Thermodynamic Role of the Pro Region of the Neurophysin Precursor in Neurophysin Folding: Evidence from the Effects of Ligand Peptides on Folding[†]

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ABSTRACT: Attention has focused recently on the role of amino-terminal precursor pro regions in protein folding, with particular emphasis on their effects on folding kinetics. We examined the kinetic and thermodynamic effects of ligand peptides on the folding of neurophysin from the reduced state; these peptides serve as analogs of the pro regions of the common precursors of the neurophysins and the hormones oxytocin and vasopressin. Folding of reduced, mononitrated bovine neurophysin-II was monitored by circular dichroism in a glutathione redox buffer. The results confirmed the ability of neurophysin to fold to a limited extent (20−25% in this system) in the absence of ligand peptides. Ligand peptides increased the efficiency of folding to 100%, the exact efficiency being dependent on peptide identity and concentration. However, the rate of folding was peptide-independent. Analysis of the folding reaction demonstrated relatively rapid conversion of the reduced state to a disulfide-scrambled state, which slowly converted (half-life of 5 h at pH 7.3) to the folded state. Native unliganded neurophysin also equilibrated with the disulfide-scrambled state in the same redox buffers. For each peptide, an equilibrium constant for the folding reaction, representing the amount of peptide bound in the folding system as a function of peptide concentration, was calculated. Comparison of this constant with the intrinsic binding constants of the native protein allowed the derivation, under conditions at or approaching thermodynamic reversibility, of the relative stability of the native and disulfide-scrambled states. The results indicate that the scrambled state, which probably represents the presence of incorrect disulfide pairs in both protein domains, is more stable than the native unliganded state by ∼1 kcal/mol in this system. The role of ligand peptide therefore is to stabilize the folded protein after it is formed, i.e., it provides a thermodynamic sink. The results contrast with the putative behavior of exogenous peptides representative of the pro regions of subtilisin and α-lytic protease, which are generally considered to facilitate folding by reaction with folding intermediates. A potential alternative view of the role of propeptides in protease folding is suggested.

Amino-terminal pro regions of protein precursors often play an essential role in precursor folding and, in this context, are considered to function as intramolecular chaperones [reviewed by Shinde and Inouye (1993)]. In the case of proteolytic enzymes such as subtilisin (Shinde et al., 1993; Strausberg et al., 1993) and α-lytic protease (Baker et al., 1992), noncovalent interactions between the pro regions and folding intermediates are postulated to diminish the activation energy required for individual steps along the folding path. In the case of bovine pancreatic trypsin inhibitor, evidence has been presented that the pro region controls folding by the participation of its single thiol in disulfide interchange reactions (Weissman & Kim, 1992).

The posterior pituitary hormones oxytocin and vasopressin are each biosynthesized as the amino-terminal segment of a precursor that also contains their carrier protein neurophysin (NP),[1] to which they are attached at their carboxyl termini via an intervening Gly-Lys-Arg sequence (e.g., Ivell & Richter, 1984). Within the precursor, hormone and neurophysin segments interact noncovalently (Ando et al., 1987). Processing cleaves the covalent bonds between hormone and NP, but retains the noncovalent interactions between the two components in the form of an intermolecular complex that can be reversibly dissociated [reviewed in Breslow and Burman (1990)]. Neurophysin contains 7 disulfide residues within a chain of ∼95 residues (Burman et al., 1989). Early studies demonstrated that these disulfides were unstable in the absence of hormone or analogous peptides that bind to the hormone-binding site, with the instability evidenced by facile reduction and marked susceptibility to disulfide interchange in the presence of low concentrations of thiol (Menendez-Botet & Breslow, 1975). One or two disulfides were initially implicated as the source of the instability, with more recent studies identifying the Cys_{10}−Cys_{54} bridge of the hormone-binding site as the most labile bridge (Huang & Breslow, 1992). These studies also demonstrated that reoxidation of fully reduced neurophysin in the absence of liganded peptides led to only limited formation of native protein, but that regeneration of native protein from the reduced state was virtually complete in the presence of ligand

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[1] Abbreviations: NP, neurophysin; BNP-II, bovine neurophysin-II; BNP-I, bovine neurophysin-I; CD, circular dichroism; Phe-$TyrNH_2$, L-phenylalanyl-L-tyrosine amide; Phe-$PheNH_2$, L-phenylalanyl-L-phenylalanine amide; Gly-$TyrNH_2$, glycyl-L-tyrosine amide; Met-Phe-$TrpNH_2$, L-methionyl-L-tyrosyl-L-tryptophan amide; DTT, dithiothreitol; GSH, reduced glutathione; GSSG, oxidized glutathione; $\bar{\nu}$, moles of peptide bound per mole of neurophysin chain; TFA, trifluoroacetic acid; HPLC, high-performance liquid chromatography; CD, circular dichroism; molar ellipticity or $[\theta]$, ellipticity per mole of polypeptide chain in units of deg·cm^2/dmol.

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peptides. Thus, the hormone segment of the neurophysin precursor, which represents its pro region, functions to control folding—possibly via direct interactions with residues 10 and 54—and can be mimicked by exogenous peptides that bind similarly to the hormone-binding site.

What is the mechanism by which ligand peptides control neurophysin folding from the reduced state? Does the peptide function purely thermodynamically by trapping the correctly folded protein after it is formed and preventing its reaction with exogenous thiol? Or does the peptide lower an energy barrier on the route to the correctly folded state, i.e, does it play a kinetic role instead of, or in addition to, a thermodynamic role? This question is addressed in the present study by an evaluation of the effects of the identity and concentration of ligand peptides on the efficiency of neurophysin folding from the reduced state and on the rate of folding. By using peptides that markedly differ among themselves in binding affinity, we present evidence for a role that is largely, if not exclusively, thermodynamic. The possible implications of this for the role of pro regions in other systems is discussed.

