Thermal Inactivation of the Protein Tyrosine Kinase of the Epidermal Growth Factor Receptor†

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ABSTRACT: It has been shown previously that the EGF-stimulable protein tyrosine kinase in a membrane preparation from A431 cells was inactivated by heat shock (45 °C), under conditions where EGF binding was unaffected [Carpenter et al. (1979) *J. Biol. Chem. 254*, 4884−4891]. A later study found that in intact cells, the protein tyrosine kinase of the EGF receptor was insensitive to heat shock [Liu & Carpenter (1992) *Biochem. J. 286*, 541−547]. We have extended these previous studies to better understand the thermal stability of the protein tyrosine kinase of the EGF receptor. We have measured the rate of inactivation of the kinase in membrane vesicles in the physiological to heat shock temperature range (37−45 °C). At 45 °C, the protein tyrosine kinase is rapidly inactivated with a rate of approximately 0.14 min^{-1}. There is, however, protection against inactivation by incubation of the EGF receptor with AMPPNP, a hydrolysis-resistant ATP analog. At 45 °C, the rate of inactivation of nucleotide-bound receptor is an order of magnitude lower than the rate of inactivation of unoccupied receptor. Analysis of the temperature dependence of inactivation between 37 and 45 °C yields an activation energy, E_a, of 42 kcal/mol, an activation Gibbs free energy, $\Delta G^{\ddagger}$, between 23 and 22 kcal/mol, an activation enthalpy, $\Delta H^{\ddagger}$, of 42 kcal/mol, and an activation entropy, $\Delta S^{\ddagger}$, of 60 cal/(K·mol). The signs and magnitudes of the thermodynamic parameters suggest that inactivation is more likely due to some local reorganization within the kinase domain than to a simple chemical process. Further, the kinetic data show that the receptor is stabilized against inactivation by binding adenine nucleotide.

The binding of EGF,[1] a ∼6 kDa mitogenic polypeptide hormone, to its plasma membrane receptor results in the activation of a protein kinase activity (Carpenter et al., 1978) specific for tyrosyl residues (Ushiro & Cohen, 1980). This EGF-stimulable protein tyrosine kinase is physically part of the receptor itself (Buhrow et al., 1982, 1983), residing in a kinase domain in the carboxy-terminal, cytoplasmically oriented portion of the receptor (Ullrich et al., 1984).

The increase in protein tyrosine kinase activity of the receptor results in an increase in autophosphorylation of the carboxy-terminal region of the receptor and an increase in phosphorylation of other cellular proteins. These phosphorylation events trigger a subsequent cascade of phosphorylation and protein−protein interactions and activations that lead to cell proliferation. These processes are tightly regulated; disruption of the signalling cascade at a number of points has been shown to result in loss of growth control as manifested in cellular transformation [reviewed in Carpenter & Wahl (1990); Ullrich & Schlessinger, 1990].

In early studies of the EGF receptor, it was shown that the EGF-stimulable kinase in a membrane preparation was inactivated at 45 °C, while the EGF binding capabilities of the membrane preparation were unaffected (Carpenter et al., 1979). A later study found that in intact cells, the EGF receptor kinase was insensitive to a 46 °C heat shock (Liu & Carpenter, 1992). The authors hypothesized that there was some component or components of the cell that prevented the kinase in intact cells from inactivation during heat shock.

In order to better understand the process that underlies the thermal inactivation observed with membrane preparations, we have measured the inactivation of the kinase in the physiological to heat shock temperature range. From these studies, we have determined the thermodynamic parameters governing the inactivation of the protein tyrosine kinase activity of the receptor. These parameters put constraints on the type of process that could cause the inactivation of the kinase. Further, we have investigated the inactivation process in the presence of a hydrolysis-resistant ATP analog, AMPPNP, and have found that at 45 °C, the nucleotide-bound kinase is inactivated an order of magnitude slower than the unoccupied kinase.

MATERIALS AND METHODS

EGF was prepared as previously described (Savage & Cohen, 1972). Membrane vesicles from A431 cells were prepared by the previously described (Rousseau et al., 1995) modifications to the method of Cohen et al. (1982).

Phosphorylation Assay. To an ice-cold suspension of membrane vesicles was added a one-fourth volume of 5× phosphorylation buffer to yield final concentrations of 20 mM HEPES, pH 7.4, 10 μM Na_3VO_4, 1 mM $MnCl_2$, 5 mM $MgCl_2$, 20 μM ATP, and 0.5 μCi [^{32}P]ATP. After a 2 min incubation on ice, the phosphorylation reactions were stopped

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[1] Abbreviations: AMPPNP, adenylyl imidodiphosphate; AT II, angiotensin II; EGF, epidermal growth factor; 5′-FSBAdo, [*p*-(fluorosulfonyl)benzoyl]-5′-adenosine; $NaDodSO_4$−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TCA, trichloroacetic acid.

