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Spectroscopic and chemical studies of the interaction between nerve growth factor (NGF) and the extracellular domain of the low affinity NGF receptor

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Abstract

Nerve growth factor (NGF) interacts with a cell surface receptor on responsive neurons to initiate a series of cellular events leading to neuronal survival and/or differentiation. The first step in this process is the binding of NGF to a low affinity and/or a high affinity receptor. In the present report, we have studied the conformation and stability of recombinant receptor extracellular domain (RED) from the human low affinity receptor and the structural basis of its interaction with NGF. Circular dichroism (CD) studies indicate that the RED is primarily random coil in nature with little regular secondary structure. Thermal stability studies have shown that this irregular conformation is a specific structure that can undergo a reversible two-state thermal denaturation with a concomitant fluorescent and CD change. During heating at 100 °C for 15 min, the structure of RED is sufficiently unfolded for a reducing agent, dithiothreitol, to inactivate the receptor toward NGF binding and cross-linking. The complex formation between the RED and NGF has been examined by differential CD measurements, and we have shown that a small, reproducible change in conformation occurs in RED or NGF upon interaction. These results are interpreted in terms of the initiation of NGF cell surface binding and possible modes of signal transduction.

Keywords: circular dichroism; conformation; fluorescence; stability; unfolding

Nerve growth factor (NGF) binds to a cell surface receptor on sympathetic, neural crest-derived sensory, and central cholinergic neurons with the resultant transduction of a signal within the cell (Heumann et al., 1981; Seeley et al., 1983; Levi-Montalcini, 1987). Two transmembrane proteins contribute to NGF binding and the initiation of signal transduction in NGF-responsive neuronal cells. The low affinity NGF receptor, LNGFR or gp75, was the first receptor protein component characterized (Grob et al., 1985), has an appropriate tissue distribution in the nervous system (Taniuchi et al., 1986; Large et al., 1989), and binds NGF with a K_d around 1 nM (Vale & Shooter, 1985). The LNGFR has a relatively small intracellular domain (Chao et al., 1986; Radeke et al., 1987) and appears to be necessary but not sufficient to mediate full biological response (Hempstead et al., 1989; Matsushima & Bogenmann, 1990; Pleasure et al., 1990; Yan et al., 1991). This nominal NGF low affinity receptor also binds the related neurotrophins, brain-derived neurotrophic factor (BDNF) (Rodriguez-Tebaz et al., 1990) and neurotrophin-3 (NT3) (Squinto et al., 1991). The second receptor component of NGF binding is the product of the protooncogene *trk*, gp140^{trk}, which includes an intracellular protein tyrosine kinase domain that initiates signaling (Kaplan et al., 1991b; Klein et al., 1991). Both LNGFR and gp140^{trk} appear to be required for high affinity binding of NGF (Hempstead et al., 1989, 1991; Pleasure et al., 1990; Kaplan et al., 1991a), although there is some controversy on this point (Klein et al., 1991).

A fundamental question in receptor research is how binding of ligand initiates signal transduction. One proposal invokes a conformational change in the extracellular domain of the receptor, analogous to conformational changes in allosteric proteins (Yarden & Ullrich, 1988). We have addressed this question by examining the de-

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tailed nature of the interactions between NGF and the extracellular domain of the LNGFR. The recombinant receptor extracellular domain (RED) of the LNGFR has previously been expressed in a baculovirus system (Vissavajjhala & Ross, 1990) in a nonglycosylated form that binds NGF with a K_d estimated to be less than 42 nM. The hydrodynamics of the RED suggest that the receptor domain has an asymmetric shape with an observed frictional coefficient of 1.7 (Vissavajjhala & Ross, 1990).

In this paper, we have used chemical and spectroscopic methods to study the protein interactions involved in the NGF binding to the RED in order to better understand the specificity and signaling aspects of receptor function. We have found from circular dichroism (CD) studies that the conformation of the RED appears to be mainly in a random coil. CD experiments also revealed a small change in conformation of the RED and/or NGF upon binding. Reduction of the RED with dithiothreitol (DTT) at elevated temperatures prevents NGF binding and cross-linking, suggesting that a disulfide bond(s) becomes accessible upon thermal denaturation.

Results

CD

The CD spectra of RED and β-NGF were obtained in 10 mM phosphate buffer, pH 6.8 (Fig. 1). The spectrum of RED is characterized by a slight maximum at about 225 nm and a strong minimum near 197 nm with an amplitude of −25,000 deg·cm^2/dmol. This spectrum is similar to protonated polylysine in the random coil state (Brahms et al., 1977) and suggests that RED in solution exists primarily in a disordered conformation with little secondary structure. A β-turn structure is the only other derived structure (Compton & Johnson, 1986) that resembles that of the RED. This spectrum and structural interpretation are markedly different than for that observed for the epidermal growth factor (EGF) receptor extracellular domain, which has significant amounts of regular secondary structure (Greenfield et al., 1989). The spectrum of β-NGF has a minimum at about 208 nm with a secondary structure that is largely β-sheet (Williams et al., 1982; McDonald et al., 1991; Timm & Neet, 1992).