Another question addressed is that of the role of the two neurophysin domains in folding. The neurophysin structure can be divided into an amino-terminal domain, containing four disulfides and the peptide-binding site, and a carboxyl-terminal domain, containing three disulfides (Burman et al., 1989). The three disulfides of the carboxyl domain and their surrounding sequences are homologous in primary structure and conformation to a large region of the amino domain (Burman et al., 1989; Chen et al., 1991). Identification of the 10-54 bridge, which is the unique disulfide of the amino domain, as the unstable disulfide, together with the distribution of trapped thiols at the terminal stage of folding or the initial stage of unfolding, suggested that the instability of the protein disulfides in the absence of ligand peptide reflected only the amino domain and that the carboxyl domain might be capable of folding independently of the amino domain or ligand peptide. This hypothesis is also explored further in the present study.

MATERIALS AND METHODS

Proteins and Peptides. Bovine NP-II and its mononitrated derivative were prepared as described previously (Sardana et al., 1987). Phe-TyrNH_2 and Phe-PheNH_2 were obtained from BACHEM. Gly-TyrNH_2 was obtained from Sigma. Met-Phe-TrpNH_2 was custom-synthesized by Peninsula Laboratories. Oxytocin was obtained from Sigma and BACHEM.

Refolding from the Completely Reduced State. Mononitrated BNP-II was completely reduced by DTT in the presence of 6 M guanidine hydrochloride at pH 8.6 as previously described (Huang & Breslow, 1992). The pH of the reduced protein was lowered with glacial acetic acid, and the solution was dialyzed exhaustively against 0.2 M acetic acid. The resulting solution was stored at 4 °C under N_2. To monitor folding, aliquots of the reduced protein were adjusted to the pH indicated (typically pH 7.3) by the addition of concentrated Tris base and mixed with fresh solutions of GSH and GSSG plus peptide as indicated, in the same Tris−acetate buffer, to give the final concentrations reported. The sample was immediately deaerated with N_2 for ~2 min and placed in a stoppered, deaerated cuvette, and its CD spectrum was monitored with time. Except as indicated, the concentrations of GSH and GSSG were each 2 mM, and the temperature was 26 °C. The cuvette was kept in the dark between readings. At the end of each run, the protein concentration was calculated from the absorption spectrum of the nitrotyrosine by using a molar extinction coefficient of 2000 at 380 nm, the isosbestic point in spectra of the ionized and un-ionized nitrotyrosine (Breslow & Weis, 1972). The range of protein concentrations used was 0.07−0.1 mM.

CD spectra were monitored by using either a Jobin-Yvon Mark 5 CD spectrometer or a Jasco J-710 spectrometer, with identical results. Each spectrum represents data accumulated over a scanning period of 10−20 min; the reported times are the end points of the accumulation periods. Because refolding was slow, no significant error was introduced by the length of time used to accumulate the data in standard refolding experiments. All results are reported as molar ellipticities (per chain basis) unless otherwise indicated.

Determination of Intrinsic Peptide-Binding Constants. Dipeptide binding affinities to 0.2 mM mononitrated BNP-II had been determined previously by CD titration at pH 6.2 in 0.1 M ammonium acetate (Breslow et al., 1991). The binding affinity of Met-Phe-TrpNH_2 had been determined similarly under the same conditions as 14 000 M^{-1} (unpublished results). Observed binding affinities are pH- and concentration-dependent, decreasing with deprotonation of the peptide α-amino group and increasing with deprotonation of the nitrotyrosine and concentration (Breslow et al., 1973, 1991). The relevant present studies were conducted at pH 7.3 in 0.2 M Tris−acetate buffer at a protein concentration of 0.1 mM. Direct measurement of the binding affinity of Phe-TyrNH_2 (amino pK_a = 7.2) by CD titration under these conditions indicated a fortuitous cancellation of the effects of pH, concentration, and buffer. Accordingly, binding affinities of Phe-PheNH_2 and Met-Phe-TrpNH_2, which are estimated to have the same α-amino pK_a values as Phe-TyrNH_2 (Breslow et al., 1971; Steinhardt & Beychok, 1964), were assumed to be the same under the pH 7.3 experimental conditions as those at pH 6.2. For Gly-TyrNH_2 (amino pK_a = 8.0), the binding affinity under pH 7.3 conditions was also determined experimentally and found to be approximately twice the pH 6.2 value, in agreement with prediction (Breslow et al., 1973).

Calculation of Folding Equilibria. For folding reactions in the presence of peptide, values of $\bar{\nu}$ (average moles of peptide bound per mole of NP) were calculated at equilibrium by comparison of observed nitrotyrosine ellipticities with the ellipticity representative of saturation. Free peptide concentrations at equilibrium were calculated from total concentrations by correction for the amount bound (Breslow et al., 1973). Saturation ellipticities were calculated from the final ellipticities obtained with the same lot of reduced protein, when folding was carried out in the presence of 10 mM Phe-TyrNH_2 at pH 7.3, a concentration sufficient to produce >95% folding at this pH. Saturation ellipticities calculated in this manner differed by no more than ±10% from values obtained upon saturating the native protein with peptide, indicating that negligible amounts of irreversibly denatured protein typically were formed during reduction and refolding under these conditions. Studies of duplicate samples indicated that the ±10% variation represents the precision of

the methods used; individual values of $\bar{\nu}$ therefore are reliable only to this extent.

Carboxymethylation and Analysis of the Partially Folded State. Reduced, nitrated BNP-II (2 mg) was allowed to refold at pH 7.3 in the presence of 10 mM Phe-TyrNH_2 and 2 mM each GSH and GSSG. After 5 h (the half-time for refolding), the sample was reacted with 0.3 M iodoacetate in the dark for 1 h at room temperature, followed by 24 h at 4 °C. The sample was subjected to gel filtration in 0.2 M acetic acid and the protein was lyophilized. A sample of protein was hydrolyzed for complete amino acid analysis. The remainder of the protein sample was used for automated gas phase sequencing before and after trypsin digestion by using procedures previously described (Burman et al., 1989). Sequencing of the intact sample gave reliable data through Cys_{44}. Trypsin treatment generated new amino termini at residues 9, 21, 44, and 67, and the sequences beginning with these termini were analyzed simultaneously as in previous work (Burman et al., 1989). The combined approach allowed a survey of the carboxymethyl-Cys content at each half-Cys position in the protein.

