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Preparation and Characterization of Spin-Labeled Derivatives of Epidermal Growth Factor (EGF) for Investigations of the Interactions of EGF with Its Receptor by Electron Paramagnetic Resonance Spectroscopy†

Lee Anne Faulkner-O'Brien,‡ Albert H. Beth,§ Ioannis A. Papayannopoulos,‖ P. S. R. Anjaneyulu,‡,⊥ and James V. Staros*,‡

Department of Biochemistry and Department of Molecular Physiology and Biophysics, Vanderbilt University, School of Medicine, Nashville, Tennessee 37232, and Department of Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

Received January 9, 1991; Revised Manuscript Received May 22, 1991

ABSTRACT: We prepared, purified, and characterized derivatives of epidermal growth factor (EGF) having a nitroxide spin-label attached covalently at the amino terminus. Characterization of these derivatives with regard to the positions of attachment of the spin-label was accomplished by a combination of peptide mapping, protein sequencing, and fast atom bombardment–mass spectrometry. One derivative was chosen for use in initial investigations by electron paramagnetic resonance (EPR) spectroscopy of receptor-bound EGF and its dissociation kinetics. This derivative was found to be equipotent with the native hormone in competitive binding assays, in activating the EGF receptor kinase, and in stimulating the formation of EGF receptor dimers in solubilized cell extracts. Upon binding to solubilized EGF receptor, the spin-labeled EGF derivative became immobilized, giving rise to a visually distinct slow-motion EPR spectrum. The resulting spectrum showed no detectable dipolar interaction between nitroxides, indicating that the nitroxide moieties of spin-labels reacted at the amino termini of receptor-bound spin-labeled EGF molecules are separated by a distance of at least 16 Å. An EPR study of the kinetics of dissociation of spin-labeled EGF in the presence of excess unlabeled EGF revealed a rapid component with a $k_{off} \approx 2 \times 10^{-2}$ s^{-1} and a less well resolved slow component.

EGF[1] was initially purified from murine submandibular glands on the basis of its property of promoting early eyelid opening in newborn mice (Cohen, 1962). It has since been shown to modulate cell growth and differentiation in a variety of target tissues (Carpenter & Cohen, 1979; Carpenter 1981). The receptor for EGF [for reviews, see Carpenter (1987),

† This work was supported by Grants PO1 CA43720, S10 RR04075, and T32 GM07347 from the National Institutes of Health. The mass spectrometry was carried out at the MIT Mass Spectrometry Facility, funded by a grant from the National Institutes of Health (RR00317 to Prof. K. Biemann). A preliminary account of some of this work was presented at the 1989 Annual Meeting of the Biophysical Society (Faulkner et al., 1989).

* Address correspondence to this author at his present address: Department of Molecular Biology, Vanderbilt University, Box 1820, Station B, Nashville, TN 37235.
‡ Department of Biochemistry, Vanderbilt University.
§ Department of Molecular Physiology and Biophysics, Vanderbilt University.
‖ Department of Chemistry, Massachusetts Institute of Technology.
⊥ Present address: Department of Cellular, Molecular and Structural Biology, Morton Building no. 4-672, Northwestern University Medical School, 320 East Superior St., Chicago, IL 60611.

[1] Abbreviations: Asp-N, endoproteinase Asp-N; BSA, bovine serum albumin; BS^3, bis(sulfo-*N*-succinimidyl)suberate; BSSDP, bis(sulfo-*N*-succinimidyl)doxyl-2-spiro-4′-pimelate; EGF, epidermal growth factor; mEGF, murine EGF as purified by the method of Savage and Cohen (1972); EGFα, the 53-residue intact form purified from mEGF; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; EPR, electron paramagnetic resonance; FAB, fast atom bombardment; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; HPLC, high-performance liquid chromatography; k_{off}, kinetic dissociation constant; $NaDodSO_4$, sodium dodecyl sulfate; PMSF, phenylmethanesulfonyl fluoride; PTH, phenylthiohydantoin; SSTPOC, sulfo-*N*-succinimidyl-2,2,5,5-tetramethyl-3-pyrolin-1-yloxy-3-carboxylate; TEA, triethylamine; TFA, trifluoroacetic acid; V_1, first-harmonic, in-phase, absorption EPR signal.

0006-2960/91/0430-8976$02.50/0

Staros et al. (1989), and Carpenter and Wahl (1990)] is a transmembrane glycoprotein that possesses an extracellular hormone-binding domain and an intracellular kinase domain in a single polypeptide chain (Buhrow et al., 1982, 1983; Ullrich et al., 1984). The binding of EGF to receptors on target cells stimulates this intrinsic tyrosyl-residue-specific protein kinase activity. The mechanism through which EGF binding results in the activation of kinase activity remains unclear, though a number of models have been proposed in which the oligomerization state of receptor molecules acts as a stimulatory (Schlessinger, 1986; Carraway et al., 1989; Staros et al., 1989) or inhibitory (Biswas et al., 1985) allosteric signal. There is evidence that the EGF receptor may exist in high- and low-affinity states (Shechter et al., 1978; King & Cuatrecasas, 1982; Kawamoto et al., 1983) in some cell types and that these states may be related to the oligomerization of the receptor (Boni-Schnetzler & Pilch, 1987; Yarden & Schlessinger, 1987).

The initial step by which extracellular hormone affects intracellular events is the binding of the hormone to the receptor to form specific hormone–receptor complexes in the plasma membranes of target cells. We have developed a new approach for the investigation of early events in this signal transduction pathway. To test the utility of this approach, we have applied this method to measurements of the kinetic dissociation constants of the EGF receptors in solution.

Intact murine EGF (EGFα) is a small protein with a mass of 6040 daltons. The EGF receptor is a transmembrane glycoprotein of $M_r \sim 170\,000$ in the monomeric form, which has been shown to dimerize upon binding EGF (Böni-Schnetzler & Pilch, 1987; Yarden & Schlessinger, 1987; Cochet et al., 1988; Fanger et al., 1989), so that an occupied receptor dimer complex has a total $M_r \sim 350\,000$. Thus the receptor complex is ~60 times larger by mass than free EGFα. The rotational diffusion of proteins in solution is determined by their relative sizes and shapes. Linear EPR, using nitroxide spin-labels at X-band microwave frequency is highly sensitive to changes in the rotational motions of spin-labeled proteins with correlation times (τ_c) from 10^{-11} to 10^{-7} s. The EPR spectrum of a spin-label attached to EGFα will reflect the rotational motion of the hormone, which is calculated to have a $\tau_c = 2.6 \times 10^{-9}$ s at 22 °C in aqueous solution.[2] Spin-labeled EGFα bound to its receptor should give an immobilized spectrum ($\tau_c > 1 \times 10^{-7}$ s) reflecting the slow rotational motion of the EGF–receptor complex. These distinct motional states allow one to differentiate and quantitate bound vs unbound hormone using linear EPR methods.

With a spin-label probe attached covalently at the amino terminus of EGFα, we were able to detect the change in rotational motion that occurs when spin-labeled EGFα interacts with its receptor. In order to apply this technique, it was necessary to satisfy the following conditions: The derivative must be prepared having the label covalently linked in such a way that the spin-label is sufficiently immobilized to give distinct EPR signals from the free and bound populations of the hormone. The spin-labeled derivative must be shown to be biologically active, indicative of normal binding to the receptor. The chemical homogeneity of the derivative must be established, which, in turn, requires that the hormone from which it is prepared be homogeneous. Moreover, it would be useful to know to which functional groups in the hormone the spin-label is covalently attached.