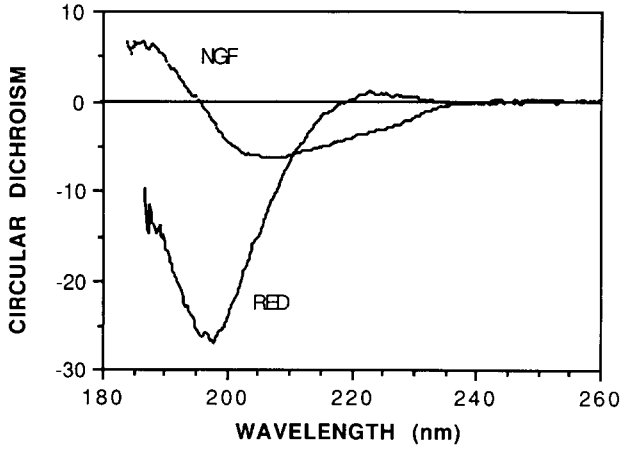

Fig. 1. CD spectra of RED and NGF. The CD spectra were recorded for RED, 0.9 μM (solid line), and for NGF, 3 μM (dashed line), in phosphate buffer, pH 6.8, in a 2.0-mm light path cell at 25 °C, averaged over 12 scans, and converted to mean residue ellipticity (deg·cm^2/dmol × 10^{-3}).

Conformational changes during interaction

Samples of NGF and RED were mixed together at a monomer molar ratio of about 3 to 1. The CD spectrum of the NGF–RED complex at neutral pH was compared (Fig. 2A) to the mathematical summation of the individual spectra that had been digitally stored in the microcomputer by the spectropolarimeter. The spectra also were compared by recording the resultant of the separate spectra in a split tandem cell and then obtaining the combined spectra after mixing (Fig. 2B). In either case the large negative trough showed a small difference in the spectral signal, characterized by a slightly more positive ellipticity below 220 nm and a slightly more negative ellipticity between 220 and 230 nm. Ordering of random coil structure into either an α-helix or β-sheet would be consistent with such CD changes.

The stoichiometry of NGF binding to the receptor is not known with any certainty. If the stoichiometry is assumed to be 1:1, then the free NGF concentration would be about 1 μM or 24-fold greater than the measured K_d (Vissavajjhala & Ross, 1990). If a single NGF dimer binds to each monomer of the RED, then the estimated free concentration of NGF is only 0.6 μM or 14-fold greater than the K_d. In any event, about 93–96% of the receptor was estimated to be in the complex in these experiments.

The overall CD spectrum of either NGF or RED did not change appreciably at pH values of 4, 4.5, or 5 compared to pH 6.8 (data not shown). After mixing, noncoincident CD curves were observed, similar to those at pH 6.8 (Fig. 2C). Although small, these spectral changes were consistently observed by both methods upon complex formation. In order to test the significance of these ellipticity changes, difference curves for eight experiments at two pH values were calculated and averaged (Fig. 2D). Although some variation exists, the difference curves are statistically significant ($P < 0.05$, Student's t-test) with a mean difference and standard deviation for three experiments at pH 4.0 of 1.19 ± 0.57 (×10^3) deg·cm^2/dmol at 204–206 nm and 0.25 ± 0.09 (×10^3) deg·cm^2/dmol at 225–227 nm. At pH 6.8 the mean for five experiments was 0.96 ± 0.20 (×10^3) deg·cm^2/dmol at 204–206 nm and 0.36 ± 0.1 (×10^3) deg·cm^2/dmol at 225–227 nm ($P < 0.01$).

