this site, reacts at one or more additional sites in the receptor kinase, the reaction of which also causes inactivation. The site modified by IAAmide is inaccessible to IAAmide when nucleotide is bound, since vesicles incubated with IAAmide in the presence of AMP-PNP and divalent cations remained sensitive to subsequent inactivation by NEM when the IAAmide and AMP-PNP were removed by washing prior to treatment with NEM (Figure 7). These data, when compared with those in Figure 3, which were obtained when AMP-PNP and NEM were coincubated with receptor, also show that the protective effect of AMP-PNP is reversible.

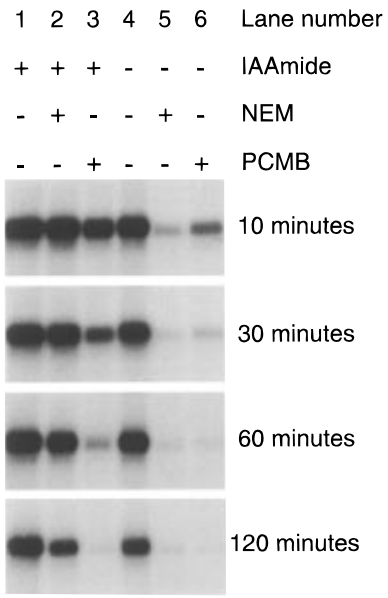

FIGURE 6: Effect of pretreatment of the EGF receptor with IAAmide on kinase sensitivity to prolonged exposure to NEM or PCMB. Vesicles were pretreated with or without 12.5 mM IAAmide, and then washed before incubation with 4 mM NEM or 100 μM PCMB for the times indicated. Results of a subsequent receptor autophosphorylation assay are shown.

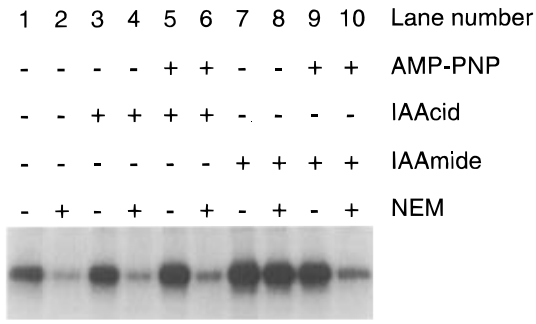

FIGURE 7: Effect of preincubation of EGF receptor with AMP-PNP on IAAmide protection of receptor from inactivation by NEM. Vesicles were pretreated with 4 mM AMP-PNP before addition of either 25 mM IAAmide or IAAcid. Vesicles were then washed and treated with 4 mM NEM as indicated; results of a subsequent receptor autophosphorylation assay are depicted.

Additional characterization of the effect of receptor modification was undertaken using steady-state kinetic analysis of angiotensin II phosphorylation. Vesicles were modified with IAAmide, washed, and solubilized, and EGF-stimulated receptor kinase activity was compared to that of unmodified EGF-stimulated receptor (Figure 8). Pretreatment with IAAmide was found to raise the $K_{m(app)}$ of the receptor kinase for ATP [$K_{m(app)} = 6.8$ μM after IAAmide treatment versus 1.5 μM for control], consistent with the evidence from protection experiments for modification of a site involving ATP binding. Interestingly, treatment by IAAmide increased the apparent k_{cat} of the receptor kinase (V_{max} increased by a factor of approximately 2 with IAAmide treatment compared to untreated control). This effect may be due to a true enhancement of the catalytic rate of IAAmide-modified receptor or to stabilization against inactivation. If stabilization against inactivation of the receptor kinase is involved, this inactivation does not appear to be the same process as that seen at physiological (37 °C) to heat shock (45 °C) temperatures (Stein & Staros, 1996), as no significant stabilization of the kinase by

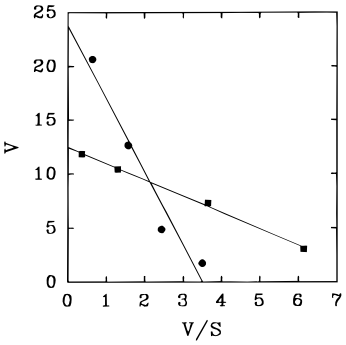

FIGURE 8: Effect of IAAmide treatment of the EGF receptor on phosphorylation of an exogenous substrate. Vesicles were incubated with (circles) or without (squares) 12.5 mM IAAmide, solubilized, stimulated with EGF, and assayed for phosphorylation of exogenous angiotensin II (1.25 mM) in the presence of various concentrations of ATP (0.5−32 μM). An Eadie−Hofstee plot of angiotensin II phosphorylation is presented, with total assay radioactivity in thousands of counts per minute (cpm $\times 10^{-3}$) plotted on the *y* axis, and (cpm $\times 10^{-3}$)/μM ATP plotted on the *x* axis. Data points shown are the averages of duplicate samples and are representative of two independent experiments.