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with the addition of a one-third volume of 4× Laemmli sample buffer (Laemmli, 1970) and heating for 4 min at 90 °C. To measure the extent of autophosphorylation, the samples were separated on a 6% polyacrylamide gel using $NaDodSO_4$−PAGE. Receptor bands were quantitated following exposure of the dried gel to a phosphorimage screen using a Molecular Dynamics phosphorimager (Model 400E).

Thermal Inactivation. At $t = 0$, A431 membrane vesicles were added to 20 mM HEPES, pH 7.4, which had been preincubated at the appropriate temperature, and the resulting suspension was vortexed. A 30 μL aliquot was immediately removed for the nominal zero time point, and subsequent aliquots were withdrawn at the times indicated in the figures. The aliquots were immediately added to a precooled EGF/20 mM HEPES solution to give a final EGF concentration of 0.5 μM. After 15 min on ice, the samples were subjected to the phosphorylation assay. Assays done in the presence of AMPPNP were carried out slightly differently due to the inhibition of the phosphorylation assay by AMPPNP. Each 30 μL aliquot was first placed in 1 mL of ice-cold 20 mM HEPES, pH 7.4, and centrifuged at 16000*g* for 5 min at 4 °C. The pellets were resuspended in 36 μL of a 0.5 μM EGF/20 mM HEPES solution and assayed as described above. This single wash step removed sufficient AMPPNP from the solution to avoid detectable inhibition of the kinase activity in the subsequent phosphorylation assay.

Exogenous Substrate Assay. This assay was carried out similarly to the thermal inactivation experiments. At $t = 0$, A431 membrane vesicles were added to 20 mM HEPES, pH 7.4, which had been preincubated at 45 °C, and the resulting suspension was vortexed. An aliquot was immediately removed for the nominal zero time point, and subsequent aliquots were withdrawn at the times indicated in the figure. The aliquots were immediately added to an EGF/20 mM HEPES solution to give a final EGF concentration of 0.6 μM and incubated on ice for 5 min. The subsequent addition of digitonin and angiotensin II (AT II) to final concentrations of 0.1% and 1.5 mM, respectively, made the final EGF concentration 0.5 μM. After a 5 min incubation on ice, a one-fourth volume of 5× phosphorylation buffer was added as above. After a 2 min incubation, the phosphorylation reactions were stopped by addition of a two-fifths volume of 12.5% TCA. Each sample was divided into two for measurement of autophosphorylation and phosphorylation of AT II. A one-third volume of 4× Laemmli sample buffer was added to half of the divided sample that had been neutralized with NaOH, and treated as above for detection of autophosphorylation. The second half of the divided sample, used to measure phosphorylation of the AT II, was centrifuged at 16000*g* for 5 min at room temperature and the supernatant was applied to a Pierce phosphocellulose unit (29520) and treated per manufacturer's instructions.

Receptor Dimerization. Trapping of receptor dimers with the cross-linker BS^3 (Staros, 1982) was carried out by the procedure of Fanger et al. (1989), with slight modifications. A431 membrane vesicles were incubated for 30 min at 45 °C or on ice and then centrifuged at 16000*g* for 5 min at 4 °C. The pellets were solubilized in 25 μL of SB (1% Triton X-100, 10% glycerol, 1 mM EGTA, and 50 mM HEPES, pH 7.4) by vortexing, incubated on ice for 10 min, vortexed, and centrifuged at 16000*g* for 5 min at 4 °C to pellet insoluble material. EGF was added to the solubilized receptor preparation to a final concentration of 1 μM and incubated for 10 min at room temperature. Cross-linking reactions were initiated by adding BS^3 (Pierce) to a final concentration of 1 mM. After incubating for 10 min at room temperature, the cross-linking reactions were terminated by the addition of 1 M glycine to a final concentration of 45 mM. Samples were then prepared for $NaDodSO_4$−PAGE on a 3−10% gradient gel by the addition of a one-third volume of 4× Laemmli sample buffer and heating for 5 min at 90 °C. Transfer to nitrocellulose (NitroBind; Micron Separations) was accomplished using a Hoefer tank transfer system (TE42) as previously described (Wang et al., 1989). Cross-linked EGF receptor dimers were detected by immunoblotting with EGFR antiserum (generously provided by Dr. G. Carpenter, Vanderbilt University) and visualized by chemiluminescence (ECL, Amersham). Transphosphorylation assays with Dc214, a truncated EGF receptor (Sorkin et al., 1992), were carried out as previously described (Coker et al., 1994).