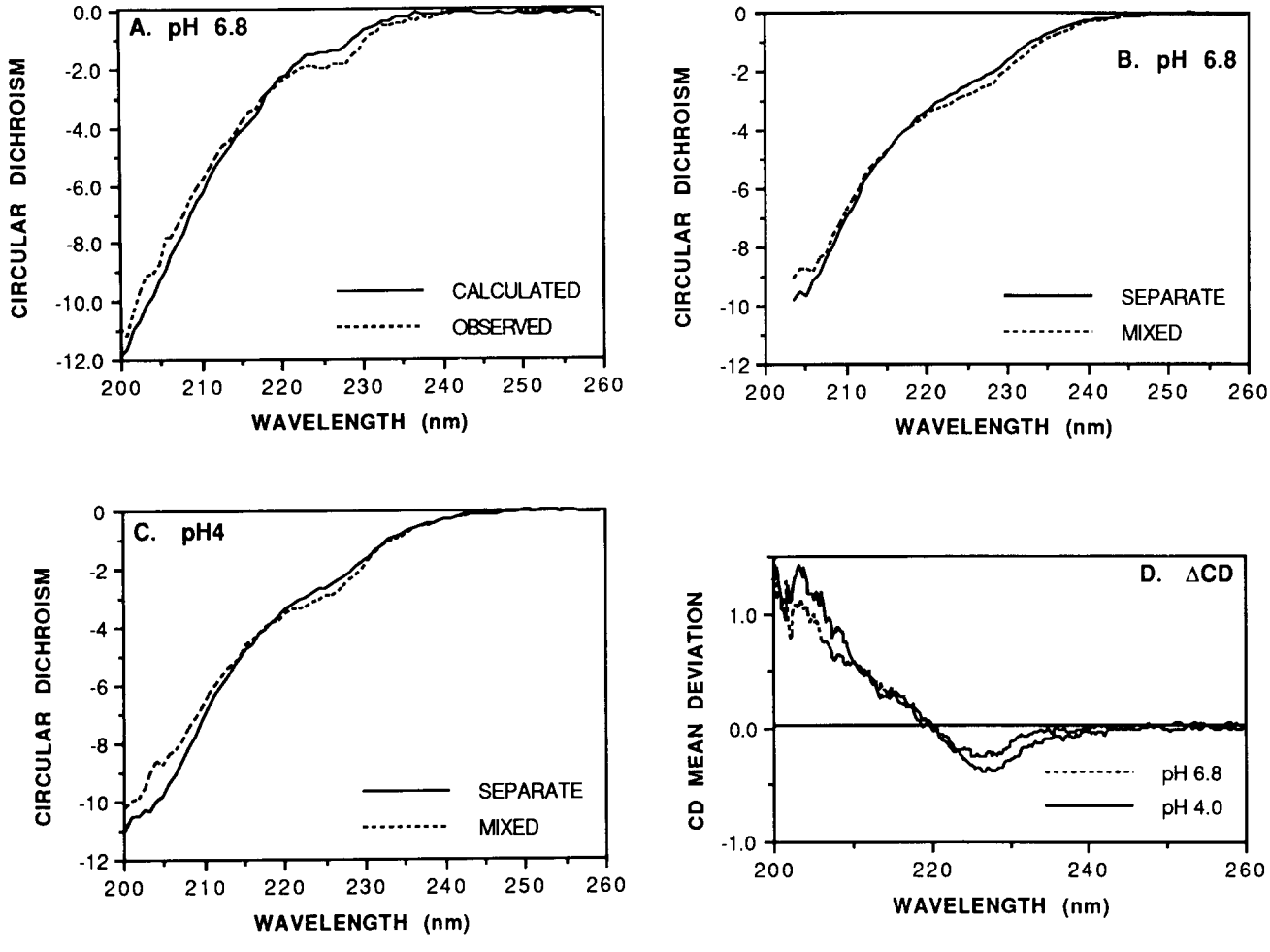

Fig. 2. CD spectra (mean residue ellipticity, deg·cm^2/dmol × 10^{-3}) for the observed and calculated NGF-RED complex. **A**: The CD spectra of 0.9 μM RED and 3 μM NGF in pH 6.8, 10 mM phosphate buffer were measured separately in a 4.5-mm path cell (cf. Fig. 1), digitally recorded in the microcomputer, and mathematically added (solid line). Equal volumes of the ligand and receptor were mixed (0.45 μM RED and 1.5 μM NGF final concentration), and the resultant CD spectrum recorded under the same conditions (dashed line). Conversion of the data to mean residue ellipticity accounted for the mixing dilution; the CD for NGF and RED are not dependent on protein concentration in this experimental range. **B, C**: The CD spectra were recorded for the optical summation of the individual spectra of 0.9 μM RED and 3 μM NGF separated in a tandem cell with a 4.5-mm path length on each side (solid line). The tandem cuvette was then inverted to allow mixing of the ligand and receptor (0.45 μM RED and 1.5 μM NGF final concentration) and the CD spectrum for the resultant solution was recorded with a 9-mm total light path (dashed line). Solutions were 100 mM phosphate buffer at pH 6.8 in panel B or at pH 4.0 in panel C. **D**: The average CD difference curves at pH 6.8 (dashed line) and at pH 4 (solid line) of NGF/RED mixtures vs. summation of individual samples calculated by either tandem cell or mathematical addition.

The specificity of the ellipticity differences and the reproducibility of the JASCO J600 were tested by mixing experiments with proteins expected to be noninteracting. Insignificant changes in spectra were observed upon mixing of ribonuclease with cytochrome c, ribonuclease with RED, or the NGF α-subunit with the NGF γ-subunit (data not shown). Insulin mixed with RED gave a small CD change that was inconsistent and irreproducible. From these series of experiments, we conclude that a small, but real, conformational change occurs in the complex between NGF and RED.

Conformation and stability of RED

Denaturation studies are quite useful in establishing the stability of a protein and some aspects of its structure in solution (Pace, 1990). Such studies of NGF are reported elsewhere (Timm & Neet, 1992). RED was examined by fluorescence and CD during denaturation by temperature or by guanidine hydrochloride. The characteristic random coil CD spectrum of RED was found to change upon heating from 23 °C to 93 °C to a distinct spectrum (Fig. 3A) that is also characteristic of a random coil (see

