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Chemical and Physical Properties of the Disulfides of Bovine Neurophysin-II†

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ABSTRACT: Bovine neurophysin-II is shown to be very susceptible to partial reduction in the absence of urea. Reduction of an average of one disulfide leads to major changes in conformation and disulfide optical activity, manifest in part by pronounced far-uv ellipticity changes, complete loss of the 248-nm ellipticity band, and a shift of the 278-nm ellipticity band to shorter wavelengths with loss of half its intensity; the reduction process generates a mixture of products and appears to be accompanied by disulfide interchange. The circular dichroism data indicate that the disulfide(s) most susceptible to reduction or interchange are either the principal contributors to the 248- and 278-nm ellipticity bands or that the optical activity of other disulfides is dependent on their integrity. Peptides that bind to the hormone-binding site of neurophysin-II protect against reduction. On reoxidation of partially reduced neurophysin-II there is only a partial return of the native circular dichroism spectrum and electrophoretic behavior. The percentage of native protein in samples reoxidized following different degrees of reduction was estimated by comparison of the circular dichroism spectra of these samples with those of the fractionated native and denatured components of monoreduced-reoxidized neurophysin. Under our reoxidation conditions, less than 50% native protein was found in monoreduced-reoxidized neurophysin and less than 10% native protein was found in completely reduced-reoxidized neurophysin. The results are interpreted with qualified reference to a model in which one or more disulfides are "strained" in the native state and in which the native protein is unstable relative to species in which the disulfides are differently paired.

The neurophysins, carrier proteins for oxytocin and vasopressin within the neurohypophyseal system, are rich in disulfides, bovine neurophysins each having seven disulfide bonds per monomer of 10,000 molecular weight; additionally, the neurophysins have a low content of aromatic amino acids, the bovine proteins containing no tryptophan, one tyrosine, and three phenylalanine residues per monomer (Rauch et al., 1969; Breslow et al., 1971; Schlesinger et al., 1972). The optical activity of neurophysin disulfides can therefore be defined with less ambiguity than with most other proteins, and two neurophysin near-uv ellipticity bands, at 278 and 248 nm, respectively, have been assigned almost exclusively to disulfide transitions (Breslow, 1970). Relationships between disulfide geometry, environment, and optical activity are inadequately understood. In particular, on the basis of existing theory, it is not possible a priori to evaluate the contribution of any single neurophysin disulfide bond to the 248- and 278-nm ellipticity bands. For example, the wavelength of an individual disulfide transition in the near-uv depends on the disulfide dihedral angle (Barltrop et al., 1954; Bergson et al., 1962; Beychok, 1965) and the sign of the longest wavelength ellipticity band depends at least in part on the disulfide screw sense (Beychok, 1965; Carmack and Neubert, 1967; Claeson, 1968; Imawishi and Isemura (1969). However, due to the compound nature of the near-uv disulfide transition, a single disulfide can exhibit one or two near-uv ellipticity bands of opposite sign, the number, position, and relative intensity of the two bands being dependent on geometry and environment (Linderberg and Michl, 1970; Casey and Martin, 1972; Sears and Beychok, 1973).

In an attempt to resolve the contributions of different neurophysin disulfides to neurophysin near-uv optical activity, we have studied the effect of varying degrees of reduction on neurophysin circular dichroism (CD) spectra. In the course of these studies, we observed that neurophysin is highly susceptible to partial reduction and we therefore additionally probed aspects of the reversibility of the reduction–reoxidation process. Studies reported here were carried out with bovine NP-II,[1] but preliminary parallel studies

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[1] Abbreviations used are: NP, neurophysin; DTT, dithiothreitol; DTE, dithioerythritol.

with bovine NP-I, which differs from NP-II in the location and pairing of one or two of its disulfide bonds (Capra et al., 1972), gave very similar results.

Materials and Methods

Materials. Bovine NP-II was prepared as previously described (Breslow et al., 1971). Dithiothreitol (A Grade) and diamide (diazinedicarboxylic acid bis(dimethylamide)) were purchased from Calbiochem; crystalline dithioerythritol and tritiated dithiothreitol (5.1 Ci/mol) were obtained from Cyclo Chemical Corp. The sodium salt of iodoacetic acid, crystalline glutathione, and iodoacetamide were obtained from Sigma Chemical Co. [1-^{14}C]Iodoacetic acid (13.45 Ci/mol) was purchased from New England Nuclear. Hormones and peptide analogs of the hormones used were those described elsewhere (Breslow et al., 1973). Urea and guanidine hydrochloride were recrystallized before use. Ellman's reagent [5,5-dithiobis(2-nitrobenzenoic acid)] was purchased from Aldrich Co. Inc.

Reduction and Reoxidation of Neurophysin. Complete reduction of NP-II was carried out by allowing the protein (2×10^{-4} *M*) to stand at room temperature for 1 hr at pH 8.0, 0.16 *M* KCl in 8 *M* urea (or 2 *M* guanidine) and 0.2 *M* mercaptoethanol or 0.03 *M* dithiothreitol. The reduced protein was separated from the other reagents either by dialysis against H_2O or, more generally, by gel filtration on a column of Sephadex G-25 in 0.1 *M* acetic acid; it was then diluted, if necessary, and allowed to air-oxidize until the -SH titer was negligible. Air-oxidation of the fully reduced protein was carried out at pH 7.5–8, using a protein concentration of approximately 5×10^{-6} *M*. The rate of reoxidation was slow, so the protein was reoxidized at room temperature for a period of 1–2 days and then kept at 4° for the remainder of the reoxidation period (approximately 1 week) to retard bacterial growth. In different experiments the composition of the reoxidation mixture was varied from 0.01 to 0.16 *M* KCl containing 4×10^{-5} *M* EDTA to 4×10^{-4} *M* EDTA, respectively. The air-reoxidized protein was concentrated by ultrafiltration to a concentration of 2 mg/ml for CD studies. No significant effects of KCl or EDTA were noted on the final product. In a single experiment, protein completely reduced by 10^{-2} *M* DTT in the absence of urea and reoxidized at 4° at a concentration of 2 mg/ml showed the same CD spectrum as that reoxidized at the lower protein concentration.

Partial reduction of NP-II was almost uniformly carried out at NP-II concentrations of 2 mg/ml, pH 8 in 0.16 *M* KCl containing 10^{-3} *M* EDTA. In a typical partial reduction study, the NP-II solution, adjusted to the desired pH, was deaerated by bubbling in N_2 for 30–60 min. Reducing agent (generally dithiothreitol or dithioerythritol) was then added and the solution kept closed until circular dichroism spectra did not change any further with time. Air was then admitted to the system and the solution allowed to reoxidize slowly, generally in the cold (4°) except for the first 24 hr and the periodic intervals when CD measurements were made at room temperature. In several studies, reoxidation was carried out exclusively at room temperature with continual stirring. Reoxidation was monitored by circular dichroism and by Ellman analysis (see below) of the total -SH content of the solution. A constant pH, generally 8, was maintained during reoxidation by periodic addition of base. In a few studies at pH 6.8, reoxidation proceeded more slowly but the final product was essentially the same as at pH 8.

In some studies, NP-II that had been partially reduced at 2 mg/ml was reoxidized at 0.08 mg/ml. The diluted reoxidized protein was concentrated by ultrafiltration or lyophilization and studied by polyacrylamide gel electrophoresis and circular dichroism. Partially reduced protein that was reoxidized under dilute conditions was more abnormal electrophoretically and spectrally than that reoxidized at 2 mg/ml.

For studies in which the -SH content of the protein was to be monitored during the reduction process, 4 mg of protein (2 mg/ml) was allowed to stand with DTT as above, adjusted to pH 3 after the specified time period to stop further reduction, and then quickly separated from DTT by passage through a 1.5 × 26 cm Sephadex G-25 column in 0.1 *N* acetic acid. The recovered protein fraction was assayed for sulfhydryl groups by reaction with Ellman's reagent (see below).