Preparation of the Carboxyl Domain from Bovine Neurophysin-II. BNP-II was reduced with 0.5 molar equiv of DTT for 10 min in 0.45 M Tris-HCl at pH 8.6. Iodoacetate (0.25 M final concentration) was added, and the sample stirred in the dark for 1 h. The mixture was chromatographed on Sephadex G-25 in 0.2 M acetic acid, and the protein peak was lyophilized. This modification has been shown to cleave the 10-54 disulfide bridge and to scramble the disulfides of the protein amino domain, with no evidence of a significant effect on the carboxyl domain (Huang & Breslow, 1992). The modified protein (binding-incompetent) was separated from residual unmodified protein by affinity chromatography (Rabbani et al., 1982), followed by dialysis and lyophilization. It was then digested with thermolysin in 0.05 M Tris−1 mM $CaCl_2$ (pH 7.6) for 2 h at room temperature using a protein to enzyme ratio of 40/1 (w/w), and the digestion was stopped by the addition of TFA (1.5% final concentration).

The carboxyl-domain peptide was isolated from the sample by reversed phase HPLC using a Beckman C18 column (250 × 4.6 mm) with the following solvent system: A, 0.1% TFA in water; B, 0.1% TFA in acetonitrile. A linear gradient of 0−60% B over 40 min was used at a flow rate of 1 mL/min. Absorption was monitored at 280 nm. A sharp peak at 27 min was isolated, lyophilized, and further purified by anion exchange HPLC using Protein Pak DEAE-5PW (Waters). The solvent system was as follows: A, 0.02 M ammonium acetate, pH 7.6; B, 0.02 M ammonium acetate, pH 4.3. A gradient of 0−10% B over 35 min at a flow rate of 1 mL/min was used. The fragment at 11.5 min was collected and lyophilized. Amino acid analysis and gas phase sequencing indicated that it represented the uninterrupted sequence 49−92, with a carboxymethyl-Cys at position 54.

RESULTS

Measurement of the Rate and Efficiency of Neurophysin Folding; General Properties of the Folding System. A CD system was developed to follow folding from the reduced state. The system utilized BNP-II that was mononitrated at its single tyrosine residue (Tyr_{49}) to take advantage of the sensitivity of the long wavelength nitrotyrosine CD band to NP conformation and binding (Breslow & Weis, 1972) and the separation of this band from contributions of other chromophores. In a typical experiment, reduced nitrated NP was mixed with glutathione redox buffer and ligand peptide and deaerated, and the optical activity of the nitrotyrosine CD band was monitored with time in a closed CD cell (Materials and Methods).

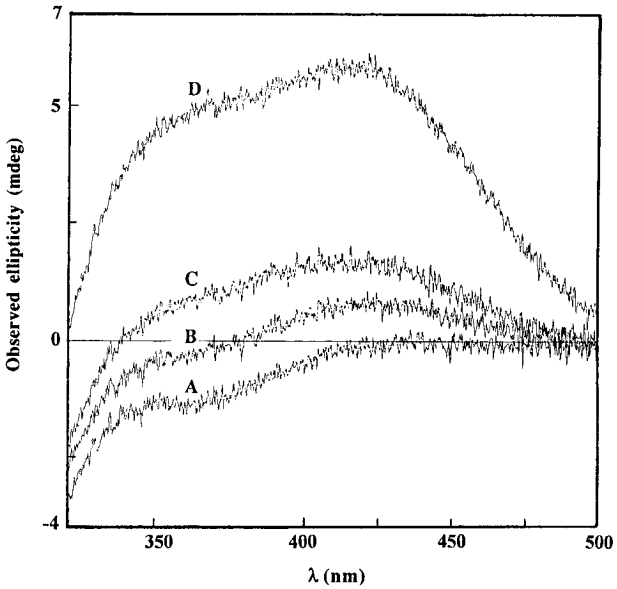

FIGURE 1: CD spectra of mononitrated BNP-II at different pH values in the unliganded state and at pH 7.3 in the liganded state. Conditions: 0.18 mM protein, 0.2 M Tris−acetate buffer, light path = 0.5 cm. Results are presented as the observed ellipticity in millidegrees. (A) Unliganded state, pH 6; (B) unliganded state, pH 7.3; (C) unliganded state, pH 8.5; (D) pH 7.3 in the presence of 10 mM Phe-TyrNH_2.

Figure 1 shows the CD spectra of unliganded, nitrated BNP-II at pH 6 (nitrotyrosine protonated), 8.5 (nitrotyrosine ionized), and 7.3 (partial nitrotyrosine ionization). The ellipticity of the unliganded state at pH 7.3 is very weak and is essentially zero in the 375−380 nm region. In the liganded state, as illustrated in Figure 1 at pH 7.3, ellipticity is markedly intensified and is positive at all pH values (e.g., Breslow & Weis, 1972). By contrast, the nitrotyrosine optical activity in this wavelength region is lost upon unfolding (vide infra). Accordingly, the 375−380 nm ellpiticity at pH 7.3 is a direct measure of the concentration of the folded, liganded state. Moreover, during refolding studies in the presence of peptide at pH 7.3, this is largely true of the optical activity at longer wavelengths, since the ellipticity of the folded, unliganded state is intrinsically weak, and this state can be calculated from the relationships established in the following to be typically present in low concentrations relative to the liganded state.