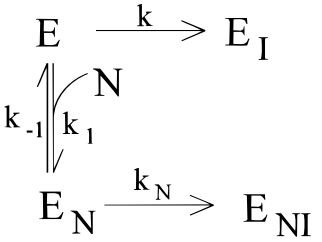

FIGURE 1: Kinetic scheme for receptor inactivation in the presence of AMPPNP. The rates of inactivation for the unoccupied and nucleotide-occupied receptors reflect one-step, irreversible processes, k and k_N, respectively. The occupied and unoccupied receptors are interconnected by the binding and dissociation of AMPPNP to the receptor, which is described by the rate constants k_1 and k_{-1}.

Kinetic Modeling of Inactivation Data in the Presence of AMPPNP. For the time courses of inactivation of autophosphorylation in the presence of AMPPNP, a simple kinetic scheme was applied (Figure 1). First, we assumed that the rates of inactivation for the unoccupied and nucleotide-occupied receptor reflect one-step, irreversible processes. The occupied and unoccupied receptors are interconnected by the binding and dissociation of AMPPNP to the receptor. The integrated rate equations describing this model were derived for the E and E_N species (see Supporting Information for details). These equations are bi-exponential and dependent on the four rate constants, k, k_N, k_1, and k_{-1}. We simultaneously fit all inactivation data for the four rate constants, k, k_N, k_1, and k_{-1}, for this model, allowing the four rate constants to vary to minimize the fits to the data (Beechem, 1992). The asymmetric standard deviations of these fit parameters were obtained as previously described (Rousseau et al., 1995). Due to the one-exponential behavior of the 1 mM AMPPNP data set, we simultaneously fit the data with and without this data set. There were little differences in the parameters from these fits; therefore, we included the 1 mM AMPPNP data in the final analysis.

Thermodynamic Analysis. The rate constants for inactivation were determined by first normalizing the time course of autophosphorylation to the zero time point. The normalized data were analyzed using a natural log plot versus time, with the slope obtained from a linear regression equal to the rate of inactivation. To obtain the thermodynamic param-

eters from these inactivation rates, two analyses were carried out.

An empirical activation energy, E_a, for the inactivation process can be obtained from an Arrhenius plot according to the relation

$$\ln(k) = -\frac{E_a}{RT} + \text{constant} \quad (1)$$

where k is the inactivation rate, R is the gas constant [1.987 cal/(K·mol)], and T is the absolute temperature.

From transition-state theory, the activation Gibbs free energy change from the initial state to the transition state for the inactivation process, $\Delta G^{\ddagger}$, is

$$\Delta G^{\ddagger} = -RT \ln\left(\frac{hk}{k_b T}\right) \quad (2)$$

where h is Planck's constant (1.584×10^{-34} cal·s) and k_b is Boltzmann's constant (3.298×10^{-24} cal/K). The activation enthalpy, $\Delta H^{\ddagger}$, and the activation entropy, $\Delta S^{\ddagger}$, for the change from the initial state to the transition state for the inactivation process can be obtained from the Eyring equation:

$$\Delta G^{\ddagger} = \Delta H^{\ddagger} - T\Delta S^{\ddagger} \quad (3)$$

Substituting eq 2 into eq 3 and rearranging yield

$$\ln\left(\frac{hk}{k_b T}\right) = -\frac{\Delta H^{\ddagger}}{RT} + \frac{\Delta S^{\ddagger}}{R} \quad (4)$$

Under the assumption that $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ are independent of T, their values are obtained from the slope and intercept, respectively, of a semi-log plot by the use of eq 4. The values for $\Delta G^{\ddagger}$ are calculated according to eq 3.