[2] Stokes–Einstein equation: $\tau_c = 4\pi\eta r_h^3/3kT$. At 22 °C for mEGF, with $r_h = 1.38 \times 10^{-7}$ cm, estimated from a calculation based on M = 6040, $\tau_c = 2.6 \times 10^{-9}$ s.

In this paper we describe the purification and characterization of EGFα modified with the bifunctional spin-label bis(sulfo-*N*-succinimidyl)doxyl-2-spiro-4′-pimelate in normal isotopic (Beth et al., 1986) and (^{15}N,$^{2}H_{16}$) isotopically substituted forms (Anjaneyulu et al., 1988), the latter to take advantage of the narrower line widths and greater signal-to-noise afforded by the use of ^{15}N,^{2}H spin-labels (Beth & Robinson, 1989). The sulfo-*N*-succinimidyl esters, which provide the reactive moieties in this reagent, react covalently with nucleophilic groups in proteins with the following order of reactivity observed at pH 7.4 and room temperature: imidazole > α-amino ≈ ϵ-amino ≫ thiolate ≈ phenolate; however, the *N*-acyl imidazole adduct formed by reaction with histidinyl residues is a transient product that undergoes either rapid hydrolysis or reaction with another nucleophile (Anjaneyulu & Staros, 1987). The high yields observed for reaction of sulfo-*N*-succinimidyl esters with proteins are attributable to their very slow rates of hydrolysis, as compared with their rates of reaction with nitrogen nucleophiles (Anjaneyulu & Staros, 1987). Since EGFα has no lysyl residues and no free cysteinyl residues (Taylor et al., 1972), the principal target for stable modification is the amino terminus of EGFα; however, tyrosyl residues within the span of the half-reacted spin-label attached to the amino terminus could react secondarily due to effectively increased local concentration of the reagent (Staros, 1988). From molecular modeling based on a solution structure of EGFα (Montelione et al., 1987), Tyr3 would appear to be the best candidate for this secondary reaction, provided that the label has first reacted with the amino terminus. Reaction with His22 could produce transient products, lowering the effective yield and specificity of the reaction.

The reaction of BSSDP with EGFα resulted in multiple products; therefore, a scheme for the purification and characterization of the various derivatives was developed. Two of the resulting derivatives will be discussed here. The major product purified from the reaction mixture by HPLC was found to be a monofunctional adduct resulting from the reaction of BSSDP with the amino terminus of EGFα and the hydrolysis of the second sulfo-*N*-succinimidyl group of the reagent. This derivative gives an EPR spectrum that indicates a partial immobilization of the label when the derivative is free in solution; however, when the derivative binds to the EGF receptor, the label becomes immobilized ($\tau_c > 1 \times 10^{-7}$ s) on the linear EPR time scale. This derivative was used in EPR measurements that allowed observation of the extent of binding and determination of the rate of dissociation of spin-labeled EGFα from solubilized receptor following addition of an excess of unlabeled mEGF. In these experiments it is important that the components of the spectrum which correspond to free and bound hormone be well resolved, a condition met by the relatively narrow line widths exhibited by this [^{15}N,$^{2}H_{16}$]-BSSDP-labeled EGFα derivative. A second product was found to be a bifunctional adduct of BSSDP and EGFα. This derivative was only partially characterized, as it was obtained in low yield and was apparently subject to breakdown to the monofunctional form; however, it gave a linear EPR spectrum indicative of a tighter motional coupling between spin-label and hormone. This type of derivative may potentially be more suitable for future investigations of the dynamics of occupied receptor complexes utilizing saturation transfer EPR techniques.

Experimental Procedures

^{125}I-mEGF was prepared as previously described (Carpenter & Cohen, 1976). [γ-^{32}P]ATP was obtained from ICN. En-

doproteinase Asp-N was obtained from Boeringer Mannheim, guanidine hydrochloride from Pierce, and iodoacetamide from Kodak, β-mercaptoethanol from Fluka, and acetonitrile and methanol were from Burdick and Jackson.

Peptide sequencing was performed on an Applied Biosystems model 470A sequencer according to the general method of Hewick et al. (1981). PTH derivatives were identified by absorbance at 269 nm following HPLC separation on a model 120A on-line analyzer with a Brownlee C-8 column.

EPR spectra were recorded with a Varian E112 spectrometer equipped with an E-238 high-volume aqueous cavity, as described (Beth et al., 1986), or a Bruker ESP 300, which was likewise equipped with an ER4103 high-volume aqueous cavity. The microwave and modulation fields at the sample for the Varian and Bruker TM_{110} cavities were calibrated with peroxylamine disulfonate (Beth et al., 1983). The sample temperature was regulated with a standard variable temperature controller by passing precooled nitrogen into the cavity through the front optical port via a specially constructed inlet. This arrangement resulted in a 1 °C temperature gradient over the active dimensions of the sample. All spectra shown are 100 G scans consisting of 1024 data points. Kinetic scans were recorded directly on the instrument's analogue recorder by setting the magnetic field sweep to zero at the peak of the low-field absorption line of unbound spin-labeled EGFα. Thus, the timed tracings gave a direct readout of the amplitude of this line as a function of time. These amplitude vs time tracings were manually digitized for data analyses.

A JEOL HX110 mass spectrometer (first half of the HX110/HX110 tandem mass spectrometer), operating at an accelerating voltage of 10 kV and 1:1000 resolution, was used to obtain the FAB–mass spectra of intact EGFα and EGFα derivatives (Biemann et al., 1986). The mass spectrometer was calibrated with $(CsI)_nCs^+$ cluster ions; the JEOL CS^+ gun was operated at 26 kV. Samples were dissolved in 2–3 μL of a 1:1 mixture of glycerol and 3-nitrobenzyl alcohol; about 0.5–0.75 μL of the solution was applied on the FAB probe and introduced into the mass spectrometer ion source. Typically, one such load was sufficient to obtain a single scan over a mass range of several thousand mass units or a number of scans over a narrower mass range of several hundred mass units. For each different sample, 5–8 scans were summed to improve the signal-to-noise ratio. All mass spectra shown are raw data profiles and were acquired with a JEOL DA5000 data system interfaced to a DEC PDP11/73 computer.

Murine EGF (mEGF) was prepared as described by Savage and Cohen (1972). This preparation was found to be heterogeneous, containing species missing one or two residues from the amino terminus. The 53 residue intact form of mEGF, EGFα, was purified with a Waters model 650 protein purification system fitted with a reverse-phase 21.4 × 250 mm, 150-Å pore size Dynamax C-18 column (Rainin). The column was equilibrated with 0.1% TFA in an aqueous/acetonitrile system. Elution by a linear gradient from 20% to 30% acetonitrile, with a flow rate of 7.5 mL/min, over 90 min achieved baseline separation. The EGFα peak was collected and then lyophilized and stored at −70 °C. Normal isotope and [^{15}N,$^{2}H_{16}$]BSSDP were prepared as previously reported (Beth et al., 1986; Anjaneyulu et al., 1988). EGFα derivatives prepared with the normal isotope spin-label were used for biological and chemical characterization, reserving the [^{15}N,$^{2}H_{16}$]BSSDP-labeled hormone for EPR spectroscopy.

Labeling of EGFα with BSSDP. Freshly prepared 10 mM solutions of either normal isotope BSSDP or [^{15}N,$^{2}H_{16}$]BSSDP in water were added to solutions of EGFα to give a 13.5–25-fold molar excess of reagent. Final concentrations of BSSDP were 1.35–2.5 mM and EGFα was 0.1 mM, in 50 mM HEPES, pH 7.6. The reaction was allowed to proceed for 12 h at room temperature (the pH was noted to remain above 7.4), then the mixture was stored frozen at −20 °C until HPLC separation.