Carboxymethylation and Carboxamidination of Partially Reduced NP-II. Following partial reduction with DTT for 60 min at pH 8 (or 20 hr at pH 6.7) as described above, NP-II was treated under N_2 with a 100-fold excess iodoacetic acid or iodoacetamide in Tris-HCl buffer containing 10^{-3} *M* EDTA (pH 8.1) for 2 hr. The protein was separated from excess reagent by gel filtration on Sephadex G-25 in 0.1 *M* acetic acid and lyophilized. The extent of carboxymethylation was determined by amino acid analysis or, when radioactive iodoacetic acid was used, by determination of the radioactivity of the modified protein. For fractionation of the different protein components of carboxymethylated NP-II, approximately 30 mg of partially reduced carboxymethylated NP-II was chromatographed on a 1.7 × 30 cm column of Sephadex DEAE A-50 using a discontinuous gradient (pH 5.9 → pH 5.5) of pyridine acetate buffer (Breslow et al., 1971). The sample was applied at pH 5.9 and elution continued with pH 5.9 buffer for 500 ml at which point pH 5.5 buffer was introduced. Protein was analyzed by the Folin–Lowry method and individual peaks were pooled and lyophilized.

Fractionation of Components of Partially Reduced–Reoxidized NP-II. NP-II (40 mg) was reduced with 1 mol of DTT/mol of NP-II at pH 8 and air-oxidized as described above. The protein was separated from residual DTT by gel filtration on Sephadex G-25, lyophilized, and redissolved to give a concentration of approximately 15 mg/ml at pH 5 in 0.16 *M* KCl. (The high protein concentration was necessary for subsequent steps, but not all the protein dissolved and insoluble protein was removed by centrifugation.) To the dissolved protein, the peptide L-cystinylbis(L-tyrosinamide), which precipitates native NP-II at this pH (Breslow et al., 1971) was added to give a concentration of 1 mg/ml. Precipitate was collected, washed with peptide-containing solution, then separated from peptide by chromatography on Sephadex G-25 in 0.1 *N* acetic acid and lyophilized. Protein that was not precipitated by peptide was also separated from peptide by gel filtration on Sephadex G-25 in acetic acid and lyophilized. Both fractions of protein were then analyzed as described in Results.

General Methods. Circular dichroism measurements were performed at room temperature on a Cary 60 spectropolarimeter equipped with a Model 6001 circular dichroism attachment as described elsewhere (Breslow et al., 1971); results in both the near- and far-uv are calculated *per mole of protein* using a molecular weight of 10,000. In some studies preliminary estimates of the amount of native protein present in samples of reduced–reoxidized protein were

carried out by circular dichroism, comparing the changes generated on binding oxytocin or small peptide analogs of oxytocin to the modified sample with those known to accompany binding to native NP-II (Breslow et al., 1971). (This was regarded as valid since denatured protein was demonstrated not to interfere with CD changes accompanying binding to native protein in mixtures of native and denatured NP-II.)

Radioactivity measurements were performed using a Nuclear Chicago liquid scintillation counter. Polyacrylamide gel electrophoresis was performed at a running pH of 9.5, using a 7.5% gel as previously described (Breslow et al., 1971); densitometric analysis of stained gels was performed using a Canalco Model G densitometer. Sodium dodecyl sulfate gel electrophoresis was also carried out in 7.5% gels, pH 7.5, according to the method of Weber and Osborn (1969); reduced–reoxidized samples were not pretreated with mercaptoethanol.

Sulfhydryl analysis of samples was performed using the method of Ellman (1964) modified by the substitution of 0.2 *M* Tris-HCl buffer (pH 8) for the phosphate buffer of the original procedure in order to improve buffering capacity. Glutathione was used as a sulfhydryl standard.

Nitration of NP-II was accomplished by the method of Furth and Hope (1970). Amino acid analyses and ultracentrifuge studies were carried out as previously described (Breslow et al., 1971, 1973).

Results

Responsiveness of Disulfide Optical Activity to Protein Conformation. Figure 1 shows the near-uv circular dichroism spectrum of native bovine NP-II compared with that of NP-II in 5 *M* guanidine and of NP-II that has been completely reduced and reoxidized. The inset shows the 248-nm band, under the same conditions, resolved from contributions from the longer wavelength band by assuming the Gaussian nature of the longer wavelength band. (Although the 248-nm band is also significantly affected by contributions from far-uv ellipticity bands, it cannot be corrected for such contributions without unwarranted assumptions.) Several features of the data for the native protein are noteworthy. First, the 248-nm band clearly represents transitions which extend to wavelengths above 260 nm, in accord with the assignment of this band to disulfides. Second, although the disulfide origin of 95% of the 278-nm band is unequivocal (Breslow, 1970), there is some fine structure in this band near 270 nm which probably represent minor contributions from aromatic chromophores.

The optical activity of neurophysin disulfides is dependent on protein conformation and on the proper half-cystine pairing. Guanidine shifts the 278-nm band to shorter wavelengths with a negative extremum at approximately 270 nm, and diminishes its intensity. The 248-nm positive band is completely lost in guanidine; tentatively this cannot be attributed to masking of the positive 248-nm band by the increased negative ellipticity in the far-uv that accompanies guanidine addition, since 248-nm ellipticity in guanidine becomes slightly *more positive* when the disulfides are completely reduced. Additionally the effects of guanidine on the 278-nm band are in the wrong direction to be attributed to loss of overlap of the 278-nm band with the positive 248-nm band. The data indicate a change in average disulfide geometry and/or environment in 5 *M* guanidine. Guanidine effects (if no reducing agent is added) are completely reversible, indicating that no disulfide interchange accompanies guanidine addition.

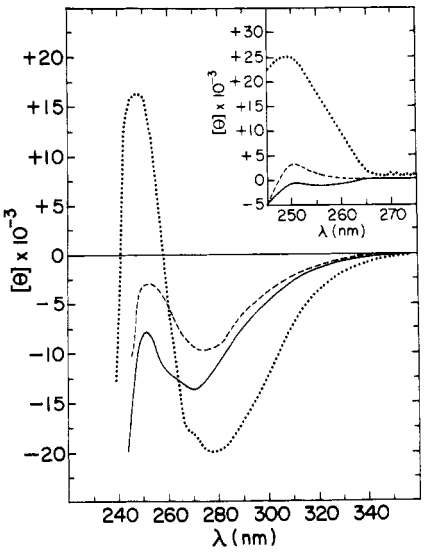

FIGURE 1: Effect of denaturation on the near-uv ellipticity spectrum of NP-II. (···) Native NP-II (pH 6)–0.16 *M* KCl; (—) NP-II in 5 *M* guanidine, pH 6; (- - -) completely reduced–reoxidized NP-II (pH 6)–0.16 *M* KCl. Inset shows the CD spectra of the same samples after subtracting out contributions from the longest wavelength band assuming the long wavelength band is a Gaussian component. (The theoretical Gaussian for the long wavelength band was calculated from its long wavelength edge.) Ellipticity is deg cm^2/dmol.

Neurophysin-II that has been completely reduced by excess mercaptoethanol in the presence of 8 *M* urea and then air-oxidized (see Materials and Methods) also shows a markedly different CD spectrum from that of the native protein (Figure 1). Again, the 278-nm band is reduced in intensity and blue-shifted, and the 248-nm band is largely lost (see inset). Differences between native and reduced–reoxidized neurophysin result from the inability of neurophysin disulfides to correctly pair on reoxidation and confirm the dependence of disulfide optical activity on molecular structure.

An additional fact which emerges from the above data, and which is reproduced in the experiments reported below, is that a diminished ratio of the positive intensity of the 248-nm band to the negative intensity of the 278-nm band is a sensitive indicator of changes in disulfide environment or geometry. Another such indicator is the ratio of ellipticity at 278 relative to that at 310 nm. An increase in the 278/310 ratio always accompanies decreases in the 248/278 ratio, suggesting that common structural features are responsible for the 248-nm band and the long wavelength ellipticity edge of the 278-nm band.