Figure 2 shows the change in 380 nm optical activity as a function of time after dilution of the reduced protein into a deaerated solution containing 2 mM each oxidized and reduced glutathione at pH 7.3 (26 °C) and the indicated concentrations of the ligand peptide Phe-TyrNH_2. The results indicate slow formation of the native liganded state with a half-time of ∼5 h under these conditions and an efficiency of folding that depends on peptide concentration. Since the binding of peptide by the unliganded, folded state is instantaneous on this time scale (Pearlmutter & Dalton, 1980), the rate-limiting step is folding. Shown in Figure 3

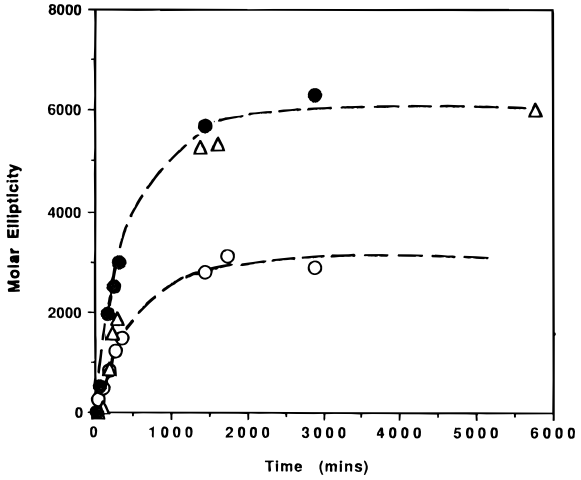

FIGURE 2: Change in molar ellipticity at 380 nm as a function of time after the dilution of reduced, nitrated BNP-II into redox buffer containing 2 mM GSH, 2 mM GSSG, and Phe-TyrNH$_2$ at pH 7.3. Peptide concentrations are as follows: ●, 10 mM; △, 2 mM; ○, 0.5 mM.

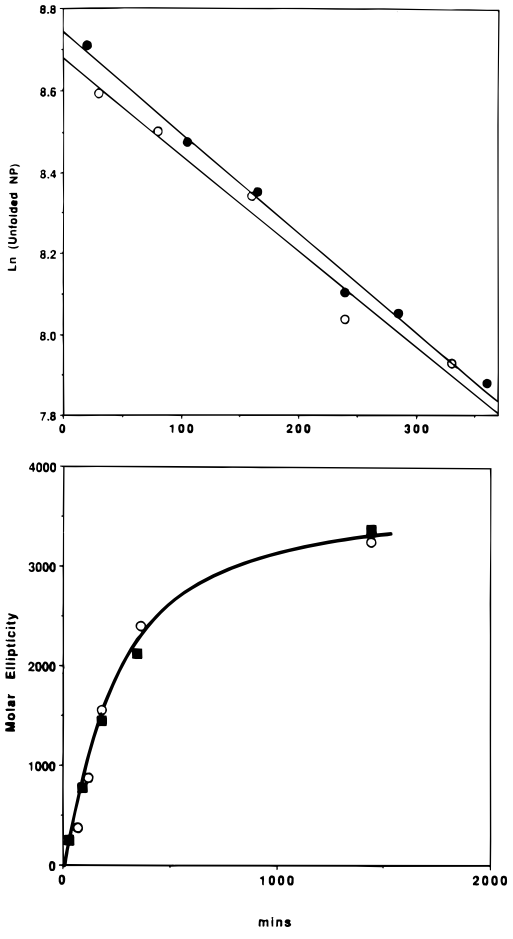

FIGURE 3: Lack of effect of peptide structure and concentration on folding kinetics. Conditions: pH 7.3 in redox buffer containing 2 mM GSH, 2 mM GSSG, and peptide as indicated. (Top) First-order refolding plots in the presence of 10 mM Phe-TyrNH$_2$ (●) and 2 mM Phe-TyrNH$_2$ (○). Data were calculated at 380 nm. Calculated first-order rate constants are 2.4×10^{-3}/min at 10 mM and 2.5×10^{-3}/min at 2 mM. (Bottom) Time course of the change in molar ellipticity at 410 nm during refolding in the presence of 0.2 mM Phe-TyrNH$_2$ (○) and 40 mM Gly-TyrNH$_2$ (■).

are first-order plots of refolding in the presence of 2 and 10 mM peptide, where folding essentially goes to completion (Table 1); the same lot of reduced protein was used for both studies. The data follow first-order kinetics with an apparent first-order rate constant of 2.6×10^{-3}/min. Increases in the glutathione concentration to 5 mM each of the oxidized and reduced states had no effect on the completeness of folding and increased the apparent first-order rate constant by only 15% (data not shown), indicating that the total glutathione concentration is not the principal limiting factor in the rate of folding under these conditions. This result is also relevant since it can be calculated that, during refolding in the 2 mM redox system, there is a change in the GSH/GSSG ratio from 1 to ~2.6 over the course of the study due to the reduction of GSSG by protein thiols; the final ratio is ~1.5 at the higher concentration of redox buffer.

Table 1: Thermodynamics and Kinetics of Neurophysin Folding in the Presence of Ligand Di- and Tripeptides[a]

peptide	concentration (mM)	$K°$ (M^{-1})	$\bar{\nu}$	K' (M^{-1})[b]	$K°/K'$	initial rate[c]
Phe-TyrNH$_2$	13	18 000	1.0			13
Phe-TyrNH$_2$	10	18 000	1.0			15 ± 3
Phe-TyrNH$_2$	2	18 000	0.9			11 ± 2
Phe-TyrNH$_2$	0.5	18 000	0.50	2150	8.4	7
Phe-TyrNH$_2$	0.2	18 000	0.38	3670	4.9	11
Phe-PheNH$_2$	10	18 000	0.9			10
Met-Phe-TrpNH$_2$	2	14 000	0.82	2280	6.1	16
Gly-TyrNH$_2$	40	180	0.41	17	10.6	10 ± 1
Gly-TyrNH$_2$	20	180	0.36	28	6.4	7

[a] Conditions: pH 7.3, 2 mM each GSH and GSSG, 26 °C. The equilibrium constant K' is calculated from the fraction of the total protein in the bound form ($\bar{\nu}$) when the folding reaction has reached equilibrium (eq 2). Data were calculated at 380 nm. Initial rates are given in units of $\Delta[\theta]$/minute. Data were calculated at 410 nm. Peptide concentrations are total concentrations and are uncorrected for the amount bound. [b] Values of $\bar{\nu} \geq 0.9$ were not used for calculations of K' because of the diproportionate sensitivity of K' to small errors in $\bar{\nu}$ in this region. [c] Rates are given as the change in molar ellipticity per minute and are calculated from 410 nm data during the linear phase of the folding reaction. The use of 410 nm data maximized sensitivity (see Figure 1), but the same trends are seen at 380 nm. Results represent single determinations except where standard errors are provided.