RESULTS

Inactivation of Autophosphorylation at 45 °C. We measured the inactivation of the EGF receptor in membrane vesicles at 45 °C in the absence and presence of increasing concentrations of AMPPNP (Figure 2). In the absence of AMPPNP, the autophosphorylation activity decays to zero, with a rate of 0.138 min^{-1}. With increasing concentrations of AMPPNP, the apparent inactivation rate is decreased, suggesting a protective effect of AMPPNP on the inactivation of EGF receptor autophosphorylation under these conditions. One possible mechanism by which AMPPNP could protect against inactivation is by directly inhibiting the process that is causing inactivation by specific binding of AMPPNP to the ATP binding site in the tyrosine kinase domain of the receptor. An alternative mechanism of protection against inactivation could be through a nonspecific inhibition of the inactivation, such as by an increase in ionic strength. To test between specific and nonspecific mechanisms of protection, we measured kinase inactivation in the presence of ITP. It has been reported that for inhibition of receptor protein tyrosine kinase activity AMPPNP has a K_i of 22 μM while ITP has a K_i of 3.1 mM (Vogel et al., 1986), suggesting that comparison of the protection against inactivation afforded by AMPPNP and ITP could be used to distinguish between specific and nonspecific mechanisms of protection. Very little protection due to ITP at a concentration of 1 mM was observed (data not shown), suggesting that the protection

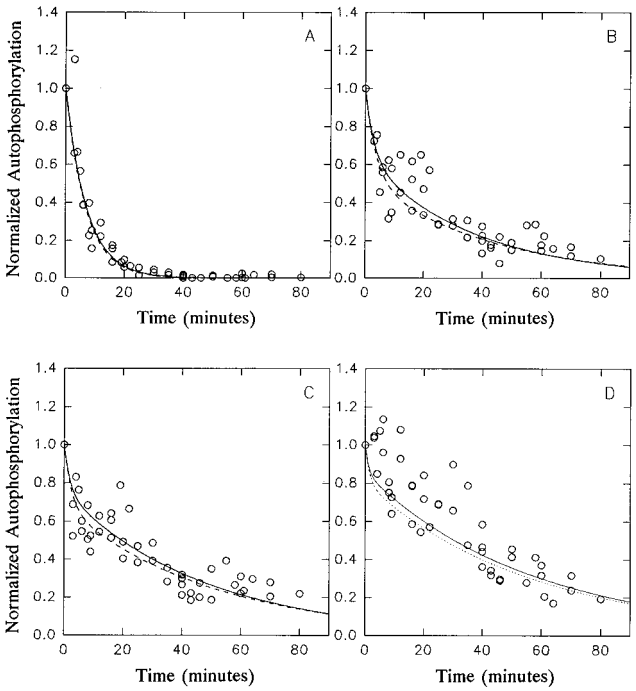

FIGURE 2: Inactivation of autophosphorylation at varying concentrations of AMPPNP. Shown is the time-dependence of the inactivation of autophosphorylation with increasing concentrations of AMPPNP: 0 (A), 0.25 mM (B), 0.5 mM (C), and 1.0 mM (D). Shown are the fits (Table 1) to the model described in the text for the data assuming no error (solid line) and 10% error for each point (dashed line).

afforded by AMPPNP is due to specific binding of nucleotide to the receptor.

We fit the inactivation data to the model proposed in Figure 1, which assumes an irreversible decay for either the unoccupied or the occupied receptor. Using this model, we find that the occupied receptor becomes inactivated with a rate constant approximately an order of magnitude lower than the unoccupied receptor, 0.012 min^{-1} compared to 0.138 min^{-1}, clearly showing the protective effect of AMPPNP on inactivation (Table 1, first row). In addition, the on and off rates of binding of AMPPNP to the receptor were determined, yielding a K_d for AMPPNP binding to the receptor on the order of 30−50 μM. This value for the dissociation constant is comparable to the K_i value, 22 μM, measured for AMPPNP inhibition of autophosphorylation (Vogel et al., 1986).

One potential problem with this analysis is that the error associated with the measured autophosphorylation at each time point is not known. This could cause two problems. One is that with each point equally weighted in the fit, the data could be biased by just a few points. The second problem is that if there is an error in the first time point, this would be reflected in all the time points for that data set, potentially creating a bias in the analysis. In order to investigate the potential effect of the error in each time point, we fit the data assuming a 10% error at each time point. In this fit, we still normalized to the $t = 0$ data point. We find that there are only slight changes in the resulting parameters, with the parameters showing the same essential behavior of the system, indicating we are not overly biasing our data because we do not have absolute values for the errors at each time point (Table 1, second row). To check the effect of normalizing to the $t = 0$ time point, we analyzed the data

Table 1: Rate Constants for Inactivation at 45 °C[a]

error[b]	normalization[c]	k (min^{-1})	k_N (min^{-1})	k_1 (min^{-1} M^{-1})	k_{-1} (min^{-1})	K_d (μM)
none	yes	0.138 (0.116,0.170)	0.012 (0.003,0.019)	668 (380,1310)	0.035 (0,0.094)	52
10%	yes	0.144 (0.135,0.158)	0.013 (0.002,0.024)	495 (243,1306)	0.021 (0,0.090)	42
none	no	0.130 (0.106,0.163)	0.016 (0.007,0.024)	600 (312,1575)	0.019 (0,0.077)	32

[a] Fit to the inactivation of autophosphorylation data at 45 °C (Figure 2) using the model outlined in Figure 1. The numbers in parentheses are the lower and upper bounds of the standard deviation of the fit parameter. [b] The error signifies the amount of error that was assumed for each data point. None assumes no error. Please see text for further details. [c] Normalization indicates whether or not the data were normalized to the $t = 0$ time point. Please see text for further details.