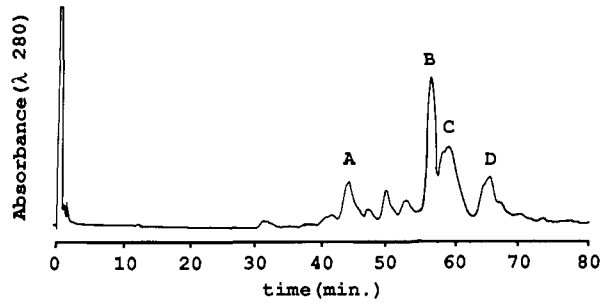

FIGURE 1: HPLC separation of spin-labeled derivatives of EGFα. In the data presented, EGFα was reacted with a 13.5-fold molar excess of [^{15}N,$^{2}H_{16}$]BSSDP, and the resulting products were separated by HPLC on a 4.6 × 220 mm Brownlee C-8 cartridge equilibrated with 20 mM triethylammonium acetate, pH 4.0/methanol (75:25 v/v) and eluted with a linear gradient of 25–45% methanol over 80 min. Fractions were collected and assayed by EPR, and fractions corresponding to peaks labeled A, B, C, and D were separately pooled.

Purification of Spin-Labeled Derivatives of EGFα. Reaction products were separated by reverse-phase HPLC using a 4.6 × 220 mm Brownlee C-8 Aquapore cartridge. The column was equilibrated with 20 mM triethylammonium acetate, pH 4.0, methanol (75:25 v/v), and the temperature was maintained at 37 °C. Elution was carried out with a linear gradient of increasing methanol, from 25% to 45%, with a flow rate of 2 mL/min, over 80 min, controlled by a Spectra Physics model SP8700 solvent delivery system. Peak fractions were detected by absorbance at 280 nm with an ISCO model V^4 detector (Figure 1). Fractions were collected and assayed for covalent incorporation of spin-label by EPR. Peak fractions (A–D) of BSSDP-labeled EGFα were pooled and reinjected and finally quantitated by absorbance at 280 nm and by spin density. Final purity was assessed by analytical reinjections on a 2.1 × 220 mm Brownlee C-8 Aquapore cartridge equilibrated in 0.1% TFA/acetonitrile (15:85 v/v). Products were eluted in a gradient of increasing acetonitrile from 15% to 50% over 80 min with a flow rate of 0.2 mL/min by using Waters 510 pumps. Absorbance at 214 and 280 nm was followed with a Waters 490E detector system fitted with microbore tubing. The system was controlled by a Maxima 820 workstation.

Biological Characterization of Spin-Labeled EGF. The relative abilities of BSSDP-labeled EGFα peak B and unlabeled mEGF to compete for binding with ^{125}I-mEGF were assayed by established methods (Carpenter, 1985). Human fibroblasts grown to confluence in 30-mm dishes were washed twice at room temperature with binding medium: Delbecco's modified Eagle's medium with the addition of 0.2% BSA. After the second wash, 0.5 mL of binding medium was added, and the dishes were chilled on ice for 15 min. To a duplicate set of four dishes was added 10, 20, 40, or 500 ng of mEGF, and to a parallel duplicate set of three dishes was added 10, 20, or 40 ng of BSSDP-labeled EGFα peak B. To each dish was then added 16.5 ng of ^{125}I-mEGF. After a 2-h incubation at 4 °C, cells were washed twice with ice-cold Hank's balanced salt solution supplemented with 0.2% BSA and then solubilized by addition of 1 N NaOH and counted. A second assay, employing A431 cell membrane vesicles prepared as in Cohen et al. (1982), was used to compare the abilities of BSSDP-

labeled EGFα peaks B and C to compete with ^{125}I-mEGF for binding. In this assay, a 20-μL aliquot of an A431 vesicle suspension (20 μg of membrane protein) was added to a total assay volume of 100 μL, containing 20 ng of ^{125}I-mEGF and 20, 40, or 80 ng of the BSSDP-labeled EGFα derivative or 600 ng of mEGF to detect nonspecific binding, each in duplicate. Following a 10-min incubation at room temperature, separation of bound from free ^{125}I-mEGF was accomplished by vacuum filtration using 0.2-μm pore size cellulose acetate filters. Following four washes with cold 20 mM HEPES containing 0.1% BSA, filters were dried and counted. In addition, BSSDP-labeled EGFα peak B was compared with mEGF in its ability to stimulate autophosphorylation of EGF receptors in soluble cell extracts as in Fanger et al. (1989). The ability of BSSDP-labeled EGFα peak B to stimulate dimerization of receptor in solution was assayed by cross-linking with bis(sulfo-*N*-succinimidyl)suberate (BS^3), following the method of Fanger et al. (1989).

Spectroscopic Characterization. EPR spectra of HPLC fractions and pooled peaks of BSSDP-labeled EGFα in aqueous buffer were recorded at room temperature with the Varian instrument. In order to assess the independent motion of the spin-label covalently bound to EGF relative to the overall motion of the hormone, EPR spectra of a 5 μM solution of [^{15}N,$^{2}H_{16}$]BSSDP-labeled EGFα peak B in 25 mM HEPES and a parallel sample containing 50% glycerol were recorded at 2 °C with the Bruker ESP300; then the glycerol-containing sample was cooled to −17 °C, and the resulting spectrum was recorded.

Chemical Characterization. Samples of EGFα and BSSDP-labeled EGFα peak B were reduced, and cysteinyl residues were modified following the method of Gracy (1977), except that iodoacetamide replaced iodoacetic acid. Reduced, carbamidomethylated peptides were desalted by HPLC in the 0.1% TFA/acetonitrile system described above. Following lyophilization, digestion was performed in 50 mM HEPES, pH 7.6, with endoproteinase Asp-N for ~36 h with an enzyme-to-protein ratio of 1:20. Digestion was allowed to proceed at 37 °C until completed, as assessed by analytical HPLC injections. Peptide mapping was performed with the 2.1 × 220 mm C8 Brownlee Aquapore cartridge column with the microbore Waters system described above, with a gradient from 10–60% acetonitrile over 80 min. Peptide fragments purified by this method were subjected to FAB–mass spectrometry. Additionally, aliquots of Asp-N total digests of both EGFα and BSSDP-labeled EGFα peak B were submitted for peptide sequencing to detect modification of the amino terminus. Asp-N digests were also performed on EGFα and BSSDP-labeled EGFα peak B in 100 mM ammonium acetate, pH 6.5, without reduction and carboxyamidomethylation. Following lyophilization, these samples, with triethylphosphine added to effect reduction of the disulfide bonds, were subjected to FAB–mass spectrometry. FAB–mass spectra of these samples and of HPLC fractions from digests of EGFα and BSSDP-labeled EGFα peak B were acquired at 1:2600 resolution as single scans at a rate of scan from m/z 200 to m/z 5000 in 2 min, with 100–300 Hz filtering. All other parameters remained as described above. Finally EGFα and BSSDP-labeled derivatives of EGFα were subjected to analysis by FAB–mass spectrometry. The measurements of the average m/z values for the protonated molecules were carried out by recording first a single-scan mass spectrum of each sample from m/z 2000 to m/z 8000. A region of about 200 mass units above and below the molecular weight of the sample thus determined was subsequently scanned repeatedly at a rate to cover, for example, m/z 6000 to m/z 6400 in 20 s, with 30-Hz filtering.