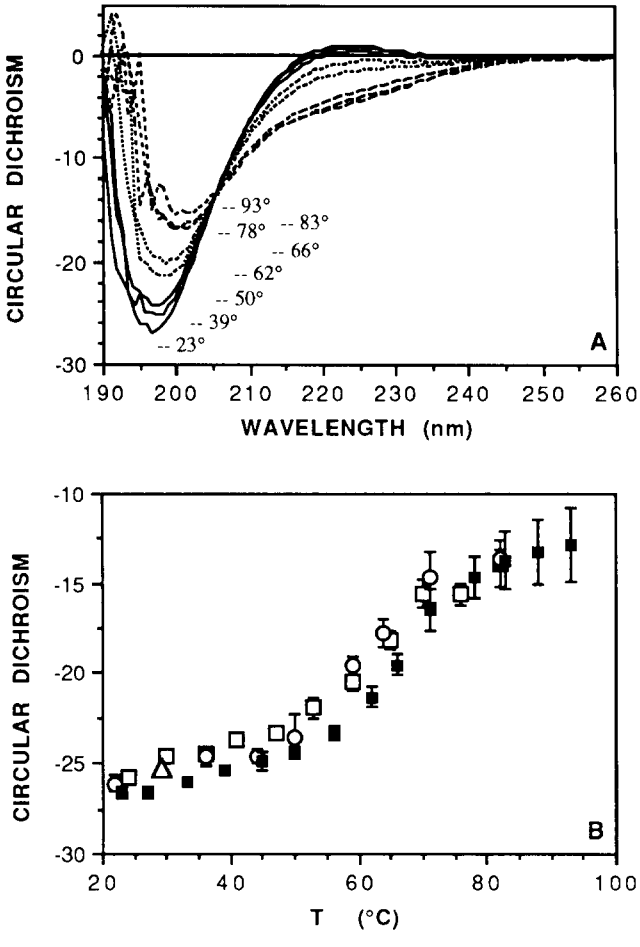

Fig. 3. CD spectra of RED as a function of temperature. **A**: The CD spectrum of 4.5 μM RED in 10 mM phosphate, pH 6.8, was recorded after 15 min equilibration at temperatures of 23 °C, 39 °C, 50 °C, 62 °C, 66 °C, 78 °C, 83 °C, and 93 °C in a 1-mm path length cell and averaged over six scans each. Mean residue ellipticity spectra (deg·cm^2/dmol × 10^{-3}) were corrected for buffer baseline at each temperature. **B**: Mean residue ellipticity values at 11 wavelengths between 197 and 199 nm (at 0.2 nm separation) were individually averaged for separate experiments and replotted against the temperature in order to construct a smoothed transition curve. ■, 4.5 μM; □, ○, 0.45 μM. △, Reversal upon recooling from 83 °C to 29 °C. Error bars are SD of wavelength averaging for a single experiment.

Discussion). The trough at 197 nm was diminished and shifted toward 200 nm, whereas the ellipticity above 210 nm became more negative. An isosbestic point at about 206 nm suggests a transition between only two states of the molecule. This denaturation process was found to be completely reversible upon cooling back to 29 °C (△, Fig. 3B). In order to smooth the noisy data at short wavelengths while accurately representing the transition, ellipticities were averaged and replotted against the temperature (Fig. 3B). Curves were constructed at two concentrations to test for the presence of associated species. A cooperative transition was observed at each concentration with a midpoint about 65 °C. Similar CD thermal transition results were obtained at pH 4.5, 0.23 μM RED, with an isosbestic point at about 202 nm and a transition midpoint of about 62 °C (data not shown). Although a small shift to the left appears with the lower concentration curve (Fig. 3B, open symbols), a buffer baseline correction was applied to obtain these results that is significant at the lower concentrations. Therefore, these low concentration CD data are considered less reliable and within the error range of replicate experiments at the other concentrations (see next section).

The fluorescence spectrum of RED was also followed during heating. The fluorescence emission peak decreased by 90% at 345 nm and shifted to about 353 nm at high temperatures (Fig. 4, inset), consistent with an unfolding of the protein and an increased exposure of the tryptophans to the solvent (Lakowicz, 1983). The relative fluorescence intensity transition appears to occur over a rather broad range of temperatures from 25 °C to 82 °C (Fig. 4). On closer inspection, the curve shows a simple linear decrease with temperature (as occurs with many proteins) up to about 55 °C and then goes through a sharp but low amplitude decrease that is over by about 75 °C. The fluorescence spectral changes were reversible upon cooling (data not shown). The midpoint of the thermal transition was assessed by taking the first derivative of the curve and was about 65 °C at both 0.16 μM or 0.72 μM. These data are clearly independent of concentration within this range and agree very well with the CD data at 4.5 μM (Fig. 3).

When denaturation by solvents was studied, we found that the RED was extremely sensitive to guanidine hydrochloride, as judged by fluorescence changes. A hyperbolic decrease in the relative fluorescence intensity at 345 nm was observed with a 25% loss to a plateau above 1 M guanidine hydrochloride (data not shown). A cooperative structural transition was not observable so this method was not pursued.