Given the dependence of the 278- and 248-nm bands on conformation, it is interesting to note that these bands are almost independent of pH between pH 0.5 and pH 11. Time-dependent changes occur above pH 12 (Breslow, 1970) where disulfide cleavage probably occurs, and minor changes in band intensity and position occur below pH 5, apparently reflecting a known conformational change in the low pH region (Breslow and Weis, 1972). Nonetheless the overall constancy of the near-uv ellipticity spectrum over such a wide pH region indicates that the only possible charge perturbation contribution to disulfide ellipticity can come from arginine residues and that conformational changes in the pH region 0.5–11 are limited in scope.

Ease of Neurophysin Disulfide Reduction. As will be

Table I: Stoichiometry of NP-II Reduction by Dithiothreitol.[a]

Moles of DTT added/ mole of NP-II	pH	Length of Time of Reduction	Moles of –SH Recovered per mole of NP-II
1	8.0	5 min	0.9[b]
1	8.0	60 min	2.05 ± 0.15[b]
			2.5[c]
			1.4 ± 0.3[d]
1	6.6	50 min	0.5[b]
1	6.6	23 hr	1.6[b]
4	8.0	60 min	6.0[b]
			6.5[d]
10	8.0	60 min	7.6[b]

[a] Protein concentration = 2×10^{-4} *M* in all studies. [b] –SH determined by Ellman assay on the protein following gel filtration. [c] Determined by carboxymethylation using [^{14}C]iodoacetic acid. [d] Determined by carboxymethylation followed by amino acid analysis of *S*-carboxymethylcysteine. Considerable variation was noted here in the values obtained in different experiments in which reduction and carboxymethylation conditions were identical. Preliminary control studies suggest that the variability in *S*-carboxymethylcysteine analysis and the generally low values obtained reflect loss of *S*-carboxymethylcysteine during hydrolysis.

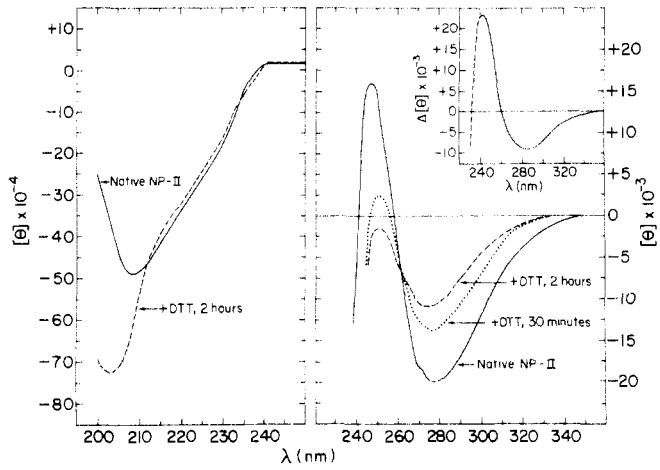

FIGURE 2: Effect of 1 mol equiv of DTT on the CD spectrum of NP-II. Conditions: 2×10^{-4} *M* NP-II; 2×10^{-4} *M* DTT (pH 8.0)–0.16 *M* KCl–10^{-3} *M* EDTA. Right: Near-uv spectrum of native NP-II and of NP-II in the presence of DTT after 30 min and 2 hr. No changes occurred after 2 hr until air was readmitted to the system. The inset shows the spectrum of native NP-II from which the 2 hr spectrum in the presence of DTT has been subtracted. Dashed line in inset indicates some uncertainty in the data below 240 nm. Left: Far-uv spectrum of native NP-II and of NP-II in the presence of DTT after 2 hr.

demonstrated below, major changes in neurophysin optical activity accompany the addition of very low concentrations of DTT, DTE, or other mercaptans *in the absence of urea*. In order to evaluate the extent of reduction of neurophysin disulfides as a function of the concentration of mercaptan added, under conditions comparable to those reported below for optical activity studies, 2×10^{-4} *M* NP-II was incubated with varying molar aliquots of DTT under the conditions indicated in Table I and the degree of protein reduction obtained in each incubation was determined either by carboxymethylation or, more typically, by separating the protein from DTT by gel filtration and measuring the –SH content of the protein fraction (see Materials and Methods). Control studies using radioactive DTT and NP-II indicated that no DTT migrated with NP-II during gel filtration. Results of these studies are reported in Table I. In sum they indicate that addition of 1 molar equiv of DTT at pH 8 almost completely reduces an average of 1 disulfide/mol within 1 hr. Reduction by the first DTT is almost 50% complete in 5 min at pH 8, but reduction rates are markedly slower at pH 6.6, presumably because of the lesser degree of –SH ionization at the lower pH. Completeness of protein reduction within the first hour (per mole of DTT added) diminishes gradually as higher molar ratios of DTT are added. After 1 hr, 3–3.2 disulfides are reduced on addition of 4 equiv of DTT and 3.8 disulfides are reduced on addition of 10 equiv of DTT. The data indicate that at least three NP-II disulfides are highly susceptible to reduction.

Effect of Partial Reduction on Neurophysin Optical Activity. Addition of 1 molar equiv of DTT to 2×10^{-4} *M* NP-II at pH 8, conditions shown above to cause the average reduction of 1 disulfide/mol, leads to rapid CD changes in both the near- and far-uv (Figure 2). The same changes are produced by DTE and by other mercaptans such as glutathione, although changes with glutathione are slower than with DTT and DTE at comparable –SH concentrations, presumably because of differences in reduction potential. The variety of mercaptans which produce the same CD effects indicates that these are a reflection of changes in the protein and not of induced optical activity in the oxidized form of the mercaptan, a conclusion also proven by the fact that the CD effects survive removal of the mercaptan.

Figure 2 shows that, in the presence of 1 equiv of DTT, within 2 hr, the 278-nm band is reduced to approximately half its native intensity and shifted to 275 nm, while the 248-nm band is almost completely lost. The 278/310 ellipticity ratio rises from 2.8 in the native protein to greater than 4 in the partially reduced protein. The resultant near-uv spectrum of the monoreduced protein is very similar to that shown in Figure 1 for completely reduced–reoxidized NP-II. Differences between the near-ultraviolet CD spectra of native and monoreduced NP-II after 2 hr of reduction are shown in the inset of Figure 2 plotted as a difference spectrum. In the far-uv, on reduction with 1 DTT, negative ellipticity near 205 nm is markedly increased and the negative extremum is shifted to shorter wavelengths; however, near 230 nm, ellipticity in the partially reduced protein is slightly more positive than in the native protein, suggesting that loss of positive ellipticity near 248 nm is not due simply to overlap with the increased negative intensity of the 205-nm band. It is possible that the 248-nm band is not really lost, but only shifted further into the ultraviolet, accounting for the increased positive ellipticity near 230 nm. No significant changes in CD occur subsequent to 2 hr of reduction until air is admitted and reoxidation begins. However, upon reoxidation under a variety of conditions, the partly reduced protein does not return to a native CD spectrum as will be discussed in greater detail below.