Preparation of Soluble Receptor. Membrane vesicles (1.5 mL) prepared from A431 cells as previously described (Cohen et al., 1982) were solubilized in 20 mM HEPES, pH 7.4, containing 5% Triton X-100, 10% glycerol, 1 mM PMSF, and 1.6 mM EGTA in a total volume of 4 mL. The suspension was mixed well during incubation at room temperature for 15 min and then was subjected to centrifugation at 200000*g* for 20 min at 4 °C. The resulting clear supernatant was added to 1.5 mL of packed wheat germ lectin agarose beads (Vector), and this suspension was incubated overnight at 4 °C in a small column with rocking. The column was then washed with 45 mL (30 volumes) of 20 mM HEPES, pH 7.4, 0.1 M NaCl, 0.02% Triton X-100, and 10% glycerol. The receptor was eluted by incubation for 30 min at 4 °C with 1.5 mL of the above buffer with the addition of 2.5 mg/mL *N,N′,N″*-triacetylchitotriose (Sigma). The solubilized receptor was then concentrated to a volume of ~0.3 mL by ultrafiltration using an Amicon CF50A Centriflow cone.

Binding of [^{15}N,$^{2}H_{16}$]BSSDP-labeled EGFα Peak B to Receptor. [^{15}N,^{2}H$_{16}$]BSSDP-labeled EGFα peak B was added to the receptor preparation, resulting in a final concentration of 0.8 μM spin-labeled EGF (5 μg/mL), and the mixture was allowed to incubate for 30 min at 4 °C. The sample was then introduced into the flat cell as described below, and a time-averaged spectrum was collected over 60 min on the Bruker ESP300.

Dissociation Kinetics. A specifically designed flat cell (Wilmad Glass, WG-812) was modified for low-volume mixing in a chamber immediately below the flat portion of the cell. Two low-volume Tygon inlet tubes, passed up through the bottom quartz support tube, were accessed via Hamilton syringes to allow the rapid addition of mEGF to the sample, which could be removed and reintroduced into the cavity without retuning. The sample was introduced into the flat cell, and a baseline spectrum was collected as described above. The proportion of bound signal was ascertained and was determined to be stable. The center field was then set on resonance with the peak of the low-field line of the free signal, and the amplitude was determined to be stable. At this point, the sample had equilibrated for ~2 h at 2 °C. The addition of a 50-fold excess of mEGF was achieved by withdrawing the sample and then reinjecting the 0.25-mL sample with the simultaneous injection of 12.5 μL of 5 μg/μL mEGF through the second inlet. In the second experiment shown, the receptor was prepared as described above, with the exception that the buffer used in the final wash and elution contained 5% glycerol, as compared with the 10% glycerol used with the first preparation. In this case, a standard flat cell equipped with a single low-volume Tygon inlet tube was used. To a 0.27-mL sample of solubilized receptor preparation, equilibrated with 0.8 μM [^{15}N,^{2}H$_{16}$]BSSDP-labeled EGFα peak B, the addition of a 100-fold excess of mEGF was achieved by drawing the sample out of the flat cell into an ice-cold syringe in which it was mixed with 30 μL of 5 μg/μL mEGF and reinjected into the flat cell. To assure complete mixing, the sample was drawn out and reinjected once more. In both protocols, as soon as the mixed sample was returned to the cavity, the microwave frequency was locked on and the change in the amplitude of the low-field line was followed. The process of mixing and relocking the frequency took ~10 s.

Results

Preparation and Purification of BSSDP-Labeled EGFα. Reaction of BSSDP with EGFα resulted in a mixture of

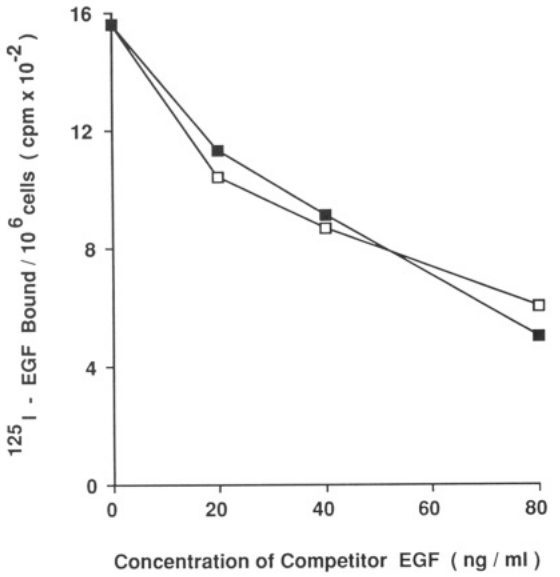

FIGURE 2: Competitive binding assay for mEGF and BSSDP-labeled EGFα peak B in the presence of ^{125}I-mEGF. Human fibroblasts were grown to confluence in 30-mm dishes. Binding assays were in 0.5 mL of DMEM with 0.2% BSA. Additions of 10, 20, or 40 ng of mEGF or BSSDP-labeled EGFα peak B and 16.5 ng of ^{125}I-mEGF were made. Following incubation for 2 h at 4 °C, cells were washed thoroughly, solubilized by addition of 1 N NaOH, and counted. The raw data were corrected for nonspecific binding, as determined by addition of a 30-fold excess of mEGF (and accounted for 12% of maximal binding).

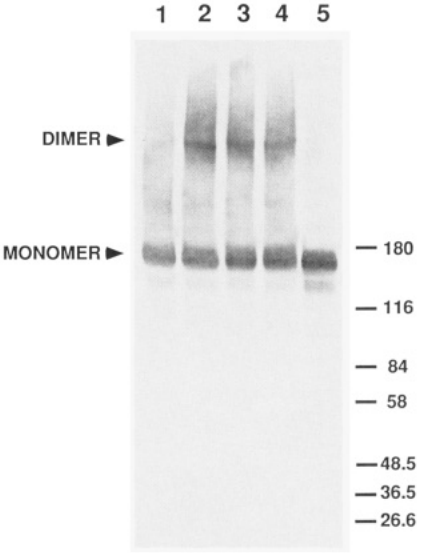

FIGURE 3: Cross-linking of EGF-induced receptor dimers. Aliquots of A431 cell extracts were incubated for 15 min in the absence of hormone (lane 1) or in the presence of 1 μM spin-labeled EGFα peak B (lane 2), mEGF (lane 3), or EGFα (lanes 4 and 5). Samples in lanes 1–4 were then cross-linked by addition of BS^3 (to 1 mM) and incubation at room temperature for 15 min. The sample in lane 5 was incubated in parallel but without the addition of BS^3. After the reactions were quenched, samples were separated by NaDodSO$_4$–polyacrylamide gel electrophoresis in 3–10% acrylamide gels under the conditions of Laemmli (1970). After electrophoretic transfer to nitrocellulose (Wang et al., 1989), bands containing the receptor were visualized by immunostaining with antiserum to the EGF receptor followed by alkaline phosphatase-linked anti-IgG. Relative molecular weight standards are indicated at the right.

products, as shown by the preparative chromatogram in Figure 1. When fractions were subjected to linear EPR spectroscopy (see below), peak A had little if any signal, peaks B and D exhibited essentially identical motional characteristics, and peak C, though not completely homogeneous, had a component with a significantly longer τ_c than the others. The major product, peak B, eluting at ~57 min, gave a homogeneous peak upon reinjection and gave a single-component EPR spectrum. Peak C, eluting at ~60 min, was obtained in relatively low yield; however, sufficient material was purified to enable a partial characterization. Since the yield of peak D was very low, it was not further characterized.

Biological Characterization of BSSDP-Labeled-EGFα. In a competitive binding assay on intact fibroblasts, BSSDP-labeled EGFα peak B was as effective as native mEGF in displacing ^{125}I-mEGF, as shown in Figure 2. BSSDP-labeled EGFα peak C competed equally with peak B in a separate binding assay that used membrane vesicles from A431 cells (data not shown). BSSDP-labeled EGFα peak B substituted effectively for native mEGF in stimulation of receptor autophosphorylation (data not shown). The ability of peak B to induce receptor dimerization, demonstrated by cross-linking with BS^3 is shown in Figure 3. Results of these experiments indicated that BSSDP-labeled EGFα peak B binds, stimulates the kinase activity, and induces dimerization of solubilized EGF receptors with activities equivalent to those of mEGF.