Thermodynamics of unfolding

The thermal transition of RED was observed both by fluorescence spectroscopy and by CD. The transition curves (Figs. 3B, 4B) were converted into fraction native for both methods and plotted against temperature (Fig. 5A). The good agreement among concentrations ranging from 0.16 μM with fluorescence to 4.5 μM with CD indicated that the denaturation was concentration independent, and, therefore, the less reliable, low concentration CD data were not used for further analysis. A simple monomer unfolding model with a two-state equilibrium transition was best suited for interpreting the data (Pace, 1990). A Van't Hoff plot was constructed from the transition data using the relationship $K_u = f_u/f_n$ to calculate the equilibrium constant for unfolding (Fig. 5B). Proteins, in general, have been found to have significant heat capacities (Privalov, 1979) and hence the Van't Hoff plot

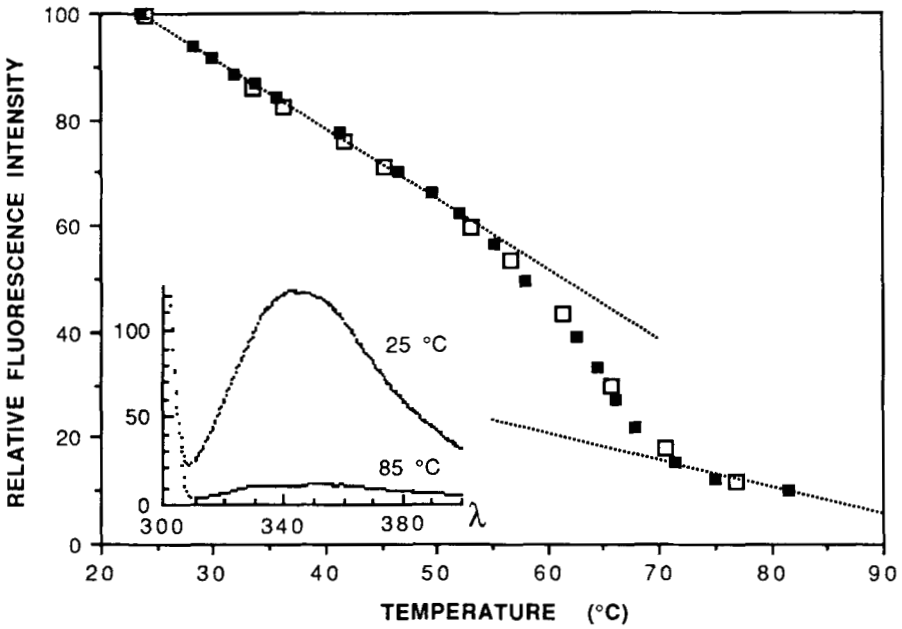

Fig. 4. Thermal transition of RED as observed by fluorescence intensity. The fluorescence spectrum of 0.72 μM RED in 10 mM phosphate buffer, pH 6.8, was recorded after 15 min equilibration at temperatures from 25 °C to 82 °C. Average values for three measurements of the relative fluorescence intensity at 345 nm are reported as a function of temperature for two separate experiments (□, ■). Dotted lines represent the linear portions outside the transition region. **Inset**: Fluorescence emission spectra for 1.56 μM RED at 25 °C and 85 °C.

should not be linear, even though it might appear to be nearly so. Therefore, we fit the data to the general relationship for the thermodynamics of unfolding (Privalov & Potekhin, 1986; Pace, 1990).

$$\Delta_D G(T) = \Delta_t H \frac{T_t - T}{T_t} - \Delta_t C_p (T_t - T) + \Delta_t C_p T \ln \frac{T_t}{T}, \quad (1)$$

where $\Delta_D G(T)$ is the free energy difference between denatured and native states at temperature T, $\Delta_t H$ is the enthalpy and $\Delta_t C_p$ the heat capacity at the transition temperature T_t. $\Delta_D G(T)$ was calculated as $-RT\ln(K_u)$ values as a function of T and Equation 1 was fit with $\Delta_t H$, $\Delta_t C_p$, and T_t as fitting parameters. The best fit (shown by the line in Fig. 5B) provided reasonable values for these parameters except that a rather high value for $\Delta_t C_p$ (2,840 cal/mol/°K) was obtained, suggesting that the RED would be marginally stable at room temperature and undergo another unfolding near 17 °C as the temperature decreased. No evidence for such behavior has been observed. Further analysis of the fitting indicated that the transition temperature and the $\Delta_t H$ were well defined at 64.6 (±0.2) °C and 69 (±2) kcal/mol but