On addition of increasing molar aliquots of DTT to NP-II, ellipticity near 248 nm becomes only slightly more negative than that observed in the presence of 1 DTT while the 278-nm band diminishes to zero gradually as the DTT/NP ratio is increased, the most major changes in the 278-nm band also having occurred on addition of the first DTT. Few changes in the far-ultraviolet CD spectrum occur subsequent to addition of the first DTT/mole. These data are shown in Figure 3 and indicate that changes induced by the first mole of DTT added are the most significant with respect to optical activity. Also to be noted is the fact that

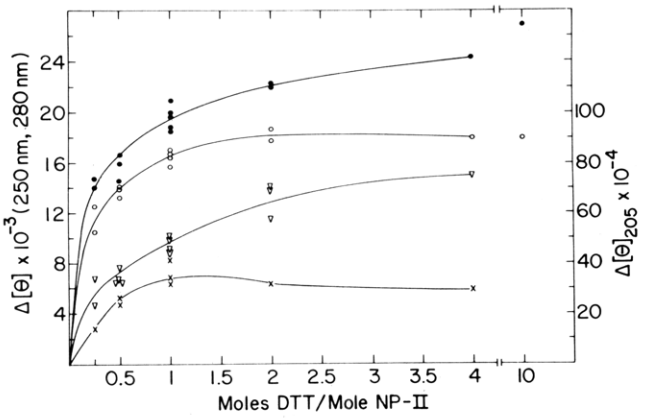

FIGURE 3: Maximum changes in NP-II ellipticity as a function of the molar ratio of DTT to NP-II. Conditions: 2×10^{-4} *M* NP-II (pH 8)–0.16 *M* KCl–10^{-3} *M* EDTA. For convenience, ellipticity changes at 280 nm are expressed as the ellipticity of the partially reduced protein minus that of the native; changes at 205 and 250 nm are expressed as that of the native protein minus that of the partially reduced protein. All changes were complete within 90 min except those at ratios of 0.25 and 0.5 which represent readings at approximately 300 and 200 min, respectively. (x) Observed changes at 205 nm; (▽) observed changes at 280 nm; (○) observed changes at 250 nm; (●) observed changes at 250 nm after subtracting out contributions from the long wavelength band as in Figure 1.

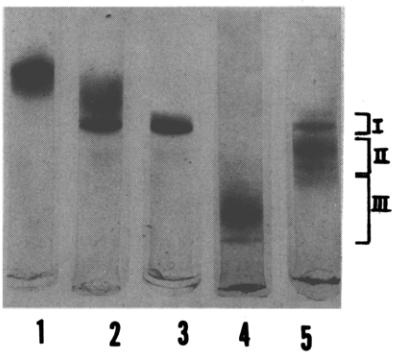

FIGURE 4: Gel electrophoresis patterns of partially reduced NP-II that has been reacted with iodoacetic acid or iodoacetamide. Conditions: 7.5% gel, running pH 9.5. Migration is from top to bottom. Gel 1: NP-II reduced with 4 DTT/mol and carboxamidinated. Gel 2: NP-II reduced with 1 DTT/mol and carboxamidinated. Gel 3: Native NP-II. Gel 4: NP-II reduced with 4 DTT/mol and carboxymethylated; Gel 5: NP-II reduced with 1 DTT/mol and carboxymethylated. Roman numerals show mobility of purified fractions I, II, and III from crude carboxymethylated mixture (see text) when electrophoresed individually. Fraction II contains both the broad band immediately after native NP-II and the narrow band ahead of it. Fraction III migrates principally as a very faint band corresponding to the diffuse region ahead of fraction II, but traces of faster material, not visible in gel 5 also appear to be present. Because of the diffuse nature of fraction III its relative percentage as observed in gel 5 is much less than its weight percentage as isolated.

changes induced by levels of DTT below one per mole are not a linear function of DTT concentration.

A plausible interpretation of the above data is that NP-II contains a single exposed and relatively strained disulfide, which is therefore particularly susceptible to reduction and which is the sole contributor to positive ellipticity at 248 nm and the main single contributor to the negative ellipticity of the 278-nm band, particularly at its long wavelength edge. The difference spectrum (inset, Figure 2) generated by the addition of 1 DTT/mol to NP-II supports such an interpretation since, for example, it resembles the CD spectrum of the single disulfide of the peptide malformin which is reported to be strained (Casey and Martin, 1972). However, changes in the far-uv suggest that general conformational changes accompany addition of the first DTT. This is supported by the electrophoretic behavior of monoreduced-carboxamidinated NP-II (see below) and our observation that reduction of nitrated NP-II (Furth and Hope, 1970) with 1 DTT leads to loss of nitrotyrosine ellipticity. The simultaneity of conformational changes with the average reduction of one disulfide suggests that near-ultraviolet CD changes induced by 1 DTT may result both from the loss of those disulfides which are actually reduced as well as changes in disulfide optical activity arising secondarily from conformational changes. Nonetheless, the question remains as to whether the first DTT selectively reduces a single strained or exposed disulfide on whose integrity the rest of the conformation depends, or whether reduction is nonselective.

Evidence for a Complex Reduction Pathway: Carboxymethylation Studies. Reaction of NP-II that has been reduced with 1 DTT/mol with iodoacetic acid or iodoacetamide leads to no change in near-ultraviolet CD spectrum although some changes are seen in the far-uv. From the near-uv results we have tentatively concluded that carboxymethylation per se does not affect the distribution of sulfhydryls generated by the first DTT and have analyzed the nature of carboxymethylated products by gel electrophoresis and by circular dichroism studies of the ion-exchange fractionated products of the carboxymethylation reaction. The product generated by carboxymethylation following reduction with 1 DTT/mole (at either pH 6.8 or 8) shows a number of bands on polyacrylamide gel electrophoresis (Figure 4); treatment of unreduced NP-II with iodoacetic acid does not lead to a change in electrophoretic mobility indicating that the heterogeneity of the reduced carboxymethylated NP-II arises from heterogeneity in the number of carboxymethylcysteine residues and not from nonuniform carboxymethylation of other residues. The slowest band in the reduced carboxymethylated mixture, barely visible in Figure 4 because of its low concentration, migrates more slowly than native NP-II and appears to be the same as a slowly moving component observed in gels of partially reduced–reoxidized NP-II (see below). The single most sharp band (approximately 30% of the total product as measured by densitometry) has the same mobility as native NP-II, and the faster components represent NP-II carrying different numbers of carboxymethyl residues. In order to estimate the degree of carboxymethylation of the different bands seen on gel electrophoresis, NP-II was reduced with 1 DTT/mol and then carboxymethylated with [^{14}C]iodoacetic acid. The ^{14}C-labeled product was isolated on Sephadex G-25 and contained 2.5 carboxymethyl groups/mol. Fractionation of the crude product into its components was achieved on DEAE-Sephadex A-50 (Figure 5). Following lyophilization of the pooled fractions, the ^{14}C content of each fraction was determined and they were electrophoresed and studied by circular dichroism (Figure 6). Fraction I, which migrates electrophoretically like native NP-II (with a trace of a slower component), is noncarboxymethylated and has a CD spectrum (Figure 6) very similar to that of native NP-II, differences probably being attributable to the minor component. On addition of peptides that bind to NP-II, fraction I shows the same changes (Breslow et al., 1971) as does native NP-II and can be assumed to be native NP-II. Fraction II, the major product, generates several bands on gel electrophoresis which move faster than native NP-II, and contains an average of 1.94 carboxymethyl

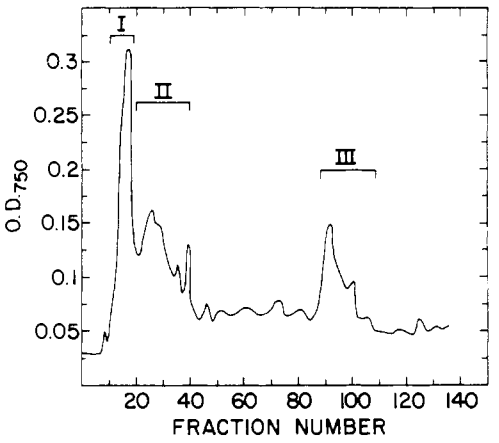

FIGURE 5: Chromatography of product of carboxymethylation following reduction of NP-II with 1 DTT/mol. Column: DEAE Sephadex A-50, 1.7 × 30 cm; fraction volume = 7 ml. Elution was begun with pH 5.9 pyridine acetate buffer and changed to pH 5.5 pyridine acetate buffer at fraction 75 (see Materials and Methods). Protein concentration was monitored by the Folin-Lowry procedure. Fractions corresponding to each peak number are shown above the peak.