Effect of pH on Folding. The rate of folding was studied as a function of pH in the region 6.2−8.6 (Figure 4). In the pH region 6.7−8.3, treatment of the resultant pseudo-first-order rate constants as a function of hydroxyl ion concentration, by using a modified Scatchard analysis, indicated the dependence of folding rate on a single group with an apparent pK_a of 8.0. This is shown more directly in Figure 4, where the effect of pH on the observed rate constants is compared with that expected for the titration of a single group with a pK_a of 8.0. Below pH 6.7, the rate of folding diminishes with pH by more than expected for dependence on a single ionizable group, indicating the intervention of other factors. At pH 6.2, the rate of folding is too low to measure accurately, with apparent half-times greater than 24 h.

The apparent pK_a of 8.0 contrasts with a pK_a of ~8.7 for the glutathione SH at this temperature (Gilbert, 1990). This difference and the relative insensitivity of folding rates to total glutathione concentrations argue against the glutathione SH as the group responsible for the pH dependence. The sole group in nitrated BNP-II that titrates with a pK_a near 8.0 at this temperature is the α-amino group (Breslow et al., 1971; Lord & Breslow, 1979). Phe-TyrNH$_2$ has no group titrating with a pK_a of 8; the pK_a of its amino group is 7.2 (vide supra). The results suggest that the pH dependence is

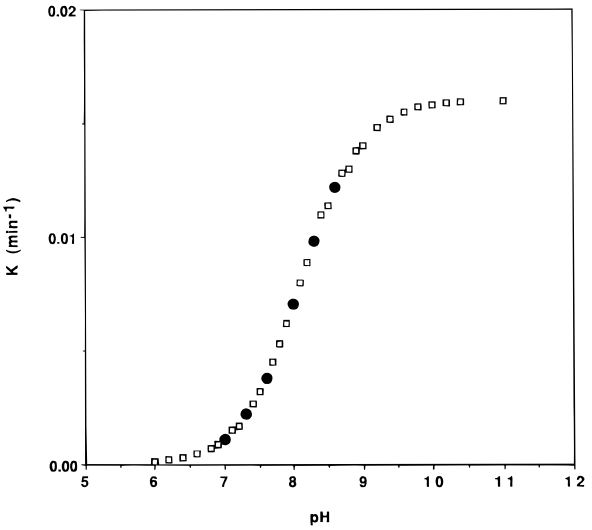

FIGURE 4: Effect of pH on the first-order rate constant for folding in the presence of 10 mM Phe-TyrNH$_2$: ●, observed first-order rate constants; □, calculated curve for the dependence of rate on the deprotonation of a single thiol, with pK_a = 8.0 and a rate constant of 1.6×10^{-2}/min in the ionized state.

determined by the protein α-amino group or by a slightly acidic thiol of the reduced protein. Preliminary folding studies of des 1-6 bovine NP-II indicate only small increases in rate relative to bovine NP-II (Zheng, Deeb, and Breslow, unpublished results), suggesting that the principal source of the pH dependence is a protein thiol.

We also carried out preliminary studies of the effects of temperature on folding. No significant temperature effects were observed other than those expected for temperature effects on thiol group ionization (e.g., Sillen & Martell, 1964) and 2-fold rate increases for each 10 °C temperature increase.

Effects of Peptide Structure and Concentration on Folding Rates and Equilibria. Figure 3 demonstrates that, under conditions where folding essentially goes to completion, a 5-fold increase in the concentration of Phe-TyrNH$_2$ had no significant effect on folding kinetics. To extend the comparison of folding kinetics to conditions in which folding is incomplete, "initial" rates of folding were calculated from the initial rate of ellipticity change (Table 1). No systematic effect of concentration on initial rates is seen between 0.2 and 13 mM Phe-TyrNH$_2$.[2] Initial rates of folding were also essentially the same in the presence of Phe-TyrNH$_2$, Phe-PheNH$_2$, Met-Phe-TrpNH$_2$, and Gly-TyrNH$_2$ (Table 1) and with oxytocin (Table 2), indicating an insensitivity of folding kinetics to the structural differences among these peptides. This is also seen in Figure 3, where the time course of folding is compared in the presence of 40 mM Gly-TyrNH$_2$ and 0.2 mM Phe-TyrNH$_2$.[2]

The peptides studied differ among themselves in some of the details of their interaction with NP. Phe-TyrNH$_2$ and Phe-PheNH$_2$ bind with comparable affinities, but the Phe in position 2 of Phe-PheNH$_2$ lacks the ability to form the phenolic hydrogen bond that characterizes interactions of Phe-TyrNH$_2$ (Chen et al., 1991). The lack of a side chain in position 1 of Gly-TyrNH$_2$ reduces its binding affinity relative to the other dipeptides by a factor of ~100 at pH 7.3 (Table 1). Met-Phe-TrpNH$_2$ binds with approximately two-thirds the affinity of Phe-TyrNH$_2$ and Phe-PheNH$_2$ (Table 1), reflecting a more weakly binding side chain in position 1 and a positive contribution to binding of the β-CH$_2$ of residue 3 (Breslow & Burman, 1990). Thus, no specific structural feature in the ligand is identified by these studies to affect the rate of folding. Oxytocin, which also gives the same kinetics, is discussed more specifically below.