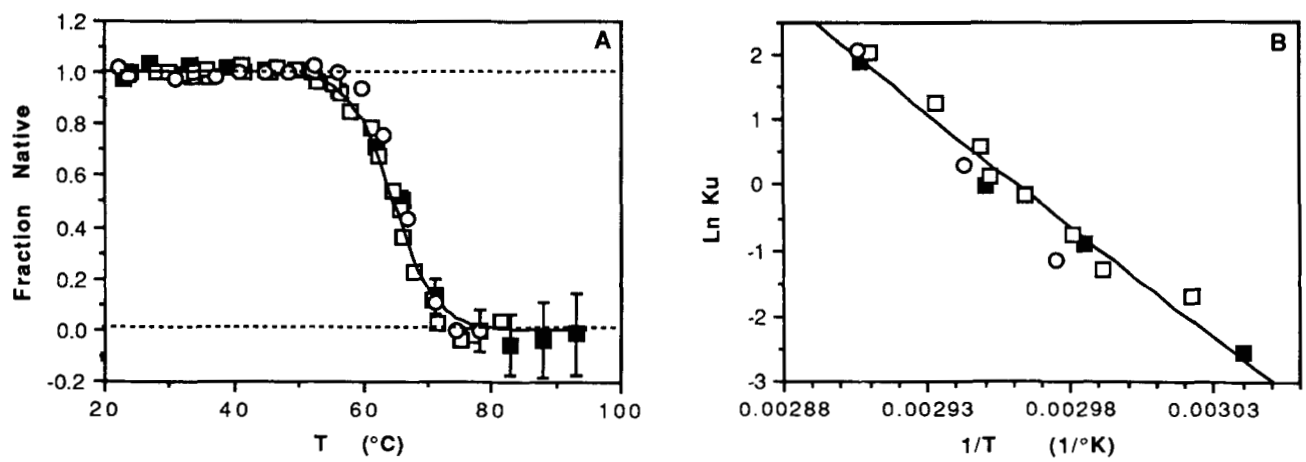

Fig. 5. Comparison of CD and fluorescence data for the thermal transition of RED. The data of Figures 3 and 4 were converted to fraction native and to the equilibrium unfolding constant, K_u (see Materials and methods). The solid line is the best fit of the fluorescence and the high concentration CD data to Equation 1. ○, Fluorescence at 0.16 μM; □, fluorescence at 0.72 μM; ■, CD at 4.5 μM. **A**: Fraction native vs. temperature. **B**: Van't Hoff plot.

that the $\Delta_t C_p$ was ill defined (not shown). Values of $\Delta_t C_p$ from 50 to 1,200 cal/mol/°K were consistent with the thermal transition data, with a reasonable stability ($\Delta_D G \sim 6$ kcal/mol) at room temperature, and gave χ^2 values only slightly larger than the best fit (0.55 vs. 0.46). The lack of an observable guanidine transition prevented us from independently determining the free energy at 25 °C to 37 °C with any more certainty. A value for $\Delta_t H$ of 69 kcal/mol is quite reasonable for a small globular protein (Privalov & Gill, 1988).

Binding of NGF by RED after thermal denaturation

The CD and fluorescence studies suggested a high degree of reversible thermal stability of the RED. This property was further tested by heating the RED with and without DTT and determining the ability to bind NGF. Binding of ^{125}I-NGF to the RED was measured by chemical cross-linking with ethyldimethylisopropylaminocarbodiimide (EDAC) (Fig. 6, lanes 1–3). The binding was judged to be specific because: (1) no cross-linking was observed in the absence of EDAC, (2) there was little cross-linking to the carrier protein albumin, and (3) addition of nonradioactive NGF prevented the cross-linking to radiolabeled NGF. Heating of RED to 100 °C for 15 min had no effect on the subsequent ability to bind NGF at 37 °C (Fig. 5, cf. lane 9 to lane 2), consistent with the spectroscopic results. We then tested whether the stability of the RED was related to its disulfide bonds. Treatment of RED at 37 °C with a 50-fold molar excess of DTT had little or no effect on binding ability (Fig. 5, lanes 4–7). However, heating RED at 100 °C in the presence of DTT completely abolished its ability to cross-link to ^{125}I-NGF (Fig 5, lane 12). This effect was not due to any residual DTT on NGF, as untreated RED added to the reduced mixture bound to the ^{125}I-NGF (Fig. 6, lane 14). Hence, one or more disulfide bonds, probably in the interior of the RED, are essential for maintenance of the NGF binding site. Heating of the protein to 100 °C disrupts the conformation of the RED and allows reduction of the disulfide bond(s).

Discussion

Interaction of NGF with the recombinant binding domain of the LNGFR, the RED, induces a conformational change, consistent with a small increase in the α-helix or β-sheet content of one or both of the proteins in the complex. The change in CD is statistically significant as shown by the analysis of difference CD curves (Fig. 2D). This result shows the power of the CD spectroscopy to generate highly reproducible and sensitive ellipticity measurements in order to observe conformational changes upon protein–protein interaction.

This change in secondary structure in the RED–NGF complex is on the same order of magnitude, i.e., 4–8% in ellipticity, as that in the EGF receptor extracellular domain upon binding of EGF (Greenfield et al., 1989). The extent of a conformational change necessary for transduction of ligand binding information from outside the cell to second messengers inside the cell is unknown. In the case of EGF, the transduction must be occurring within the same polypeptide to activate the tyrosine kinase activity of the intracellular domain. With NGF, no activity has been directly ascribed to the LNGFR, so that the participation of another membrane protein in the transduction of the signal is needed (Kaplan et al., 1991b; Klein et al., 1991). The similarities in the NGF and EGF