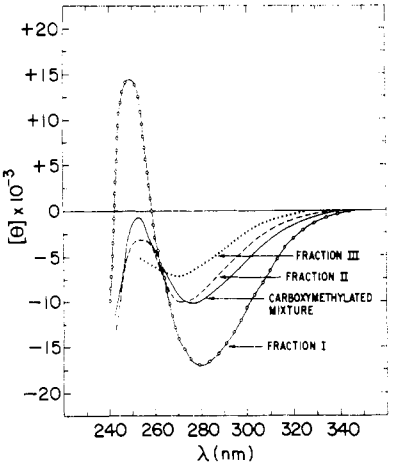

FIGURE 6: CD spectra of carboxymethylation product of monoreduced NP-II and of its fractionated components. The carboxymethylated mixture contains 2.5 carboxymethyl groups per NP-II; fractions I, II and III contain 0, 1.9, and 3.7 carboxymethyl groups per NP-II, respectively. Conditions: pH 6.2, 0.16 *M* KCl.

groups/mol; its near-uv CD spectrum is more denatured than that of the unfractionated carboxymethylated product (Figure 6), showing more negative ellipticity at 248 nm and a more blue-shifted 278-nm band. *The data for fraction II indicate that reduction of a single disulfide is sufficient to cause complete loss of the 248-nm ellipticity band.* Fraction III, which moves as a very diffuse band on electrophoresis, has an average of 3.7 carboxymethyl groups/mol and a CD spectrum which is very similar to that of NP-II reduced with 2 DTT/mol. The ratio of fraction I:II:III, estimated from the integrated curves in Figure 5 with the aid of a standard Folin curve, is 1:1.1:0.72. Allowing for protein migrating between fractions II and III (Figure 5) and not isolated, the integrated Folin analysis indicates that fraction I, which principally represents unreduced NP-II, represents 30% of the total protein, in good agreement with previous (see above) estimates of its concentration from densitometric analysis of the polyacrylamide gels.

When NP-II is reduced with 4 DTT/mol and then carboxymethylated, the resultant gel electrophoresis patterns (Figure 4) show that all components have a higher degree of carboxymethylation than those seen following reduction with 1 DTT, indicating that reduction does not proceed in an "all-or-none" fashion. Reduction of NP-II followed by reaction with iodoacetamide leads to the formation of carboxamidinated products which move more slowly on electrophoresis than NP-II (Figure 4) indicating that the reduction process is accompanied by unfolding and/or polymerization.

The heterogeneity of products resulting from carboxymethylation of NP-II that has been reduced with 1 DTT/mol can, in principle, arise via two general mechanisms: (a) disulfide interchange and (b) the simultaneous or nearly simultaneous reduction of more than one disulfide. The latter can occur either because initial selective reduction of a single disulfide renders a second disulfide in the monoreduced molecule more susceptible to reduction or because more than one disulfide in the native state is equally susceptible to reduction. Our data suggest that mechanisms (a) and (b) may operate simultaneously.

Several lines of evidence support the existence of disulfide interchange although its rate relative to other processes is uncertain. First, the nonlinear relationship at low DTT/NP ratios (Figure 3) between CD changes and the degree of NP-II reduction is most simply explained[2] by assuming that DTT can alter the structure of NP-II both by direct reduction and by setting into motion a limited interchange process in which monoreduced NP-II is reoxidized to a denatured form concurrent with the reduction of a second NP-II; reoxidation of monoreduced NP-II proceeds largely to a denatured form as will be demonstrated below. Second, when partially reduced NP-II is mixed with mononitrated NP-II at pH 8, the CD of the mixture is not the sum of the isolated components, showing significant intensity decreases in both the 250- and 280-nm disulfide peaks and in the nitrotyrosine band. The nonadditivity of the CD spectrum does not occur at pH 3 suggesting its association with an intermolecular disulfide interchange mechanism which affects the structure of nitrated NP-II rather than, for example, noncovalent complex formation between reduced and nonreduced molecules. Finally the existence of an *intra*molecular disulfide exchange mechanism is suggested by the fact that monoreduced NP-II is air-oxidized to a product that contains a significant fraction of monomer in which the disulfides appear to be incorrectly paired (see below). Alternatively some indirect evidence supporting overlap in the rates of reduction by the first DTT of at least two disulfides is found in the fact that the carboxymethylated monoreduced NP-II mixture contains 30% native NP-II. If only one disulfide had been directly reduced by the first DTT, the generation of products with more than 1 reduced disulfide/mol could only arise via intermolecular exchange between two monoreduced molecules such that one was left reoxidized and the other left in the direduced state. Our studies on the reoxidation properties of partially reduced NP-II (see below) indicate that less than 50% recovery of native structure accompanies reoxidation of partially reduced NP-II. Thus, unreduced NP-II which has been gen-

[2] Potential alternate explanations of this effect are: (1) noncovalent complex formation between partially reduced NP-II and native NP-II leading to a CD change of native NP-II and (2) overlapping susceptibilities of several disulfides to reduction with opposing CD consequences of the different cleavages. (1) has been ruled out (see text) and (2) can be shown to necessitate the assumption of unreasonably large ellipticities for single disulfide bonds.

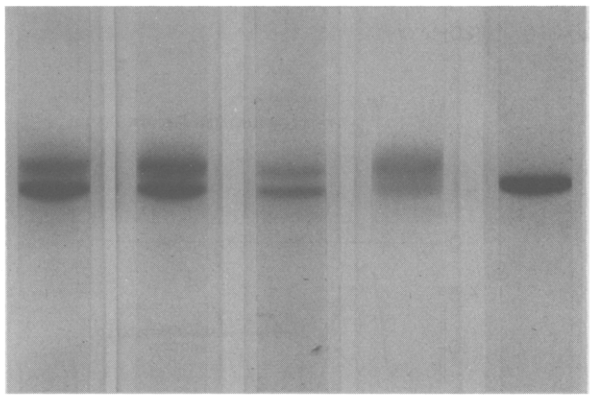

FIGURE 7: Gel electrophoresis patterns of reduced–reoxidized NP-II. Conditions: Running pH 9.5. Direction of migration is to the bottom of the gel. From left to right: (1) NP-II reoxidized after reduction with 0.5 DDT/mol; (2) NP-II reoxidized after reduction with 1 DTT/mol; (3) NP-II reoxidized after reduction with 2 DTT/mol; (4) NP-II reoxidized after complete reduction; (5) native NP-II.

erated by reoxidation of reduced NP-II should be half-denatured whereas the observed unreduced NP-II is almost exclusively native; this suggests the simultaneous reduction of two or more disulfides.

The simplest explanation to account for both the complete loss of the 248-nm CD band in neurophysin fractions containing a single reduced disulfide and for the heterogeneity of the monoreduced protein would invoke initial *selective* reduction of a critical disulfide followed by disulfide interchange. Our data do not establish this mechanism unequivocally.

Properties of Completely Reduced–Reoxidized NP-II. As shown in Figure 1, NP-II that has been reoxidized under our conditions following complete reduction has a near-uv circular dichroism spectrum that differs significantly from native NP-II. The completely reduced–reoxidized NP-II also has an altered far-ultraviolet CD spectrum and shows essentially none of the CD changes that accompany peptide or hormone binding to native NP-II. On gel electrophoresis it shows two very diffuse bands, a minor component moving identically with native NP-II and a major slower moving component (Figure 7). The same two components are present in mixtures of partially reduced–reoxidized protein (Figure 7) and their significance will be discussed below. It is noteworthy that the failure to reoxidize reduced NP-II to a product that shows more native characteristics is not due to destruction of H_2O_2-sensitive residues by any H_2O_2 that might be produced (Trotta et al., 1974) from the added mercaptan. H_2O_2 at concentration orders of magnitude higher than could be produced in these studies has no effect on NP-II, and addition of catalase during reduction and reoxidization did not change the CD spectrum of the reoxidized NP-II. Additionally, the failure to regain the normal CD spectrum on reoxidation is probably not due to the failure of the protein to refold correctly in the presence of properly paired disulfides. In contrast to that of unreduced NP-II, the CD spectrum of reduced–reoxidized NP-II is not "native" after cycling through 5 *M* guanidine.