Spectroscopic Characterization. V_1 EPR spectra of fractions corresponding to [^{15}N,^{2}H$_{16}$]BSSDP-labeled EGFα peaks B and C are shown in Figure 4. BSSDP-labeled EGFα peak B exhibits a single-component EPR spectrum from which τ_c was estimated to be $\sim 0.7 \times 10^{-9}$ s at 22 °C. This relatively rapid τ_c is indicative of some degree of freedom between the label and EGF. The spectrum of BSSDP-labeled EGFα peak C is characterized by two motional components, one of which is significantly slower than that of BSSDP-labeled EGFα peak B. The slow component in this product peak was estimated to have a $\tau_c \approx (2\text{–}3) \times 10^{-9}$ at 22 °C, in agreement with the theoretical value for EGF calculated by using the Stokes–Einstein equation.[2] The faster component varied in proportion between preparations and increased during storage at −20 °C.

The properties of [^{15}N,^{2}H$_{16}$]BSSDP-labeled EGFα peak B were further examined in a low-temperature spectroscopic study. At low temperatures in 50% glycerol, BSSDP-labeled EGFα peak B exhibited slowed tumbling motions reflected in characteristic changes occurring in the line shapes of the V_1 spectra shown in Figure 5. In the V_1 spectrum acquired with the sample containing 50% glycerol at −17 °C, the spin-label was at the no-motion limit. This is suggestive of significant, though not complete, motional coupling between the nitroxide and the peptide backbone. For comparison, in preliminary experiments in which mEGF was modified with a monofunctional spin-label with the same reactive group, sulfo-*N*-succinimidyl-2,2,5,5-tetramethyl-3-pyrrolin-1-yloxy-3-carboxylate (SSTPOC), a V_1 EPR spectrum of this product in 50% glycerol at −19 °C indicated significantly greater motional freedom of the nitroxide from the polypeptide backbone (Faulkner, 1991) than that indicated in the lower spectrum in Figure 5.

Chemical Characterization. Samples of BSSDP-labeled EGFα peak B and unmodified EGFα were reduced and treated with iodoacetamide and then desalted and digested with endoproteinase Asp-N; and the resulting peptides were separated by HPLC (Figure 6). Three of the five expected peptide fragments (I–III) in the control digest (Table I) were identified by FAB–mass spectroscopy. Only peptides I, II, and III contain functional groups that are potentially reactive with BSSDP (Anjaneyulu & Staros, 1987). A comparison of Figure 6B, the control EGFα map, with Figure 6A, the elution profile resulting from digestion of BSSDP-labeled EGFα peak B, shows a shift of the peak identified as the amino-terminal

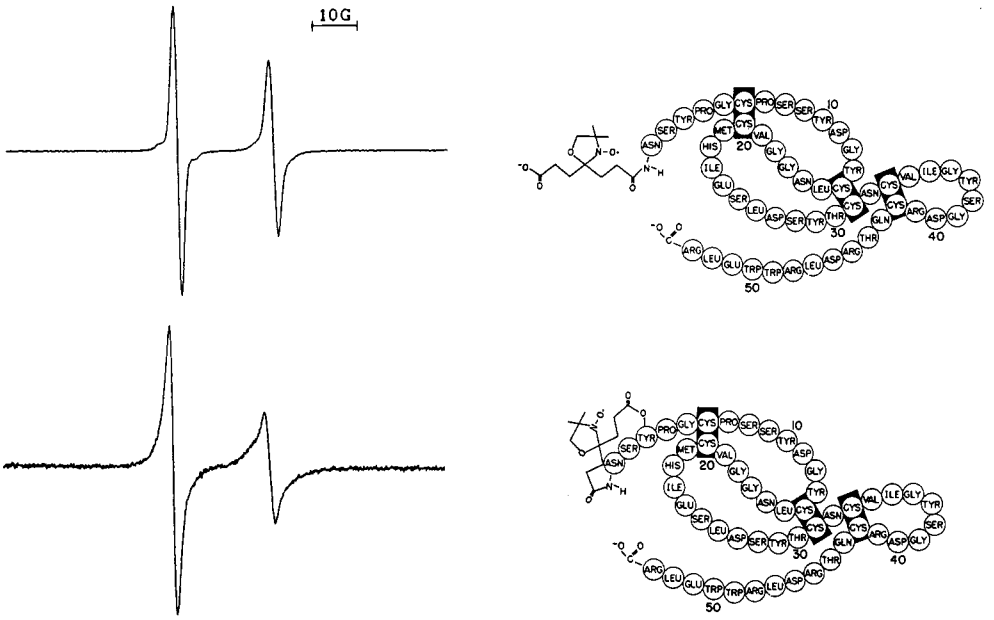

FIGURE 4: V_1 EPR spectra of [^{15}N,$^{2}H_{16}$]BSSDP-labeled EGFα peaks B and C. Spectra were obtained from concentrated peak fractions corresponding to labeled peaks B and C (Figure 1) at 22 °C with the Varian E112 with a modulation amplitude of 1.0 G and a microwave power of 5 mW. Spectra of peak B (upper) and peak C (lower) are shown on the left. The panels on the right represent the proposed structures of peak B (upper) and the slow component of peak C (lower).

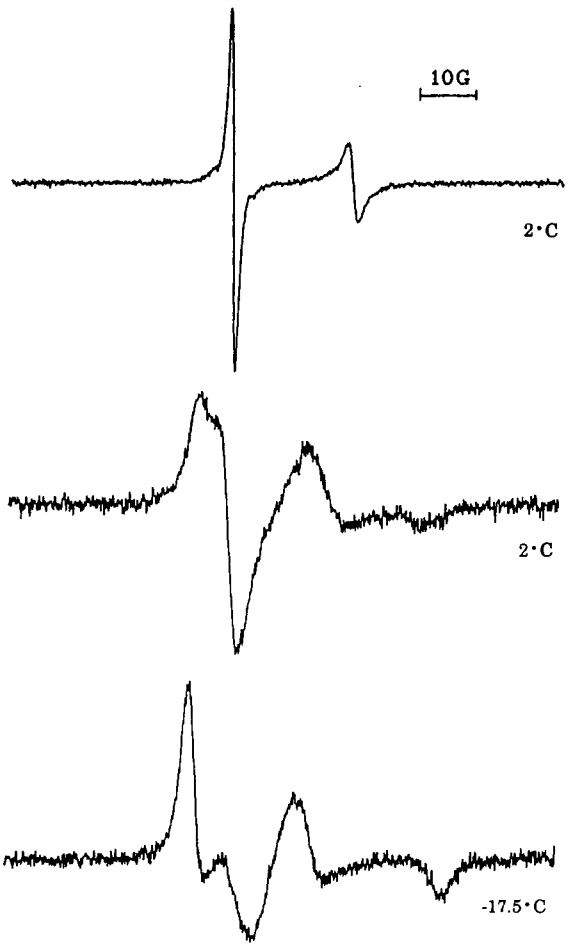

FIGURE 5: Effect of viscosity on V_1 spectra of [^{15}N,$^{2}H_{16}$]BSSDP-labeled EGFα peak B. Spectra of [^{15}N,$^{2}H_{16}$]BSSDP-labeled EGFα peak B in 25 mM HEPES at 2 °C (upper) and in 25 mM HEPES with 50% glycerol at 2 °C (middle) and at −17 °C (lower) were obtained with the Bruker ESP-300 with a modulation amplitude of 0.5 G and a microwave power of 10 mW.