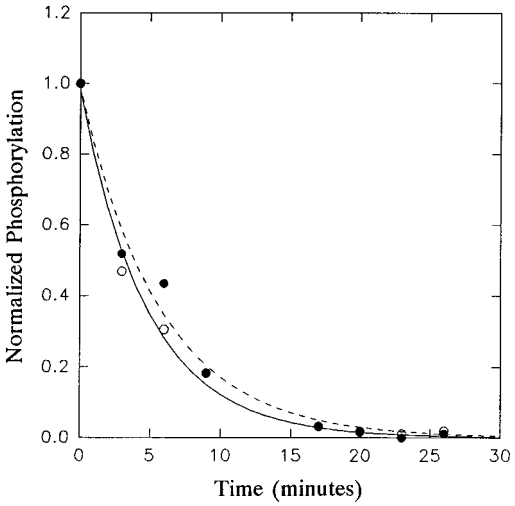

FIGURE 3: Comparison of autophosphorylation to phosphorylation of exogenous substrate by A431 membrane vesicles incubated at 45 °C. At each time point, aliquots were removed and were assayed for autophosphorylation (○, solid line) and phosphorylation of angiotensin II (●, dashed line). The rates of inactivation of autophosphorylation and phosphorylation of angiotensin II observed in this experiment were 0.208 ± 0.024 and 0.175 ± 0.032 min^{-1}, respectively.

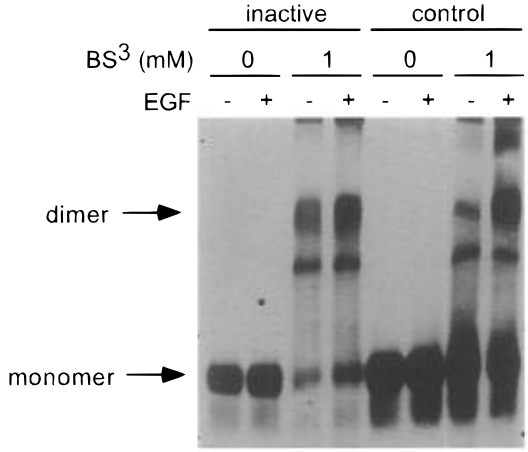

FIGURE 4: EGF-dependent dimerization of thermally inactivated receptors. Vesicles which had been incubated at 45 °C (left four lanes) or on ice (right four lanes) were solubilized, incubated ±EGF and ±BS^3. Following NaDodSO$_4$−PAGE, samples were transferred to nitrocellulose and blotted with receptor antisera as described under Materials and Methods. This experiment is representative of four independent trials. There is a cross-linker-specific increase in high molecular weight bands and an EGF-dependent increase in receptor dimer band.

without normalizing to this time point. Again, we find that there are only small changes in the resulting parameters, with the parameters still indicating the 1 order of magnitude difference in inactivation of the unoccupied and occupied receptors (Table 1, third row).

The experiments described above used receptor autophosphorylation as the measure of the kinase activity of the receptor. In order to test whether autophosphorylation activity is a reliable measure of the intrinsic kinase activity of the receptor, we compared the decay of autophosphorylation activity with the decay of kinase activity toward angiotensin II, a well-studied, relatively nonspecific substrate for protein tyrosine kinases (Wong & Goldberg, 1983) (Figure 3). The autophosphorylation activity of the receptor and the kinase activity of the receptor toward exogenous substrate decay in parallel, indicating that under these conditions receptor autophosphorylation is a reliable measure of the kinase activity of the receptor.

Receptor Dimerization. The above experiments measured the decrease of EGF-stimulated autophosphorylation of the EGF receptor. To measure whether the loss of activity was due to a loss of EGF-induced dimerization, the extent of receptor dimerization of active and inactivated receptor was determined in experiments where receptor dimers are trapped via cross-linking. While EGF- and BS^3-dependent receptor dimers are seen (Figure 4) in both the heat-inactivated (left four lanes) and control (right four lanes) samples, significant differences are seen between the two samples. First, there is an increase in EGF-*independent* receptor dimers in the heat-inactivated sample relative to the control (compare lanes 3 and 7). In addition, in the heat-inactivated samples there is a loss of receptor antibody-staining material from the migration positions of monomeric and dimeric receptor, with an increase in higher molecular weight bands (in part out of range of the blot shown in Figure 4).