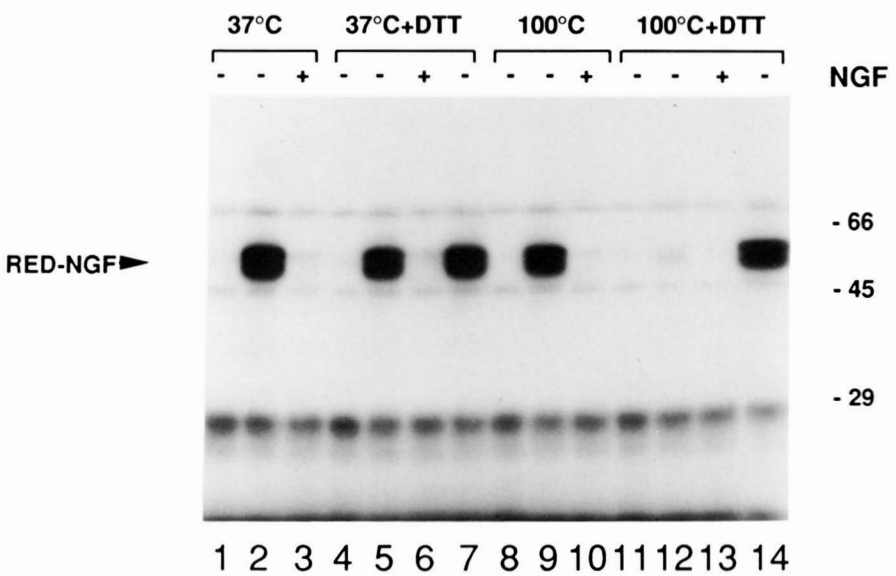

Fig. 6. Significance of the disulfide bonds in the thermal stability of RED as measured by ^{125}I-NGF binding and cross-linking. RED (3.5 μM) was incubated at 37 °C (lanes 1–7) or 100 °C (lanes 8–14) with 175 μM DTT (lanes 4–7 and 11–14) or without DTT (lanes 1–3 and 8–10) for 15 min. After passage through a Sephadex G-50 column, the RED was tested for the binding of ^{125}I-NGF by chemical cross-linking using EDAC. The samples were analyzed as described in Materials and methods. Lanes 1, 4, 8, and 11 were controls devoid of EDAC. As an additional control, the same amount (0.18 μM) of RED that had been heated at 37 °C (lane 7) and 100 °C (lane 14) without DTT was added prior to cross-linking.

systems, however, suggest that consideration of small tertiary structural changes or push–pull mechanisms within the membrane may be required. The results with EGFR–LNGFR chimeras (Yan et al., 1991) support the requirement for signal transduction from the extracellular domain to the intracellular domain of the LNGFR and further implicate the transmembrane region of the LNGFR as being important. Extension of the current spectroscopic studies to recombinant constructs containing more of the LNGFR than just the RED will be extremely interesting.

The secondary and tertiary structure of NGF and RED were retained at low pH, and the same changes in CD of the complex formed were observed at pH values down to 4.0. One might have expected that dissociation of the ligand–receptor complex would have occurred at this pH, and no difference in the CD spectra would have been observed. However, it is clear that the conditions for stripping NGF from its receptor (Bernd & Greene, 1984; Kasaian & Neet, 1988) include high ionic strength as well as low pH. When measurements have been directly made under acidic conditions, good binding of NGF to cells or to partially purified receptor still occurs at lower pH (Vale & Shooter, 1985; Stach et al., 1987). Thus, the retention of small conformational changes in the complex between pH 4 and 7 are consistent with studies of NGF binding to the LNGFR in this pH region.

Studies of the conformation and stability of recombinant RED in solution are simplified by the absence of carbohydrate even though NGF binding ability is retained (Vissavajjhala & Ross, 1990). The RED conformation was found by CD to be mostly random coil in solution with little ordered regular α or β structure. This result is reasonably consistent with predictions from the sequence data. Using the Chou–Fasman (1978) or the Garnier–Osguthorpe–Robson (1978) algorithms, α-helix predictions range from a minimum of 6% to a maximum of 16% (residues within 96–108 and 170–190) and β-sheet from 4% to 11% (residues within 71–77 and 87–93). The CD result should be interpreted as a specific tertiary structure, not a randomly distributed population of molecules in solution. Indeed, different distinct random conformations may display varying magnitudes of the characteristic trough at 195–197 nm; a 15% difference in ellipticity has been observed between poly(Lys^+) and poly(Pro-Lys^+-Leu-Lys^+-Leu), both believed to be in random states (Brahms et al., 1977). Such a random coil structure is also consistent with the hydrodynamic properties of the RED (Vissavajjhala & Ross, 1990) and does not mean that the RED is unstructured. For example, wheat germ agglutinin is organized into four cysteine-rich domains that are folded into globular structures lacking α-helix or β-sheet (Wright, 1977).

The structured nature of the RED random coil is also emphasized by the sharp, reversible transition of the tertiary structure upon thermal denaturation. This transition appears to be two state and is characterized by a ΔH of stabilization in the range for other globular proteins. The extracellular domain of the receptor has a repeating primary structural motif of four Cys-rich domains of about 40 amino acids each (Johnson et al., 1986). Thus, 162 of the 183 residues of our recombinant RED are contained within these domains. The homology among the domains is at most 40–50%, including the conserved Cys residues, with the two Trp residues occurring at position 155 in the fourth domain and at 167 nearer the C-terminus. This domain structure is highly conserved among species (Radeke et al., 1987; Large et al., 1989). Two models for the unfolding of the RED are (1) that the four domains unfold independently, or (2) that the four domains are tightly coupled and unfold as a single cooperative unit. The first model is discredited because the four domains do not appear to be similar enough (Johnson et al., 1986) to unfold independently yet with identical transitions, as would be required by a two-state transition. The cooperative two-state model is supported by the demonstration of an isosbestic point in the CD data and the agreement between the CD and the fluorescence data. This latter agreement is particularly striking in light of the asymmetric distribution of the tryptophans toward the C-terminal end (Johnson et al., 1986); the fluorescence indicates increased solvent exposure of the tryptophans in the C-terminus and exactly corresponds with the CD transition that relates to the whole structure. A tight coupling of the unfolding domains might result from the packing of the four domains to form a specific folded structure, as has been observed for the EGF receptor (Lax et al., 1991). A strong interaction among the four domains would be consistent with the measured frictional coefficient (Vissavajjhala & Ross, 1990) if each of the 4–5-kDa domains, itself, has a similar asymmetry. This model predicts that the RED exists as a compact globular protein consisting of a polypeptide backbone randomly coiled into four domains and unfolds upon heating into a more expanded flexible random coil.