Reoxidation of Partially Reduced NP-II. Upon air-reoxidation of NP-II that has been partially reduced with DTT, there is a partial return of the CD spectrum to that of native NP-II; i.e., the negative 278-nm band increases in intensity and there is a partial return of the positive 248-nm band. The extent to which the CD spectrum of the product

Table II: Circular Dichroism Properties of Reduced–Reoxidized NP-II.[a]

Reduction Conditions	Molar Ellipticity of Reoxidized Protein		Calculated % "Native" Protein in Reoxidized Samples[b]	
	280 mm	250 mm	280 mm	250 mm
	−18,750	+15,600	100	100
0.25 DTT/mol of NP-II	−14,500 ± 500	+4,250 ± 750	60	42
0.5 DTT/mol of NP-II	−13,750	+4,000	53	41
1 DTT/mol of NP-II	−12,833 ± 1277	+2,867 ± 570	44	35
2 DTT/mol of NP-II	−12,500 ± 0	+1,830 ± 837	41	30
4 DTT/mol of NP-II	−11,000	0	27	20
10 DTT/mol of NP-II	−12,250	+250	39	19
20 DTT/mol of NP-II	−10,000	−950	18	16
Complete reduction (8 *M* urea + mercaptoethanol)	−8,100 ± 770	−2,750 ± 333	0	7

[a] All partially reduced samples were reduced using 2×10^{-4} *M* NP-II and allowing reduction to proceed until circular dichroism changes were complete before air was readmitted to the system. CD spectra were taken periodically during and after reoxidation; CD spectra of the completely reoxidized samples were monitored for at least 1 week after oxidation was complete. Completely reduced samples were monitored by circular dichroism only after reoxidation was complete (see Materials and Methods). All results are reported without correction for the water content (generally 6%) of the samples. [b] Calculated from the relationship: $[\theta]_{obsd} = \text{fraction}_{native} \cdot [\theta]_{native} + (1 - \text{fraction}_{native})[\theta]_{denatured}$, where $[\theta]_{native}$ at 250 and 280 nm are the values for native protein and $[\theta]_{denatured}$ are the ellipticities of the nonprecipitated fraction of Figure 9, corrected for a 10% contamination of this fraction with native NP-II (see text).

resembles that of native NP-II varies inversely with the degree of reduction as shown in Table II. There is some preliminary indication that samples air-oxidized exclusively at room temperature, and hence more rapidly oxidized than those partly stored in the cold during the reoxidation process (see Materials and Methods) have slightly more native characteristics than those oxidized partially in the cold, but the difference is small. In a single experiment, the addition of 2×10^{-3} *M* diamide to speed the oxidation process did not increase the degree to which the native CD spectrum was regenerated.

Figure 8 shows the near-ultraviolet CD spectrum and –SH content of a solution of NP-II in the presence of 4 molar equiv of DTT during reduction and air-reoxidation. Under the conditions used, loss of ellipticity was maximal at 5 min. As reoxidation occurred, ellipticity near 248 nm did not become significantly more positive until the number of –SH in the system decreased to less than two per molecule of NP-II. (Sulfhydryl content was monitored on the entire solution. Since 1 mol of DTT had previously been shown to stoichiometrically reduce 1 NP-II disulfide, the two demonstrable –SH groups can be assumed to be on NP-II and not on DTT.) Similarly the most major changes near 278 nm occurred when the –SH content decreased to less than two

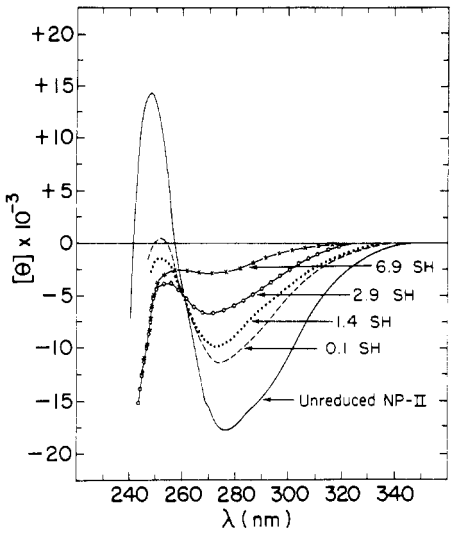

FIGURE 8: Ellipticity changes during reoxidation of NP-II that had been reduced with 4 mol equiv of DTT. Conditions: 2×10^{-4} NP-II, 8×10^{-4} *M* DTT (pH 8.2), 0.16 *M* KCl, and 10^{-3} *M* EDTA. NP-II and DTT were mixed in the absence of air and spectra taken until no further changes occurred. Air was admitted and CD spectra and the total -SH content of the solution were simultaneously monitored until reoxidation was complete. Numbers shown on the spectra represent the -SH content of the solution expressed as moles of -SH per mole of NP-II. For reference, the spectrum of the same sample of NP-II prior to reduction is shown.

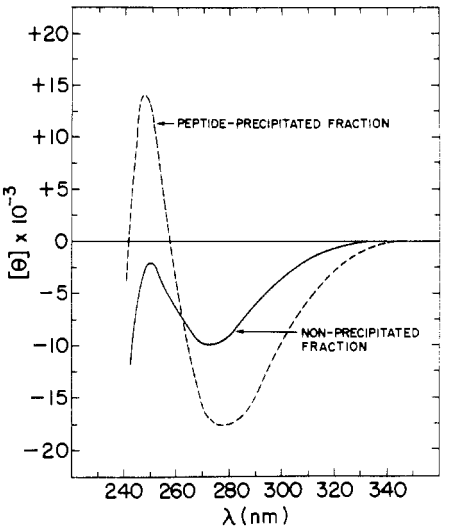

FIGURE 9: Fractionated components of monoreduced-reoxidized NP-II. Conditions: pH 6.2, 0.16 *M* KCl. The peptide-precipitated fraction is that which precipitates with L-cystinyl-bis(L-tyrosineamide) (see text) and behaves like native NP-II on gel electrophoresis. The nonprecipitated fraction contains 10% native NP-II when examined by gel electrophoresis.

per NP-II. Since the principal changes at 248 and 278 nm occurring on reduction of NP-II accompany reduction of the first disulfide, these results indicate that there are common aspects of the reduction and reoxidation pathways; i.e., the first disulfide to be reduced and the last disulfide to be regenerated during reoxidation are directly or indirectly responsible for the 248-nm positive ellipticity and most of the 278-nm ellipticity. Similar reoxidation patterns of the recovery of ellipticity as a function of -SH content were obtained following all partial reductions.

The degree of spectral recovery following partial reduction (Table II) indicates that, under the conditions used, a large fraction of the reoxidized protein is nonnative, even when the initial reduction is with only 0.25 DTT/mol. This is also indicated by gel electrophoretic analysis of the partially reduced-reoxidized protein (Figure 7). Two distinct bands are seen, one with the mobility of native NP-II, and a slower moving component, the relative proportion of the two bands being only slightly dependent on the reducing conditions at ratios of DTT/NP below 2; however, the fraction of the retarded component seen on partial reduction is less than that seen with completely reduced-reoxidized protein. Sedimentation velocity studies of partially reduced-reoxidized NP-II also indicate that it contains a high fraction of nonnative protein. At pH 6.2, the weight average $s_{20,w}$ of native NP-II is 1.87 at a protein concentration of 5 mg/ml. Under identical conditions the $s_{20,w}$ of protein that has been reoxidized following reduction with 2 DTT/mol is 1.45.