We also measured the ability of the inactivated receptor to be transphosphorylated by a mutated EGF receptor, Dc214, that is truncated at N972 and thus lacks all five known autophosphorylation sites (Sorkin et al., 1992). Given its lower MW and the absence of significant autophosphorylation, the use of the Dc214 receptors allows for detection of transphosphorylation of the heat-inactivated receptor by Dc214. The inactivated receptor was able to be transphosphorylated by Dc214 in an EGF-dependent manner, though to a lesser extent than a kinase negative receptor (D813A) (Coker et al., 1994) control (data not shown).

Temperature-Dependent Inactivation. It is evident that AMPPNP has a protective effect on inactivation of autophosphorylation of the receptor at 45 °C. It still does not explain the process that is occurring to inactivate the receptor. In order to better understand the process that is involved in inactivation of receptor autophosphorylation, we measured the temperature dependence of inactivation in the absence

Table 2: Inactivation Rates in the Temperature Range of 37−45 °C[a]

T (°C)	37	39	41	43	45
k (min^{-1})	0.025	0.047	0.059	0.116	0.138

[a] Shown are the thermal inactivation rates of autophosphorylation in the range of 37−45 °C. Please see text for further details.

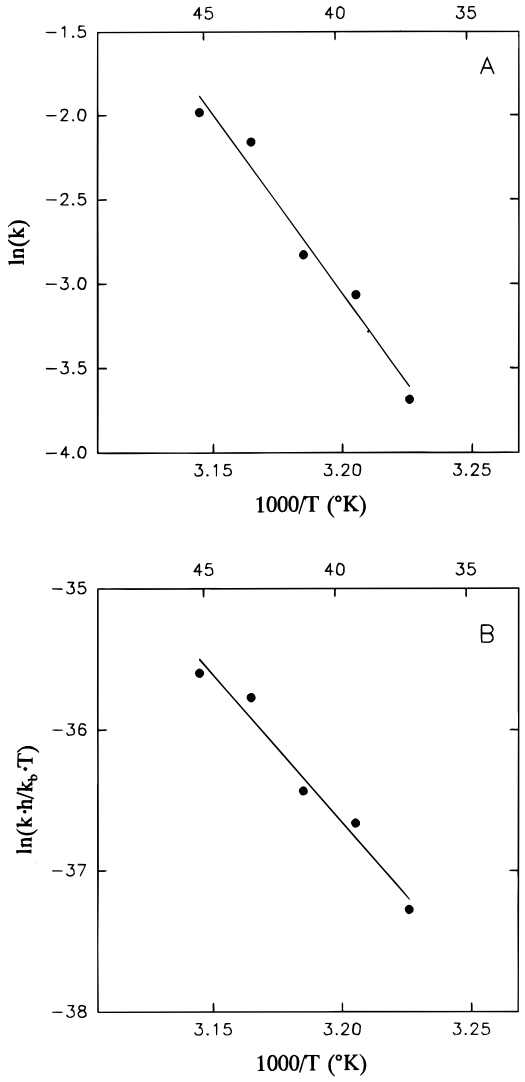

FIGURE 5: Calculation of thermodynamic parameters for the inactivation of autophosphorylation. The rates of inactivation at each temperature are shown in Table 2. (A) Arrhenius plot yielding an empirical activation energy, E_a, of 42.3 ± 4.2 kcal/mol. (B) Eyring plot yielding an activation enthalpy, $\Delta H^{\ddagger}$, of 41.6 ± 4.2 kcal/mol and an activation entropy, $\Delta S^{\ddagger}$, of 60.5 ± 0.3 cal/(K·mol).

of AMPPNP between 37 and 45 °C (Table 2). From the Arrhenius plot (Figure 5A), we find that the activation energy, E_a, is 42.3 ± 4.3 kcal/mol. From the Eyring plot (Figure 5B), we find that the activation enthalpy, $\Delta H^{\ddagger}$, for the inactivation process is 41.6 ± 4.2 kcal/mol and the activation entropy, $\Delta S^{\ddagger}$, is 60.5 ± 4.2 cal/(K·mol), yielding activation Gibbs free energies, $\Delta G^{\ddagger}$, between 22.9 ± 4.3 and 22.4 ± 4.3 kcal/mol for the temperature range of 37−45 °C.