The NGF cross-linking studies support the thermal stability and further show that binding activity is lost upon reduction of disulfides in the heated, unfolded RED. At this time the arrangement, or even the number, of disulfides formed within the RED by the six Cys residues per domain is unknown. Disulfide bonds are common in extracellular proteins and frequently are buried in the interior of the protein (Thornton, 1981). The disulfide cross-links in the RED stabilize the protein in the folded form by decreasing the conformational entropy of the unfolded state (Pace et al., 1988); a term not included in the calculated ΔH. We interpret these data to mean that the irregular but specific folding of the polypeptide chain of the native RED, further stabilized by disulfide bonds, is sufficient to confer thermodynamic stability and ligand binding abilities. A small increase in the α-helix or β-structure in either the RED or in the NGF upon specific complex formation is entirely consistent with such a view.

Materials and methods

Reagents

NGF was purified from mouse submaxillary gland as the 7S complex, subunits separated as previously described (Smith et al., 1968; Stach et al., 1977; Woodruff & Neet, 1986), and stored at pH 4, −20 °C. Recombinant RED was purified by immunoaffinity and Mono-Q chromatography from *Spodoptera frugiperda* (Sf9) cells after infection with recombinant baculovirus AcH1-3 encoding the extracellular domain of the LNGFR (Vissavajjhala & Ross, 1990). Both NGF and RED used for the spectroscopic studies were essentially single bands on Coomassie blue-stained sodium dodecyl sulfate (SDS) polyacrylamide gels. The 2.5S NGF (from Bioproducts for Science) was iodinated by the lactoperoxidase method (Sutter et al., 1979) and used in the cross-linking studies. All other reagents were the highest quality obtainable from commercial sources.

Reduction of disulfide bonds of RED and cross-linking to NGF

RED (3.5 μM) was incubated at 37 °C or 100 °C with or without DTT (175 μM) for 15 min, in order to reduce the disulfide bonds. The samples were run through a Sephadex G-50 spin column to eliminate DTT before testing for ^{125}I-NGF binding. RED (180 nM final concentration) was incubated with ^{125}I-NGF (1 nM) for 45 min at 37 °C either with or without unlabeled NGF (385 nM). Ethyldimethylisopropylaminocarbodiimide (EDAC) was added to a final concentration of 30 mM for 2 min at room temperature. The samples were then reduced, boiled, electrophoresed on a 12% SDS-polyacrylamide gel, and visualized by autoradiography (Vissavajjhala & Ross, 1990).

Fluorescence

Steady state fluorescence of RED was measured in a Perkin-Elmer LS-5B spectrophotofluorometer in a 300-μL microcuvette with excitation at 280 nm, emission at 345 nm, and recorded on a Perkin-Elmer GP-100 graphics printer. For thermal measurements, the temperature was raised in 2–3 °C increments in a water-jacketed compartment and allowed to equilibrate for 15 min when the new fluorescence level was steady. The fractions native (f_n) and unfolded (f_u) were calculated by linear extrapolation of the sloping baselines into the transition region.

CD

CD spectra were recorded on a Jasco J600 spectropolarimeter with nitrogen flushing to reach lower wavelengths. The CD spectral cut-off at low wavelengths was determined from the photomultiplier tube voltage (about 500–600 V) and depends on cell path length, buffer concentration, pH, and protein concentration; therefore different low wavelengths were attained in different experimental setups (cf. Fig. 2). Data were processed on a Wyse (IBM compatible) microcomputer with software provided by Jasco. All spectra were digitally stored and averaged over 3–12 scans. Scan speeds were 20–100 nm/min with a time constant of 0.5–2.0 s. For thermal measurements, the temperature was raised in 5 or 10 °C increments in a water-jacketed compartment and allowed to equilibrate for 15 min. The spectra presented were corrected for apparent buffer ellipticity by digital subtraction of baseline at each temperature. The f_n and f_u were calculated by linear extrapolation of the sloping baselines into the transition region. Molarities of RED and NGF were calculated on the basis of their monomer molecular weights, 22,355 and 13,300, and extinction coefficients $\epsilon^{0.1\%}$ at 280 nm of 1.0 and 1.6, respectively.

Data analysis

The curves for thermal transitions with either CD or fluorescence were fit by nonlinear regression with three variable parameters to Equation 1 using the Marquardt gradient-analytical search algorithm (Bevington, 1969) implemented in BASIC for the Macintosh computer in the program BASICFIT.

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