Reduced-reoxidized NP-II can be shown to be a mixture of native and two classes of denatured protein. (1) The electrophoretic component whose mobility is the same as that of NP-II has been identified as native NP-II. (2) The electrophoretic component whose mobility is slower than that of native NP-II has been demonstrated to be monomeric NP-II of a different conformation from native, presumably altered because of incorrect disulfide pairing. (3) A polymeric component is present that migrates between the native and slow components on gel electrophoresis as such a diffuse band that is not clearly observable (except that it gives a diffuse character to the two other electrophoretic bands) until separated from the other two components. Identification of these components was achieved by fractionating a sample of monoreduced-reoxidized NP-II using the peptide L-cystinylbis(L-tyrosineamide), which appears to bind to the hormone-binding site (Breslow et al., 1971), as the fractionating reagent (see Materials and Methods). Protein that precipitated with the peptide migrated identically with native NP-II on gel electrophoresis, had the same CD spectrum as native NP-II (Figure 9) and showed CD changes typical of the native protein (Breslow et al., 1971) on addition of peptide analogs of oxytocin. Protein that was not precipitated by cystinylbis(L-tyrosineamide) behaved electrophoretically as the diffuse slow band of Figure 7 contaminated with a trace of native NP-II; its near-uv ellipticity spectrum (Figure 9) is almost identical with that shown in Figure 1 for completely reduced-reoxidized NP-II. On dodecyl sulfate gel electrophoresis in the absence of pretreatment with mercaptoethanol (see Materials and Methods) the nonprecipitated protein showed several components, the major and fastest component identifiable as monomer and the other components identifiable as oligomers. In a separate experiment, the oligomeric components of partially reduced-reoxidized NP-II were separated from the native and denatured monomeric components by chromatography on Sephadex G-50 and shown to migrate electrophoretically on polyacrylamide gel as a very diffuse component between native NP-II and the slow component of Figure 7; the CD spectrum of the oligomeric components was the same as that of the total denatured protein shown in Figure 9. Denatured monomeric components migrated identically with native protein on Sephadex G-50 and as the slow-moving electrophoretic component of Figure 7 except that they gave a sharper electrophoretic band once separated from denatured oligomers.

A puzzling feature of these data is that the slow-moving

electrophoretic component of reduced–reoxidized NP-II is denatured monomer while the oligomeric components migrate between the slow and fast bands. We have looked for other alterations in the slow-moving band (such as changes in amino acid composition) that might account for the reduced mobility, but as yet have found none.

Estimation of Degree of Recovery of Native NP-II Following Reduction. The isolated components (native and denatured) of partially reduced–reoxidized NP-II give additive CD spectra. Thus if these components are invariant in their characteristics and are the only proteins present in all samples of reduced–reoxidized NP-II, it should be possible to interpret the CD spectra of different samples of reduced–reoxidized NP-II as the simple sum of different proportions of native and denatured protein. In Table II, the estimated fraction of native NP-II in protein that has been reoxidized after different degrees of reduction has been calculated in this manner at two key wavelengths from the CD spectrum of the reoxidized protein. Data at intermediate wavelengths give similar results. There is a disparity between the two wavelengths in the calculated fraction of native protein, calculated recoveries at 250 nm generally being somewhat lower than those calculated at 280 nm; this is true for computations done on most individual spectra as well as for the averaged data presented in Table II. Preliminary estimates of the percentage of native protein in these samples using other criteria, such as changes in CD spectra on binding oxytocin relative to that of native protein (see Materials and Methods), are in somewhat better agreement with calculations based on the 250-nm data. The reason for the disparity between calculations at the two wavelengths is uncertain. A contributory factor might be the selective loss during the procedure used to fractionate native from nonnative protein of material with different CD characteristics, since protein was not quantitatively recovered (see Materials and Methods); therefore, the CD spectrum of the isolated nonprecipitable protein component of reduced–reoxidized NP-II may not be a true reflection of all the nonnative protein present. While for this reason, the calculated values in Table II should be regarded as approximate, the data at both wavelengths show a trend which indicates a lower percentage of native protein in those samples that have been reduced to the greatest extent.[3] This is in part a reflection of differences among the samples in the fraction of protein that has reacted with DTT during the reduction step; cf. 30% native NP-II is present prior to reoxidation in samples reacted with 1 DTT/mol (see above), while essentially none is present in samples reacted with 4 DTT/mol, as evidenced from carboxymethylation patterns (Figure 4); thus the *regenerated* native protein in samples reduced with less than 2 DTT/mol is significantly less than the *total* native protein while this is not true for the more highly reduced samples. An upper limit of about 40% can be specified as the extent of proper refolding of a partially reduced molecule under our conditions. It is relevant that similar yields of native protein were obtained following exposure of NP-II to very low concentrations of other mercaptans such as glutathione.

Effect of Hormone Analogs on Reduction and Reoxidation. Peptides that bind to the hormone-binding site of NP-II (Breslow et al., 1973), such as Met-Tyr-PheNH_2, give partial protection to NP-II from reduction by DTT at pH values where significant peptide binding can occur. For example, at pH 6.7 in 0.05 *M* phosphate buffer, ellipticity changes at 250 and 280 nm in the presence of 10^{-3} *M* Met-Tyr-Phe NH_2 occurred at less than half the rate than when peptide was absent and proceeded only to about 50% completion relative to values obtained in the absence of peptide under the same conditions, despite prolonged standing in the absence of air and under conditions under which no net reoxidation could be demonstrated. Carboxymethylation (prior to reoxidation) of protein partially reduced in the presence of peptide reveals the same heterogeneity of products as found following reduction in the absence of peptide except that a much greater percentage of protein is noncarboxymethylated.

Although binding of peptide protects significantly against reduction, it has not, in our hands, significantly increased the percentage of material that is reoxidized back to the native structure following either partial or complete reduction. Reoxidation of completely reduced protein at pH 7.5 in the presence of 10^{-3} *M* *S*-methyl-L-cysteinyl-L-Tyr-L-Phe-NH_2, a peptide that binds to the hormone-binding site (Breslow, et al., 1973), gave only a completely denatured product. The CD of protein reduced with only 1 mol of DTE/mol and reoxidized at a concentration of 2×10^{-4} *M* in the presence of a fivefold excess of oxytocin at pH 8 (conditions under which significant oxytocin-binding occurs to native NP-II) is not significantly different from that reoxidized in the absence of oxytocin.

Discussion

The two disulfide near-uv ellipticity bands seen in neurophysin differ markedly from the near-uv disulfide ellipticity spectrum of L-cystine in solution, the latter characterized by a single much weaker negative band located near 250 nm; differences between the two systems can be attributed to the fact that cystine, in solution, equilibrates to a mixture of components that differ in disulfide screw sense (Beychok, 1965) while the disulfides in a native protein are largely fixed in a conformation dictated by that of the polypeptide chain. In this context, an effect of denaturation on neurophysin disulfide ellipticity is to be expected. It is also to be expected that disulfide ellipticity spectra will differ in different proteins and where disulfide CD contributions have been characterized differences are evident. For example, in pancreatic RNase, the resolved disulfide ellipticity contributions to the longest wavelength band are centered at 270 nm, 5 nm below the true position of the 278-nm NP-II band (located at 275 nm by resolving it from the 248-nm band) and, in additional contrast to NP-II, no RNase disulfide contributions are evident above 305 nm (Horwitz et al., 1970). Also, the second longest wavelength CD band in RNase differs in position from that of NP-II and may not be assignable to disulfides (Sears and Beychok, 1973). On the basis of current theory (Linderberg and Michl, 1970; Sears and Beychok, 1973) one interpretation of the relatively long wavelength at which the 278-nm NP-II band is centered and of the 30-nm separation between the two near-uv bands, is that the average disulfide dihedral angle in NP-II differs from the 90 to 100° value characteristic of linear unconstrained disulfides; i.e., one or more of the disulfides in