DISCUSSION

Inactivation in Membrane Preparations. It was shown previously that in membrane preparations the EGF-stimulable protein tyrosine kinase was inactivated at 45 °C (Carpenter et al., 1979). We measured the rate of inactivation of autophosphorylation activity of the receptor in a membrane preparation at 45 °C (Figure 2A) and found that the inactivation rate is approximately 0.14 min^{-1} (Table 1). While these observations of heat inactivation of the kinase measured autophosphorylation of the receptor, it is possible that the kinase may have remained active toward other substrates, but was unable to phosphorylate tyrosyl residues in the carboxy-terminal region of the receptor. However, we observed essentially no difference in the rate of inactivation of autophosphorylation *versus* phosphorylation of an exogenous peptide substrate, angiotensin II (Figure 3). These data indicate that inactivation involves a loss of kinase activity and does not occur solely through a structural change in the phosphate-acceptor portion of the receptor.

In principle, the loss of activity seen in these membrane preparations could have been due to a loss of stimulation by EGF of the kinase activity, rather than to a decrease in the intrinsic kinase activity of the receptor. Two pieces of evidence argue against this possibility: (1) the rate of inactivation of the basal activity was the same as the rate of inactivation for the EGF-stimulated activity (Carpenter et al., 1979); and (2) the rate of inactivation was the same, whether EGF was or was not present during the inactivation process (data from this study, not shown). While these data suggest that the inactivation is not likely due to a loss of dimerization, we tested this hypothesis by directly looking at the ability of EGF to induce receptor dimerization (Figure 4). Although heat-inactivated receptors are not as efficiently converted to receptor dimers as active receptors, they are still capable of undergoing significant EGF-dependent receptor dimerization, suggesting that the loss of kinase activity at 45 °C is not due to loss of EGF-dependent receptor dimer formation. Therefore, inactivation is not likely due to a loss of EGF stimulation of the protein tyrosine kinase of the receptor, but rather due to a loss of tyrosine kinase activity *per se*.

Protection against Inactivation. Unlike receptor membrane preparations, there was no loss of receptor autophosphorylation activity under heat shock conditions, 46 °C, in intact cells, and there was also no loss of phospholipase Cγ (PLCγ) phosphorylation (Liu & Carpenter, 1992). These results suggested that some component or components within the cell stabilize the receptor from being inactivated at heat shock temperatures. There are many components of the cell that might not be present in the membrane preparations. One such component is ATP. In testing whether ATP might be one of the components that protect against inactivation in intact cells, we used a hydrolysis-resistant analog of ATP, AMPPNP. It is clear that the apparent rate of inactivation in membrane preparations is decreased in the presence of AMPPNP in a concentration-dependent manner (Figure 2). This protection is achieved with a concentration of AMPPNP comparable to the physiological concentration of ATP (millimolar). Based on the kinetic model in Figure 1, the protection obtained from nucleotide appears to occur not through decreasing the rate of inactivation of the unoccupied receptor, but by binding to the receptor and the nucleotide-bound receptor having a 10-fold decrease in its rate of inactivation (Table 1). In addition to the rates of inactivation of the unoccupied and occupied receptor, fitting the experimental data to our model also yields values for the association and dissociation rate constants for the interaction of

AMPPNP with the receptor (Table 1). While the ratio of these rates yields values of K_d that are reasonable when compared with the published value for the K_i of AMPPNP, the absolute values of the association and dissociation rates are somewhat problematic. These values were derived with the assumption that the local concentration of nucleotide is the same as that added to the vesicle suspension. Since the actual local concentration cannot be determined, the emphasis of this analysis is on the inactivation rates of occupied and unoccupied receptor, not on the rates of binding and dissociation of AMPPNP.

The data with AMPPNP indicate that while nucleotide binding may not be the only factor accounting for the thermal stability of the receptor in intact cells, it is possibly one of the factors involved. Further evidence that the inactivation process involves a direct effect on the kinase domain arises from the ability of the kinase to react with [*p*-(fluorosulfonyl)benzoyl]-5′-adenosine (5′-FSBAdo). This ATP analog contains a *p*-(fluorosulfonyl)benzoyl group in place of the phosphates that forms a covalent adduct with Lys 721 in the kinase domain of the EGF receptor (Russo et al., 1985). Thermal inactivation of the receptor (15 min at 45 °C) inhibits the ability of the receptor to be labeled with 5′-FSBAdo (Buhrow et al., 1982), consistent with a structural rearrangement of the receptor involving the nucleotide binding site.