Table 2: Thermodynamics and Kinetics of Folding in the Presence of Oxytocin[a]

redox system	peptide (mM)	$\bar{\nu}$	K' (M^{-1})	$K°/K'$ [b]	initial rate
2 mM GSH, 2 mM GSSG	1.2	0.7	1900	21	12
5 mM GSH, 5 mM GSSG	1.0	0.7	2300	17	15

[a] Conditions: pH 7.3, redox system as indicated, 26 °C. The value of $K°$ used for oxytocin at pH 7.3 is 4×10^4 M^{-1} (Breslow & Walter, 1972; Nicolas et al., 1980). Other calculations are the same as in Table 1. [b] The value of $K°$ used is probably a minimum estimate since it represents data obtained using nonnitrated BNP-II at this pH. Ionization of the nitrotyrosine (pK_a = 7.3 in Tris−acetate) increases binding by a factor of ~4 (Breslow et al., 1973). Moreover, a value of $K°$ for the nitrated protein of $\sim 1 \times 10^5$ M^{-1} at pH 7.3 can be calculated from data for the nitrated protein at other pH values (Breslow et al., 1973). Accordingly, values of $K°/K'$ are conservative.

By contrast with the insensitivity of folding kinetics to peptide concentration and structure, these variables had a profound effect on the apparent efficiency of folding, as measured by $\bar{\nu}$, the final concentration of the liganded state relative to the total protein concentration (Table 1). For Phe-TyrNH$_2$, $\bar{\nu}$ decreased from ~1 at 10 mM peptide to ~0.4 at 0.2 mM peptide. For Gly-TyrNH$_2$, $\bar{\nu}$ was only 0.4 at 40 mM concentration. Differences among the peptides are reasonable when viewed in the context of their different binding affinities (vide supra), but absolute values of $\bar{\nu}$ attained for each peptide upon folding were less than expected on the basis of their binding constants in the absence of the glutathione buffer system.

The binding of peptide to native neurophysin in the absence of the glutathione redox buffer system is defined by the equilibrium constant, $K°$, where

$$K° = \frac{[\text{NP}-\text{peptide complex}]}{[\text{NP}][\text{peptide}]} = \frac{\bar{\nu}}{(1-\bar{\nu})[\text{peptide}]} \quad (1)$$

and NP represents the folded protein. This can be compared with the degree of folding in the redox system by formulating an apparent binding constant in this system, K', as follows:

$$K' = \frac{[\text{NP}-\text{peptide complex}]}{[\text{NP}]_t[\text{peptide}]} \quad (2)$$

where $[\text{NP}]_t$ represents the total concentration of unliganded neurophysin and includes both the folded and unfolded states. Table 1 compares values of $K°$ and K' for several peptides in different refolding experiments. Although there is some spread to the data (Materials and Methods), values of K' are typically one-seventh that of $K°$ for each peptide. The

[2] Rates of ellipticity change, which represent the data in Table 1, are responsive only to the amount of liganded state present. Under conditions in which the folded state is partially dissociated (as per eq 1), measured rates of folding will be less than true rates. Accordingly, for Phe-TyrNH$_2$, the measured initial rates should be ~10% and 20% lower at 0.5 and 0.2 mM peptide, respectively, than at 10 mM peptide. Similarly, rates measured at 40 and 20 mM Gly-TyrNH$_2$ should be ~10% and 20% lower than true rates. Trends are seen, but the effects are largely within the noise of the data.

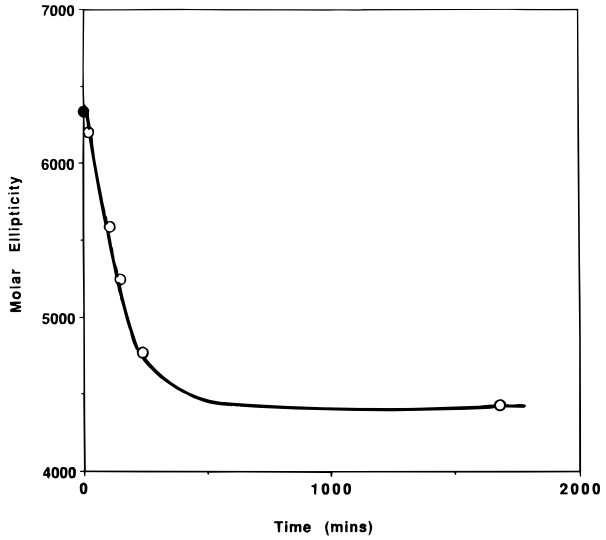

FIGURE 5: Effects of 2 mM GSH/2 mM GSSG on native, nitrated BNP-II in the presence of peptide at pH 7.3. The CD spectrum was obtained of a solution containing 0.1 mM protein and 0.5 mM Phe-TyrNH$_2$ at pH 7.3 in 0.2 M Tris−acetate buffer. NP is 90% in the liganded state under these conditions. The solution was adjusted to a final concentration of 2 mM each GSH and GSSG (by the addition of a concentrated stock solution of redox buffer), and CD spectra were taken as a function of time. Results are reported at 410 nm. The initial point (●) represents the observed molar ellipticity before the addition of the redox components, corrected for the small dilution associated with the addition of these components. Other points (○) represent readings after the addition of redox buffer. The final equilibrium position represents $\bar{\nu}$ = 0.62 and gives an apparent binding constant of 3400 M^{-1}.

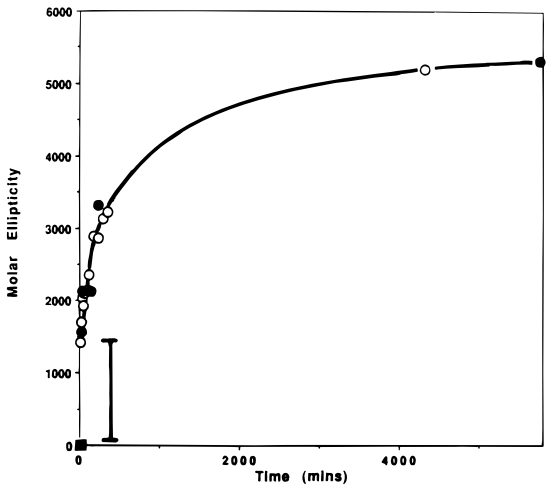

FIGURE 6: Molar ellipticity at 380 nm as a function of time after the addition of Phe-TyrNH$_2$ (final concentration = 10 mM) to either reduced (●) or native nitrated BNP-II (○) that had been equilibrated for 24 h with 2 mM each GSH and GSSG at pH 7.3. The initial point (■) represents readings before peptide addition. The magnitude of the initial increase upon peptide addition (represented by the vertical bar) represents folded, unliganded NP present before peptide was added.

weaker binding constants under refolding conditions do not reflect affinity differences between the native and refolded states, since purification of the refolded protein by gel filtration and affinity chromatography (Rabbani et al., 1982) indicates that it is identical to the native protein in binding affinity (data not shown).