[3] In principle, differences in CD spectra among samples reoxidized following different degrees of reduction could also arise if there were a constant fraction of native protein in all samples accompanied by a constant fraction of denatured protein whose CD spectrum was dependent on the degree of reduction. However, such an interpretation is at variance with gel electrophoresis patterns of fully reduced–reoxidized NP-II (which show little native NP-II) and with estimates of native protein content based on the effects of hormones and hormone analogs on the CD spectra of reoxidized protein samples (see text).

the native state are strained. Using this assumption, the shift of the 278-nm band to shorter wavelengths in guanidine and in the completely reduced–reoxidized protein can be explained as a relaxation of the dihedral angle in the denatured state toward that preferred by linear disulfides. The qualitative resemblance of the near-uv difference CD spectrum generated on reduction by 1 DTT (Figure 2) to that of the strained disulfide of malformin, which shows a negative band at 280 nm and a positive band at 235 nm (Casey and Martin, 1972) is in accord with the loss or normalization of strained disulfides on partial reduction. This difference spectrum can also be generated by comparison of the CD spectrum of the completely reduced–reoxidized protein with that of the native, and as cited previously, it may reflect changes in one disulfide that is particularly abnormal in the native state or it may reflect smaller contributions from several disulfides.

The apparent disappearance of the 248-nm band on denaturation and monoreduction is also in accord with a model in which the average disulfide dihedral angle changes from an abnormal value in the native protein to a value closer to normal in the modified protein. This follows because, according to current theory (Linderberg and Michl, 1970; Sears and Beychok, 1973), as the dihedral angle approaches 90–100°, the separation between the two oppositely signed near-uv transitions of a given disulfide will decrease, leading to internal cancellation and loss of ellipticity. Thus, the decrease in intensity of the 278-nm band and loss of the 248-nm band could arise from such internal cancellation, particularly if all of the 248-nm band and half of the 278-nm band are assigned to a single disulfide. However, as indicated previously, it is not absolutely certain that the 248-nm band really disappears on partial reduction; there is a small possibility that it is only shifted further into the ultraviolet. Additionally other factors may contribute to changes in the 248-nm band. Recent computer analyses of the CD spectra of proteins (Saxena and Wetlaufer, 1971; Chen et al., 1972) suggest that peptide bonds in the β conformation may contribute a positive ellipticity band centered at 237 nm. Analysis of the far-ultraviolet CD spectrum of NP-II (Breslow, 1974) suggests that it has 40% β structure. While the shape of the 248-nm band of NP-II, as resolved from the 280-nm band (Figure 1), continues to support the assignment of major disulfide contributions to the 248-nm band, the possibility of peptide bond contributions must now also be considered. Therefore changes in the 248-nm band under denaturing conditions may reflect both changes in disulfide and in peptide bond transitions.

With reference to reduction–reoxidation properties, the principal observations made here are: (1) partial reduction of bovine NP-II proceeds very easily and with only a limited degree of reversibility; reoxidation of completely reduced NP-II yields almost no native protein under the conditions used; (2) reduction or interchange of only a single disulfide bond produces conformational changes and major changes in disulfide optical activity. The marked susceptibility of NP-II to reduction and the low degree of reversibility are not simply the reflection of the high disulfide content which would, for example, be expected to increase the opportunities for disulfide interchange. Some of the trypsin inhibitors with an equally high disulfide content tentatively do not appear as susceptible to reduction (L. S. Gennis and C. R. Cantor, personal communication) and the equally disulfide-rich cobra toxins reoxidize back to the native conformation after complete reduction (Yang, 1967). Additionally, the fact that conformational changes attend reduction of the first NP-II disulfide differs from the observation made with many other disulfide-containing proteins (including those with a much lower disulfide content) that a few disulfide bonds can be broken without important conformational effects (Sperling et al., 1969; Schechter et al., 1973).

An attractive hypothesis can be advanced to explain many of the reduction–reoxidation properties of NP-II, as follows. In contrast to proteins such as pancreatic ribonuclease which are less readily reduced than NP-II and which can be easily reoxidized back to the native state following complete reduction (Anfinsen et al., 1961), NP-II, as isolated, is not in its lowest free energy state with respect to disulfide pairing. In this respect NP-II is regarded as analogous to proteins such as insulin (Givol et al., 1965; Steiner and Oyer, 1967) in which the reversibility of reduction is also limited, and it is assumed that the disulfides of NP-II are paired during biosynthesis when NP-II (like insulin) exists as part of a larger precursor molecule whose subsequent cleavage leaves the disulfides in a metastable state. This precursor can also be envisioned as a precursor of vasopressin (or oxytocin) in which case the noncovalent interactions between hormone and NP-II in the isolated hormone–NP-II complex are assumed to be vestiges of similar interactions within a common precursor. (It is important to note that neither the existence of a neurophysin precursor or a hormone precursor has been strictly proven, but a common precursor for vasopressin and neurophysin has been postulated to account for a number of other experimental observations [Sachs et al., 1969, 1971; Breslow, 1974]). A contributing factor to the metastability of the isolated NP-II molecule arises from the unfavorable bond angles of specific disulfides. Addition of mercaptan leads to a more stable conformation both by direct reduction of strained disulfide bonds and by providing an electron carrier that mediates disulfide interchange and allows equilibration to a different distribution of conformations of lower free energy and more favorable disulfide geometry. Peptides that bind to the hormone-binding site of neurophysin protect against reduction by lowering the free energy of the native protein relative to that of the reduced or "disulfide-interchanged" protein, since the peptide-binding site is lost upon reduction or disulfide interchange.

The above model was suggested earlier in a preliminary report (Menendez-Botet and Breslow, 1972) and is similar to that recently invoked by Chaikin et al., (1975). It is in agreement with the ease of NP-II reduction, its apparent susceptibility to disulfide interchange, the failure to regenerate native protein after complete reduction, the protecting effects of peptide, and the ellipticity changes that accompany disulfide reduction and interchange. However, there are serious limitations in the degree to which the model fits the data. In its simplest form, it assumes that a true equilibrium is achieved both during reduction (partial and complete) and reoxidation, and this is not so. Peptide analogs of oxytocin and vasopressin offer some degree of protection against ready reduction but do not (in our hands) demonstrably increase the yield of native protein generated by reoxidation of partially reduced protein. The composition of a sample of reduced–reoxidized protein is dependent on the degree of initial reduction. Thus there is evidence that the final conformation(s) attained by NP-II after exposure to mercaptan are dependent on path and, for a given experiment, may partly represent accessible metastable state(s) rather than a true equilibrium state. Another qualification to be stressed

is that, at least following partial reduction, a significant fraction of native NP-II is regenerated on reoxidation and this fraction is much greater than the value of 1/135, 135 allowed on the basis of random pairing of the seven disulfides. Thus, if the presence of a true equilibrium is assumed during partial reduction and reoxidation (and not on reoxidation from the completely reduced state) the data suggest that the native conformation differs only marginally in stability from the sum of conformations with rearranged disulfides. Finally, it remains possible that the limited regeneration of native protein following partial or complete reduction represents, at least in part, covalent changes in the protein, other than reduction, in the presence of added mercaptan. We have consistently observed that, subsequent to addition of DTT to neurophysin, a substance is released that has the odor of a mercaptan; it is not formed on addition of DTT to ribonuclease or insulin but is also formed when mercaptans other than DTT are added to neurophysin. It can therefore be presumed that some sulfur is lost from neurophysin during reduction. Preliminary attempts to quantitate the amount of sulfur released suggest that it represents quantities too small to account for any significant fraction of the irreversibility of NP-II reduction, but further studies of this phenomenon are in progress. In sum, the data support the concept that some of the driving force behind the ease of neurophysin reduction arises from the unfavorable geometry of one or more of the disulfides in the native state, but the free energy of the native state relative to that of conformations containing different half-cystine pairs remains uncertain.

Acknowledgments

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