peptide I. In order to confirm the position of attachment within this peptide, equivalent samples of BSSDP-labeled

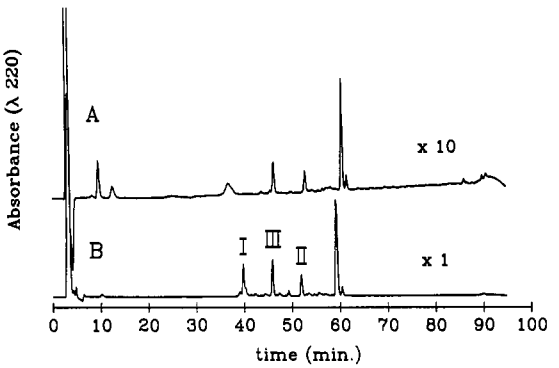

FIGURE 6: Peptide maps of EGFα and spin-labeled EGFα peak B digested by Asp-N. Asp-N digests of reduced, carbamidomethylated (A) spin-labeled EGFα peak B and (B) unlabeled EGFα were analyzed by reverse-phase HPLC. Elution was by a linear gradient from 10–60% acetonitrile over 80 min. Peptides expected to result from Asp-N digests of EGFα and their masses are shown in Table I. Peptide fragments I–III, as identified by FAB–mass spectrometry, are indicated in panel B.

Table I: Peptides Resulting from Asp-N Digestion of EGF

		calcd m/z^b	
fragment	amino acid sequence[a]	reduced[c]	Cys modified[d]
I	NSYPGCPSSY	1075.1	1132.2
II	DGYCLNGGVCMHIESL	1712.0	1826.1
III	DSYTCNCVIGYSG	1382.5	1496.6
IV	DRCQTR	788.9	835.9
V	DLRWWELR	1174.3	1174.3

[a] Expected amino acid sequence of Asp-N cleavage products, based on the known sequence (Savage et al., 1972) and Asp-N specificity (Drapeau, 1980). [b] Calculated m/z values for singly protonated expected Asp-N cleavage products obtained by using averaged isotopic abundances. [c] Cysteines reduced. [d] Cysteines reduced and carbamidomethylated.

EGFα peak B and unmodified EGFα were subjected to 10 cycles of Edman degradation. The sample of unmodified EGFα yielded the expected sequence in all 10 cycles with the exception of cycle 6, in which the unprotected Cys, as expected, yielded no identifiable residue. In contrast, the BSSDP-labeled

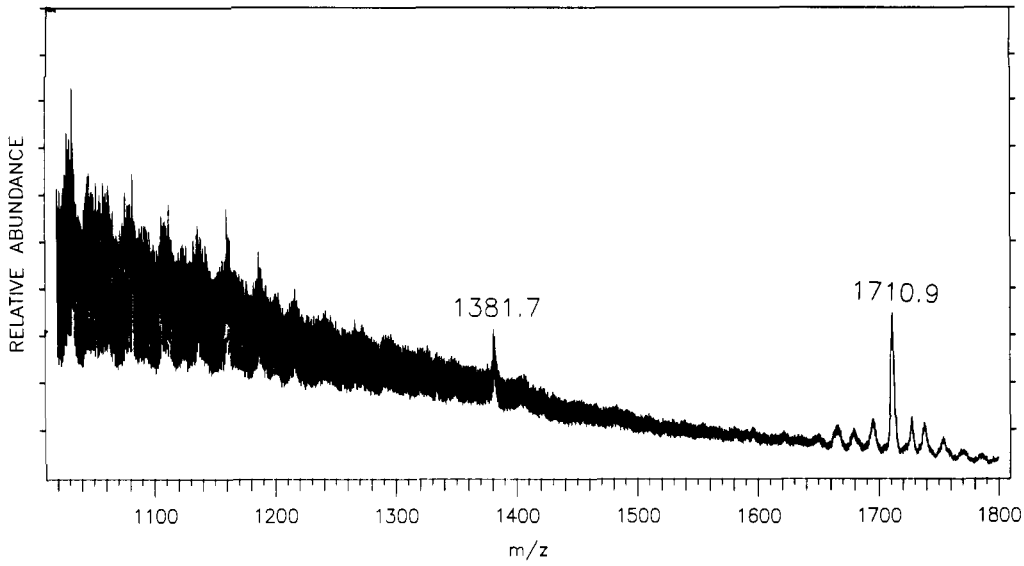

FIGURE 7: FAB–mass spectrum of the digest of BSSDP-labeled EGFα peak B with Asp-N. Disulfide bonds were reduced with Et_3P. The section of the mass spectrum shown reveals molecular ions at m/z 1710.9 and 1381.7, corresponding to unmodified peptides II and III (see Table I).

EGFα peak B sample yielded essentially nothing in cycles 3–10. In cycles 1 and 2, Asn and Ser were detectable but were not the predominant peaks, suggesting that they originated from contamination of the sample rather than from EGFα with an unmodified amino terminus. These data support the hypothesis that the amino terminus in BSSDP-labeled EGFα peak B is blocked by reaction with BSSDP. Evidence that the amino-terminal peptide is the sole site of modification in this product was obtained by FAB–mass spectrometry performed on an aliquot BSSDP-labeled EGFα peak B, digested with Asp-N in ammonium acetate buffer to minimize Na^+ contamination. The detection of peaks at m/z = 1710.9 and m/z = 1381.7 (Figure 7) demonstrates that peptides II and III are present and unmodified in BSSDP-labeled EGFα peak B.[3]

To address the question of whether BSSDP was attached to the spin-labeled EGFα derivatives at one or two sites, intact EGFα and derivatives prepared with normal isotope BSSDP were subjected to FAB–mass spectrometry. Figure 8 shows the results of these studies. The intact EGFα gave a molecular ion m/z = 6040.8, which agrees well with the calculated mass for protonated unmodified EGFα (6040.6). BSSDP-labeled EGFα peak B gave a molecular ion m/z = 6282.9, consistent with the calculated mass of the monofunctionally modified hormone (6282.8). Product peak C gave a mixture of molecular ions. The major ion was at m/z = 6264.9, consistent with the reagent being attached at two points in this product (calculated mass 6264.8). There was also a molecular ion at 6282.9, which is likely to represent the product of hydrolysis of the ester linkage to the side chain of Tyr3. This result would also explain the appearance of a relatively fast (~1 ns) component in the V_1 spectrum. Schematic models of the proposed structures of BSSDP-labeled EGFα peak B and the slow bifunctionally labeled component of peak C that are consistent with these data are presented in Figure 4.