Effects of the Glutathione Redox System; Evidence of Formation of a Common State from Either Native or Reduced Neurophysin in the Presence of Glutathione. The lower apparent binding affinity under refolding conditions reflects unfolding effects of the redox components on native NP. When the mixed redox components are added to a solution of nitrated native BNP-II in the presence of Phe-TyrNH$_2$ at pH 7.3, a time-dependent decrease in binding occurs until a final equilibrium position is reached (Figure 5). This position represents an apparent binding constant of 3400 M^{-1}, a value within the range of values of K' calculated upon refolding from the reduced state in the presence of Phe-TyrNH$_2$ (Table 1). The reduction in binding reflects an effect on protein conformation, as can be demonstrated by the fact that addition of the redox buffer to native, nitrated NP in the absence of ligand leads to the loss of nitrotyrosine ellipticity and to the nonadditivity of protein and GSSG disulfide ellipticities (data not shown). The extent to which binding is lost can be demonstrated by equilibrating native, nitrated NP with the redox system in the absence of peptide for 24 h and measuring the *immediate* ellipticity change that accompanies the addition of saturating concentrations of Phe-TyrNH$_2$ (Figure 6). The magnitude of the initial jump (Figure 6, legend) indicates ∼25% binding relative to the calculated ellipticity at saturation. Significantly, the initial jump is followed by a slower time-dependent increase indicative of peptide-induced refolding (Figure 6). Similar results were obtained at both 2 mM GSH/2 mM GSSG (Figure 6) and 5 mM each component (data not shown). The data argue for partial reduction and/or disulfide interchange of unliganded NP mediated by the glutathione redox system.

The state to which unliganded, native NP is changed in the glutathione redox system is similar or identical to the state to which the reduced protein is converted by this system in the absence of peptide. As demonstrated previously in an air−mercaptoethanol system (Huang & Breslow, 1992), reduced NP can refold to a limited extent in the absence of peptide. Similar results are obtained with the glutathione system (Figure 6). In this study, the reduced protein was equilibrated in the glutathione system without peptide for 24 h at pH 7.3, after which the solution was made 10 mM in Phe-TyrNH$_2$. On average, the magnitude of the immediate ellipticity change indicates 20−25% refolding in the absence of peptide. As before when the starting material was the native protein, this immediate ellipticity increase is followed by slow time-dependent ellipticity increases indicative of peptide-induced refolding. The results obtained, starting with either the native or the reduced protein, are remarkably similar and suggest that both states of the protein are equilibrated in the glutathione system to a new common state. By averaging over eight studies, the results indicate that this state consists of 20−25% native protein and 75−80% binding-incompetent protein.

Refolding Rates in the Absence of Peptide. Studies analogous to those in Figure 6 were used to assess the rate of folding of reduced NP in the absence of peptide. Two samples were allowed to refold at pH 8[3] in the absence of peptide for 3 and 24 h, respectively, followed by peptide addition. The results indicated that the immediate CD

[3] These conditions were chosen in the hope that the increased molar ellipticity of the unliganded folded protein at pH 8 relative to that at pH 7.3 (Figure 1) would permit folding to be monitored in the absence of peptide addition. CD changes prior to peptide addition appeared to occur rapidly, but were too small to be monitored accurately.

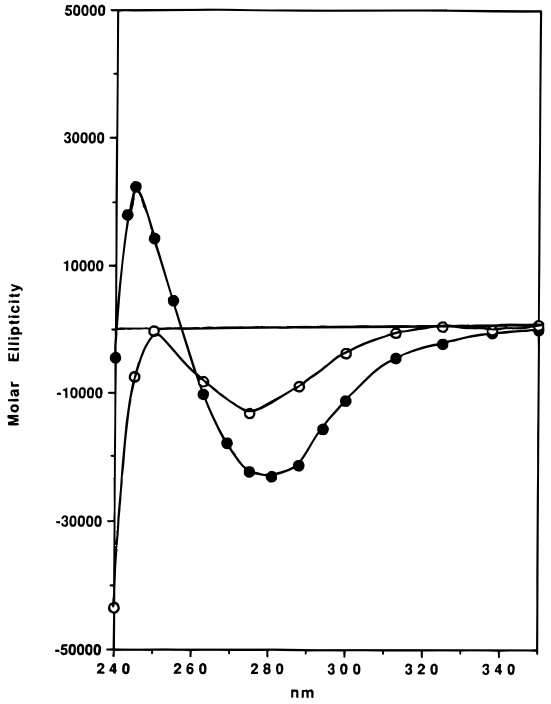

FIGURE 7: CD spectrum of reduced, nitrated BNP-II equilibrated for 24 h at pH 7.3 in a redox buffer containing 2 mM each reduced and oxidized cystamine (○). The spectrum of native, nitrated BNP-II in the same wavelength region is shown for comparison (●).

increase upon the addition of peptide was the same at 3 and 24 h. Since the half-time for refolding in the presence of peptide at pH 8 is ~1.5 h (see Figure 4), the results suggest that refolding kinetics are similar in the presence and absence of peptide—in accord with the lack of an effect of peptide concentration on kinetics.