Thermodynamics. There are several possible mechanisms of inactivation, including chemical modification and protein unfolding. To better address the mechanism of receptor inactivation, we have determined thermodynamic parameters for the inactivation process. One analysis of the inactivation rate data is an Arrhenius plot, the slope of which yields an empirical activation energy, E_a, which for reactions in solution is essentially equivalent to the activation enthalpy, $\Delta H^{\ddagger}$ (Tinoco et al., 1985). An Arrhenius plot of the inactivation rate data (Figure 5A) yields a value for E_a of 42.3 ± 4.2 kcal/mol, a value slightly above the high end of that reported for most readily occurring chemical reactions (Barrow, 1973). This suggests that inactivation is not likely due to a simple chemical modification, such as oxidation. On the other hand, the activation energy of 42 kcal/mol is comparable to the low end of previously measured activation energies for unfolding of soluble proteins (Segel, 1975).

If one makes the simplifying assumption that the inactivation process occurs through a single transition state, transition state theory can be applied, which yields not only the activation enthalpy but also the activation entropy, and, from these, the activation Gibbs free energy of the process. An Eyring plot of the rates of inactivation observed in the 37−45 °C temperature range (Figure 5B) yields an activation enthalpy, $\Delta H^{\ddagger}$, of 41.6 ± 4.2 kcal/mol and an activation entropy, $\Delta S^{\ddagger}$, of 60.5 ± 0.3 cal/(K·mol). From these parameters, the activation Gibbs free energies, $\Delta G^{\ddagger}$, are calculated to be (22.9 ± 4.3) to (22.4 ± 4.3) kcal/mol for the 37 to 45 °C temperature range studied.

The thermodynamic parameters of kinase inactivation yield insights concerning the nature of the inactivation process. The observation that the activation Gibbs free energy, $\Delta G^{\ddagger}$, which is a measure of the spontaneity of the inactivation process, is approximately half in magnitude of the activation enthalpy, $\Delta H^{\ddagger}$, emphasizes the importance of the positive entropic contribution to the activation step of the inactivation process. The relatively large increase in entropy from the ground state to the transition state of the inactivation process is consistent with a local disordering of the kinase.

Mechanism of Inactivation. As outlined above, chemical and thermodynamic data suggest that the inactivation occurs through a local disordering of the kinase. Further evidence for a structural rearrangement of the kinase is seen in the cross-linking studies. While there is an EGF-dependent formation of receptor dimers, there is also an EGF-independent aggregation of the inactive receptor. This heat-induced aggregation does not appear to be covalent, as suggested by the lack of higher aggregates in the absence of cross-linker (Figure 4, compare lanes 1 and 2 to lanes 3 and 4). While it is not certain whether the observed aggregation contributes to or is simply an effect of the inactivation of the kinase, it is possible that the (partially) unfolded kinase domain associates with other molecules of the receptor or with other proteins in the membrane causing the formation of noncovalent, high molecular weight aggregates that are trapped by cross-linking. Based on the protection against inactivation seen with AMPPNP, it appears that the nucleotide binding region of the kinase domain might be involved in this process.

Conclusion. We found that at 45 °C the protein tyrosine kinase of the EGF receptor in a membrane preparation is rapidly inactivated and that there is protection against inactivation by incubation of the EGF receptor with AMPPNP. The rate of inactivation of nucleotide-bound receptor is an order of magnitude lower than the rate of inactivation of unoccupied receptor (0.012 min^{-1} vs 0.138 min^{-1}). Analysis of the temperature dependence of inactivation yields an activation energy, E_a, of 42 kcal/mol, an activation Gibbs free energy, $\Delta G^{\ddagger}$, of 22−23 kcal/mol in the temperature range examined, an activation enthalpy, $\Delta H^{\ddagger}$, of 42 kcal/mol, and an activation entropy, $\Delta S^{\ddagger}$, of 60 cal/(K·mol). These parameters suggest that the *initial* process in the inactivation of the kinase more likely occurs via some local reorganization within the kinase domain, than via a simple chemical modification. Further studies need to be carried out to better define the perturbations of the tyrosine kinase domain that lead to inactivation and the role that these perturbations might play in the cellular function and regulation of the EGF receptor.

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SUPPORTING INFORMATION AVAILABLE

The derivation of the integrated rate equations for the model depicted in Figure 1 is given. A description of how these equations are used to describe the time-dependence of autophosphorylation activity is also presented (3 pages). Ordering information is given on any current masthead page.

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