Binding of BSSDP-Labeled EGFα Peak B to Receptor. The spectra of [^{15}N,$^{2}H_{16}$]BSSDP-labeled EGFα peak B in the presence of soluble receptor before and after the addition of excess mEGF are shown in Figure 9. The upper spectrum is composed of well-resolved components resulting from

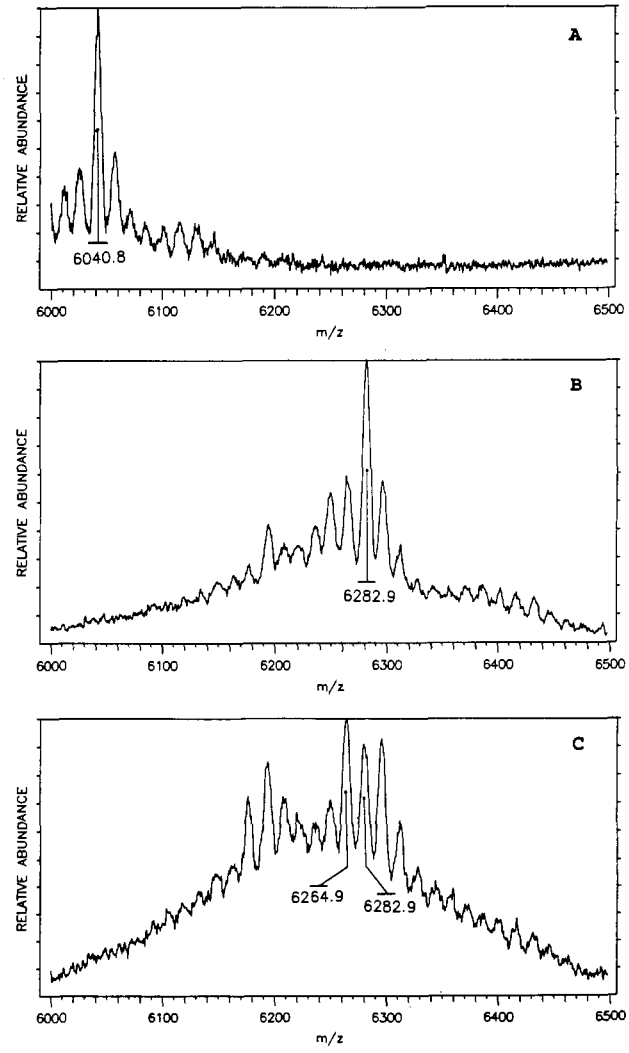

FIGURE 8: FAB–mass spectra of unmodified EGFα and BSSDP-labeled EGFα derivatives. Unmodified EGFα (panel A) and normal isotope BSSDP-labeled EGFα peak B (panel B) and peak C (panel C) were subjected to positive ion FAB–mass spectrometry, and the resulting major ions were interpreted as detailed in the text.

BSSDP in two distinct motional environments that are likely to represent bound and unbound spin-labeled hormone.[4] The

[3] In FAB–mass spectra of peptide mixtures, not infrequently some of the component peptides go undetected. This may be due to competition between peptides for the matrix surface (Naylor et al., 1986).

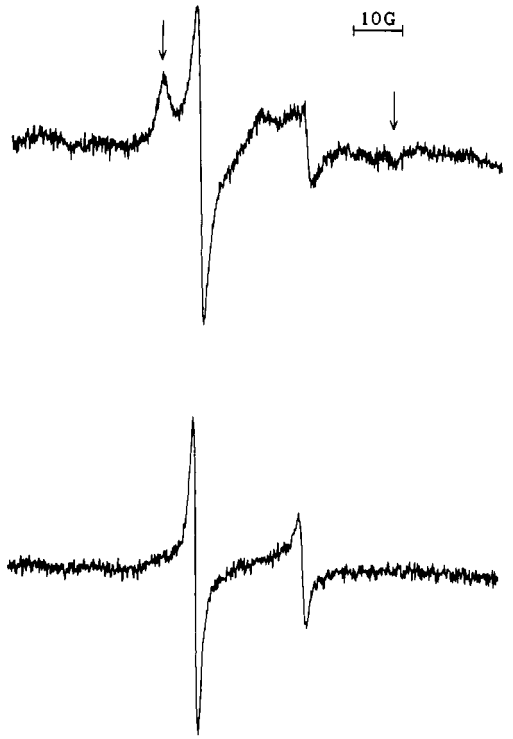

FIGURE 9: Binding of [^{15}N,^{2}H$_{16}$]BSSDP-labeled EGFα peak B to the solubilized receptor. The upper V_1 spectrum was obtained from spin-labeled EGFα peak B at 2 °C, in the presence of solubilized partially purified EGF receptor. Arrows indicate spectral lines that are unique to the immobilized component. Note the absence of dipolar splitting in the immobilized component. The lower V_1 spectrum was obtained from the same preparation following the addition of excess unlabeled mEGF and reequilibration for ~1 h at 2 °C. Both spectra were recorded with the Bruker ESP-300 with a modulation amplitude of 2.0 G and a microwave power of 10 mW. Spectra are shown normalized.

slower motion, bound signal, represents approximately 80% of the spin-labeled EGF, as estimated by comparison of the line heights before and after dissociation. This line shape exhibits a splitting of 48 G, indicative of a rotational motion of the probe with a $\tau_c > 1 \times 10^{-7}$ s (Beth & Robinson, 1989). With this degree of immobilization and with the narrow line width afforded by the [^{15}N,^{2}H$_{16}$]BSSDP label, dipolar interactions should be observed if the two labels are within 16 Å (Beth et al., 1984). The absence of detectable dipole–dipole interactions in this spectrum indicates a minimum separation of 16 Å between nitroxide moieties of the spin-labeled EGF molecules bound to solubilized receptors. The bound component disappeared following the addition of a 50-fold excess of unmodified mEGF, as demonstrated by the lower spectrum. This process was further examined as described below.

Kinetics of Dissociation of Spin-Labeled EGFα Peak B in the Presence of mEGF. The change in amplitude of the free signal vs time, following addition of excess unlabeled mEGF in the experiments described above, is plotted in Figure 10. A nonlinear analysis of these data (Beechem et al., 1985) requires two exponentials to fit these dissociation curves. According to independent analyses, the faster component had a lifetime of 0.84 min in the first experiment and 1.1 min in the second. The linked analysis of these two experiments yielded a well-defined value, i.e., a well-defined minimum in

[4] That the slower motion component did not result from the presence of detergent micelles was shown by a spectrum of BSSDP-labeled EGFα peak B in HEPES buffer containing 1% Triton X-100 and 10% glycerol obtained at 2 °C, in which only a single fast component was detected.

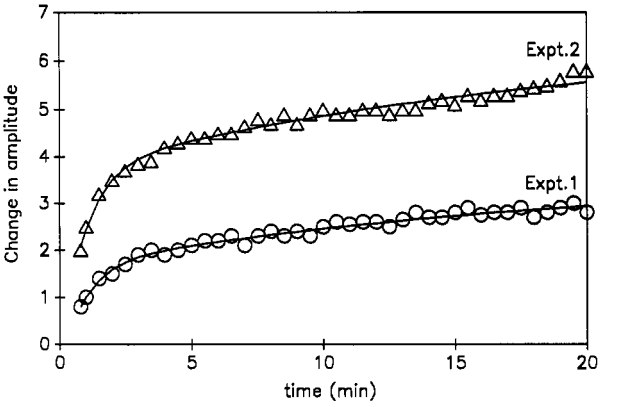

FIGURE 10: Kinetics of dissociation of [^{15}N,^{2}H$_{16}$]BSSDP-labeled EGFα peak B in the presence of excess unmodified mEGF. Dissociation of [^{15}N,^{2}H$_{16}$]BSSDP-labeled EGFα peak B was followed by continuously monitoring the low-field line corresponding to the unbound hormone. The change in amplitude of that line is plotted vs time. The different symbols represent the two independent experiments described in the text. The curves represent the results of a linked exponential analysis of these data.

the calculated variance, of 0.95 min for the faster component. The longer component was more problematic, as it was not possible to collect data for the long times necessary to establish an accurate value for this component, as minor fluctuations in ambient temperature affect instrument stability, and the magnitude of this effect becomes critical as the rate of change in amplitude decreases. However, the data that were collected suggest that this second component has a lifetime >20 min.