IAAmide is observed under conditions of heat shock (data not shown).

DISCUSSION

Previous studies have demonstrated that receptor autophosphorylation is inactivated by treatment with the relatively sulfhydryl-specific reagent NEM, and that NEM treatment prevents subsequent reaction with the nucleotide affinity label 5′-FSBA (Buhrow *et al.*, 1982), suggesting that NEM may react with a residue in the nucleotide binding site. A puzzling aspect of these observations is that since they were first made, much work has been published on the catalytic mechanism of protein kinases, and no evidence has been reported for the involvement of a Cys residue in the catalytic mechanism. The specificity of NEM for sulfhydryl groups is, however, only relative, and reaction with amino groups is known to occur (Brewer & Riehm, 1967; Knight & Offer, 1978). Considering amino groups as potential targets for NEM, one candidate residue of the EGF receptor kinase for reaction with NEM is Lys721, the residue with which 5′-FSBA reacts (Russo *et al.*, 1985), and a residue which by homology with Lys72 of cAPK is critical for ATP binding (Zheng *et al.*, 1993). In our present studies, NEM and other relatively sulfhydryl-specific reagents were employed to investigate the mechanism of inactivation of the EGF receptor kinase.

These studies indicate that the kinase activity of the EGF receptor is inactivated by NEM and PCMB through modification of two or more sites within the receptor kinase, with at least one of these in the nucleotide binding site or allosterically coupled to nucleotide binding. IAAmide also modifies a residue (or residues) that affect(s) nucleotide binding; however, modification by IAAmide results in receptor which retains catalytic activity but with an increased $K_{m(app)}$ with respect to ATP and with decreased susceptibility toward inactivation by other reagents. The charge difference between IAAmide and IAAcid appears to preclude the reaction of IAAcid at the site in the EGF receptor modified by IAAmide, likely due to charge repulsion from acidic residues bordering the site.

The protection from both the inactivating sulfhydryl reagents and IAAmide observed in the presence of AMP-PNP may be due to direct protection of sulfhydryl groups by bound AMP-PNP. Alternately, the protective effect of AMP-PNP may be indirect, due to a conformational change that the receptor kinase undergoes upon nucleotide binding that results in occlusion of cysteinyl residues. However, comparison of the closed (with bound nucleotide) and open (without bound nucleotide) structures of the related cAPK demonstrates only subtle (<1 Å) shifts in the relative positions of active site residues; least-squares superposition of the Cα atoms of these structures has been reported to give rise to a rms deviation of 0.55 Å (Zheng *et al.*, 1993). Accordingly, though the allosteric mechanism cannot be excluded, the most straightforward interpretation of the experimental data is that NEM, PCMB, and IAAmide react at one or more residues that can be directly blocked by bound nucleotide.

We examined the crystal structures of the insulin receptor kinase domain (Hubbard *et al.*, 1994) and the ternary complex of cAPK with bound Mg^{2+}-ATP and inhibitor peptide (Zheng *et al.*, 1993) in conjunction with the sequence-based (Hanks *et al.*, 1988) and structure-based (Hubbard *et al.*, 1994) alignments of the EGF receptor with various kinases. The kinase domain of the EGF receptor contains six cysteinyl residues (Ullrich *et al.*, 1984). Alignment of the EGF receptor kinase with cAPK demonstrates that two of these, Cys751 and Cys773, are much closer to bound nucleotide than the remaining four. Cys773 of the EGF receptor is homologous to Glu127 of cAPK and Asp1083 of the insulin receptor kinase. In the cAPK ternary complex, Glu127 is hydrogen-bonded to the 2′-hydroxyl of the ATP ribose (Zheng *et al.*, 1993). Cys751 of the EGF receptor is homologous to Val104 of cAPK and Val1060 of the insulin receptor kinase. In the cAPK ternary complex, Val104 is close to the adenine ring of bound ATP, but does not have a major contact surface with it (Zheng *et al.*, 1993). While it is not known whether Cys773 of the EGF receptor kinase directly interacts with the ATP ribose,[2] it seems possible that modification of either Cys751 or Cys773 by NEM or PCMB could disrupt nucleotide binding. Both of these are relatively bulky reagents that are likely not to be well accommodated within the nucleotide binding pocket. However, a smaller modification at either site (e.g., by IAAmide) might permit retention of kinase activity.

It appears that charge differences in the haloacetates lead to the differences in reactivity observed at the site modified by IAAmide. A likely mechanism by which this might occur is through charge repulsion of the negatively charged IAAcid by acidic residues in proximity to the site of modification by IAAmide. Using computer modeling, the residues in the insulin receptor kinase corresponding to Cys751 and Cys773 of the EGF receptor were mutated to cysteine, with little effect on the structure of the kinase as a whole or on residues involved in nucleotide binding in particular. Acidic residues of the EGF receptor kinase near the sulfur atoms of the two

[2] After this work was submitted for publication, a study appeared by Singh *et al.* (1997) in which 2′-thioadenosine was shown to be a potent inhibitor of the EGF receptor kinase. The interpretation of the authors was that the 2′-thiol group of the nucleoside formed a disulfide bond with Cys773 (Cys797 in their numbering system, which counts the residues of the signal sequence of the receptor), resulting in inactivation, and that this disulfide is reduced by dithiothreitol, reactivating the kinase.

cysteinyl residues were examined. The carbonyl oxygens of four such residues were located within approximately 12 Å of the sulfur atom of Cys773, with carbonyl oxygens of both Asp813 and Asp776 approaching within approximately 8 Å of the sulfur atom. In contrast, a single acidic group is located near the sulfur atom of Cys751, namely, Asp831; the distance to the carbonyl oxygens of this residue is approximately 11 Å. From this modeling study, we can speculate that the pattern of reactivity of the haloacetates is most consistent with Cys773 as the site of reaction with IAAmide.

IAAmide protects the kinase from inactivation after even long incubations with NEM, suggesting that IAAmide and NEM share a site of modification. Protection experiments using AMP-PNP indicate that this site is occluded by bound nucleotide. The hypothesis of a shared site of modification by NEM and IAAmide is consistent with previous work that also suggested that NEM exerts its inhibitory effect at the nucleotide binding site (Buhrow *et al.*, 1982).

The initial protection from PCMB observed upon pretreatment of receptor with IAAmide may be due to blocking of a relatively facile inactivating reaction of PCMB at the site modified by IAAmide. However, the protection of the kinase from PCMB by IAAmide is relative, and can be overcome by prolonged incubations of the EGF receptor kinase with PCMB. This argues for the modification by PCMB of an additional Cys residue (or residues) that does not react with IAAmide, at least under the conditions utilized in these studies.

Although no Cys residues have been implicated in the catalytic mechanism of protein kinases, these results are intriguing, especially in the context of other experiments involving kinase inactivation. Compounds containing disulfide groups have been developed as potent inhibitors of tyrosine kinases, with exchange of kinase thiol groups with inhibitor disulfides thought to lead to kinase inhibition (Palmer *et al.*, 1995). Modification of the EGF receptor by NEM at a site (or sites) in the cytoplasmic domain has also been reported to lead to the disappearance of high-affinity EGF binding (van Belzen *et al.*, 1991).

An unexpected observation in these studies was that the apparent k_{cat} of the IAAmide-treated receptor preparation was approximately 2-fold higher than that of the untreated control. For cAPK, the release of ADP is rate-limiting (Adams & Taylor, 1992), and product release as the rate-limiting step for the EGF receptor kinase is consistent with available kinetic evidence (Guyer *et al.*, 1994). The enhanced catalytic activity observed in IAAmide-modified receptor could be due to an alteration in binding of the metal ion/nucleotide complex that allows more rapid dissociation of reaction products. Altered nucleotide binding is certainly consistent with the significantly higher $K_{m(app)}$ of IAAmide-modified receptor for ATP.

Studies are currently underway in which site-directed mutagenesis is being used to test the roles of Cys751 and Cys773 in kinase inhibition by NEM and protection by IAAmide. Mutants with measurable kinase activity will also be useful for testing hypotheses concerning the increase in $K_{m(app)}$ with respect to Mg^{2+}-ATP and the increase in k_{cat} observed for the IAAmide-modified wild-type receptor.

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