Discussion

We reacted BSSDP with EGFα and purified the products by HPLC (Figure 1). The major product, BSSDP-labeled EGFα peak B, was thoroughly characterized and found to have biological and spectroscopic properties appropriate for its application to kinetic studies. BSSDP-labaled EGFα peak B was found to be equipotent with mEGF in competition binding studies and in stimulating autophosphorylation and dimerization of the receptor (Figures 2 and 3). We characterized BSSDP-labeled EGFα peak B as a monofunctional adduct having BSSDP reacted with the amino terminus of EGFα (Figures 6–8). Freely tumbling in aqueous solution at 22 °C, it gave a linear EPR spectrum indicating relatively fast ($<1 \times 10^{-9}$ s) rotational motion (Figure 4); however, at low temperatures in 50% glycerol, it exhibited slowed tumbling motions that were reflected by characteristic changes occurring in the V_1 spectra shown in Figure 5. In the spectrum acquired with the sample at −17 °C, the spin-label was at the no-motion limit. This is indicative of significant immobilization of the probe on EGF. In the context of our preliminary studies using mEGF modified with SSTPOC, which indicated greater mobility of the nitroxide under comparable conditions, it would appear that the carboxylate resulting from the hydrolysis of the second sulfo-*N*-succinimidyl ester of BSSDP interacts e.g., by hydrogen bonding, with the EGFα molecule to which the other end of the reagent is covalently attached.

In the presence of a detergent-solubilized preparation of EGF receptors, [^{15}N,^{2}H$_{16}$]BSSDP-labeled EGFα peak B binds and becomes immobilized on the linear EPR time scale. This change in motion is reflected in the composite spectrum with a decrease of the relatively sharp absorbance at 3475 G and appearance of a broader downfield absorbance at 3468 G (Figure 9). When this spin-labeled EGFα derivative is bound to the receptor, the carboxylate moiety of the hydrolyzed second arm of the reagent presumably makes noncovalent

interactions with the hormone–receptor complex, resulting in the observed immobilization of the spin-label. This speculation is supported by experiments in which SSTPOC-labeled mEGF exhibited much less immobilization of the spin-label on EGF when it was bound to receptor (Faulkner, 1991).

Using [^{15}N,^{2}H$_{16}$]BSSDP-labeled EGFα peak B, we first obtained data on the binding of EGF to its solubilized receptor at equilibrium, demonstrating that binding occurs and is in stable equilibrium at 2 °C (Figure 9). Experimental spectra were obtained that indicated immobilization of ~80% of the spin-label on the linear EPR time scale. The absence of dipolar interactions in these spectra indicates a minimum separation of 16 Å between the doxyl moieties at the amino termini of spin-labeled EGFα molecules bound to solubilized receptors, under conditions in which a significant fraction of receptors is presumed to be dimeric. Fluorescence measurements by Carraway et al. (1988) in A431 membrane vesicles suggest that the distance between binding sites in receptor aggregates is <100 Å. Further investigations using solubilized receptor preparations are underway that will attempt to more precisely determine this distance.

The addition of a 50- or 100-fold excess of unlabeled mEGF to our preparations allowed observation of the dissociation of [^{15}N,^{2}H$_{16}$]BSSDP-labeled EGFα peak B, which appeared to have two components, the faster of which displayed a k_{off} = 1.8×10^{-2} s^{-1} (Figure 10). This is the first study of which we are aware in which EPR spectroscopy has been used to measure the interaction of a polypeptide hormone with, and its dissociation from, its receptor. The study of Mayo et al. (1989) included measurement of the dissociation kinetics of ^{125}I-mEGF that was bound to receptor on human fibroblasts in monolayer culture at 4 °C. Fibroblasts were incubated in the presence of ^{125}I-mEGF. At various times before and after reaching equilibrium, unlabeled mEGF was added. Dissociation was found to be biphasic with an initial fast component, $k_{off} = 1.5 \times 10^{-2}$ s^{-1}, and a slow component, $k_{off} = 5.6 \times 10^{-5}$ s^{-1}. The slow component increased proportionally with longer equilibration times. The two-component dissociation that we observe in a solubilized receptor preparation and the value that we obtain for the fast component are in reasonable agreement with Mayo's studies on whole cells.

Investigations of binding kinetics show a substantial variation in reported values for k_{off}, which may in part be attributed to methodological differences. Problems such as inhomogeneous ligand, difficulties in ascertaining the position of equilibrium prior to the dissociation experiment, and the effects of temperature on ligand internalization and degradation often make these data difficult to interpret. Various monophasic k_{off} values have been obtained for fibroblasts of 1.2×10^{-2} s^{-1} (Knauer et al., 1984) and 3.6×10^{-3} s^{-1} (Wiley, 1988) for human cells and 2×10^{-3} s^{-1} for fetal rat lung fibroblasts (Waters et al., 1990). For membranes prepared from A431 cells, Wiley (1988) obtained a limiting k_{off} value of 1.8×10^{-3} s^{-1}. In investigations by van Bergen en Henegouwen et al. (1989) two components of dissociation were noted to occur in A431 cells with k_{off} values of 1.1×10^{-3} and 3.5×10^{-5} s^{-1}, and biphasic dissociation was also observed in A431 cytoskeleton preparations. It was noted in these latter studies that a 2-h preincubation with EGF appeared to cause the association of a low-affinity receptor population to cytoskeletons prepared from pretreated cells. There also appeared to be a significant increase in the proportion of receptors on these cytoskeleton preparations showing fast dissociation. A possible relationship between low- and high-affinity states of the receptor and fast and slow dissociating populations of EGF is intriguing, but as yet unclear.

BSSDP-labeled EGFα peak C was not completely characterized, but on the basis of FAB mass spectrometric data, peak C most likely represents the product of bidentate attachment of the label at the amino terminus and at the side chain of Tyr3. If this is the case, it would appear from the FAB–mass spectrometric data presented in Figure 8 that the ester linkage to the side chain of Tyr3 is subject to hydrolysis, as the mass spectrum includes a prominent component with m/z identical with that of BSSDP-labeled EGFα peak B. When peak C was bound to receptor, it also became immobilized ($\tau_c > 1 \times 10^{-7}$ s); however, the spectral line shape from unbound peak C was less well resolved from that of the bound hormone (Faulkner, 1991). Thus, although BSSDP-labeled EGFα peak C is the product in which the motion of the spin-label best reflects the motion of the hormone, it is not as suitable as peak B for measuring dissociation rates. Such a bidentate derivative would, however, be useful for studies of rotational dynamics of the hormone–receptor complex, and we are currently working on the preparation of a site-directed mutant of EGFα that would provide a more stable linkage with the side chain of residue 3.

We describe the application of a novel method for observing hormone–receptor interactions. Linear EPR spectroscopy, sensitive to submicromolar concentrations of spin-labeled molecules and rotational motions in the range of τ_c = 10^{-11}–10^{-7} s, has been shown to be useful in observing the interaction of EGF with its receptor. In this application, linear EPR was used to observe the relative concentrations of free and bound BSSDP-labeled EGFα peak B molecules in the presence of solubilized receptor, before and after the addition of excess unlabeled mEGF, and to obtain values for the dissociation constants of solubilized receptor. In future studies, this method may also be applied to membrane preparations and whole cells. Saturation transfer EPR methods [reviewed in Beth and Robinson (1989)] are sensitive to slower motions in the range of τ_c = 10^{-7}–10^{-3} sec. These methods, coupled with the appropriate derivatives, can potentially allow one to observe the motion of receptors that have bound spin-labeled hormone, thus yielding new information on the dynamics of hormone–receptor complexes.

Acknowledgments

We thank Dr. J. M. Beechem for assistance with the kinetic analyses, Dr. J. E. Nieves for many helpful discussions, Dr. K. Balasubramanian for assistance in the graphical display of spectral data, and M. Jayne for assistance in the preparation of the manuscript.

Registry No. BSSDP, 102260-45-7; EGF, 62229-50-9; BSSDP-EGF, 135339-26-3.

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