Evidence That the Formation of Scrambled Disulfide Pairs Occurs More Rapidly Than Folding. We followed the formation of cystine pairs upon refolding from the reduced state in the absence of peptide by monitoring the near-ultraviolet CD spectrum in a redox buffer in which reduced and oxidized cystamine (which also permits refolding) were substituted for glutathione. Since cystamine is optically inactive and the reduced protein is optically inactive in the near UV, near-UV optical activity generated during refolding represents either internal NP disulfide pairs or possibly mixed disulfides with cystamine. Figure 7 compares the CD spectrum after 24 h of refolding with that of native, nitrated protein. The single negative 275 nm band seen in the initially reduced protein contrasts with the two banded disulfide spectrum characteristic of the native protein and is similar to that seen (Menendez-Botet & Breslow, 1975) in disulfide-scrambled NP. Monitoring of the CD spectrum with time during refolding indicates that formation of the 275 nm band is ~80% complete in 20 min at pH 7.3.

We also assessed the states of reduction and mixed disulfide formation with glutathione at the half-time for refolding (5 h) in the presence of 10 mM Phe-TyrNH_2 at pH 7.3. Free SH groups were trapped by iodoacetate, and the protein was subjected to amino acid analysis and sequencing (Materials and Methods). The results indicated 0.4−0.5 mol/mol of (carboxymethyl)cysteine and no measurable additional Gly, Glu, or Cys residues from glutathione. Since half of the protein is folded at this stage, the results suggest an average of ~1 free SH per unfolded chain.

We attempted to locate the carboxymethylated Cys residues. Gas phase N-terminal sequencing of the intact protein and trypsin-digested samples indicated traces (0.05 ± 0.02 residues per mol of total protein) of carboxymethyl-Cys at residues 10 and 13, with still smaller quantities scattered among several other half-Cys residues in amounts that were not clearly above background. The results suggest that almost all of the Cys residues of the unfolded protein are internally paired, with trace amounts of thiol located among a subset of residues that includes Cys_{10} and Cys_{13}. Collectively, these and CD results suggest that neurophysin is converted to a disulfide-scrambled state under these conditions more rapidly than it folds.

General Model for the Refolding Pathway. Figure 8 represents a general folding model in the glutathione redox system that is consistent with the preceding results. Specifically, upon refolding from the reduced state, the data suggest relatively rapid conversion to a disulfide-scrambled state, followed by slow formation of unliganded, folded protein in a reaction that does not involve peptide. The unliganded, folded protein and the scrambled protein are in a relatively slowly established equilibrium characterized by the constant K_c, where

$$K_c = [\text{scrambled}]/[\text{folded, unliganded}] \qquad (3)$$

In the presence of peptide, the folded protein is stabilized by rapid formation of the peptide complex in an equilibrium governed by the intrinsic peptide-binding constant, K^o (eq 1). With this model, the equilibrium constant (K') governing the fraction of peptide−protein complex formed during folding (eq 3) is equivalent to

$$K' = \frac{[\text{NP}-\text{peptide complex}]}{[\text{scrambled} + \text{folded, unliganded NP}][\text{Peptide}]} \qquad (4)$$

and is related to K^o and K_c by the following relationship:

$$K' = K^o/1 + K_c \qquad (5)$$

These relationships allow the calculation of K_c from the data in Table 1, with the results indicating an average value of 6.3 with an uncertainty of ~30%. The results indicate, under conditions approaching thermodynamic reversibility, that the disulfide-scrambled state is thermodynamically more stable than the folded, unliganded state and provide an estimate of the difference in stability of ~1 kcal/mol. This estimate can be compared with the value of ~0.8 kcal/mol calculated from the fractional content of folded protein present at equilibrium in the redox buffer in the absence of peptide (20−25%).

A relevant feature of this thermodynamically controlled model is that it predicts that the total concentration of folded protein at equilibrium is greater than the concentration of the complex—a natural consequence of the dissociation of the complex (eq 1). This disparity will increase at low values of $\bar{\nu}$. In accord with this, the addition of saturating concentrations of peptide to protein refolded at low peptide concentration can be shown to lead to small immediate increases in ellipticity, representing binding by the folded, unliganded protein present. This is one of the features that distinguish the model from that developed for protease refolding (Discussion).

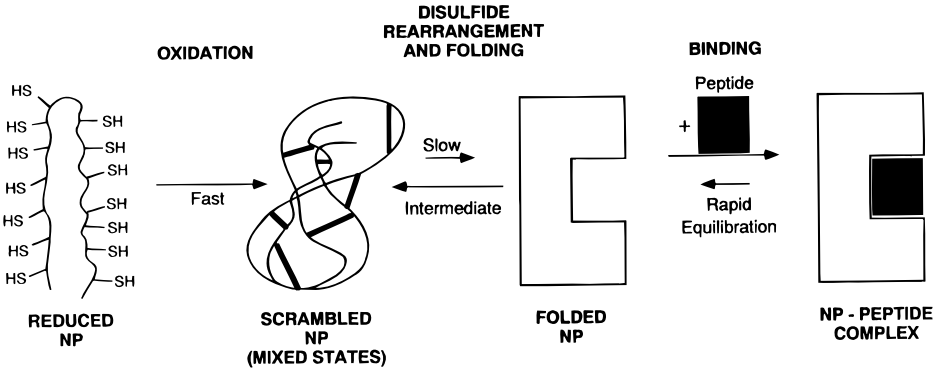

FIGURE 8: General model for the folding of BNP-II from the reduced state in the presence of 2 mM GSH, 2 mM GSSG, and peptide at pH 7.3. The reduced state is assumed to convert relatively quickly (compared to the total time course of folding) to an oxidized but disulfide-scrambled state, probably containing a mixture of species. This state is converted slowly and reversibly by disulfide rearrangement to the folded, unliganded state, which is instantaneously trapped by peptide. The equilibrium between the folded, unliganded state and the scrambled state is defined by the equilibrium constant K_c. The equilibrium between the folded, unliganded and liganded states is defined by the intrinsic binding constant of the folded state, K^o.

Folding in the Presence of Oxytocin. Analysis of the effects of oxytocin on folding were complicated by ring-opening effects of the glutathione redox system on the oxytocin disulfide, as reported elsewhere (Rabenstein & Yeo, 1994) and also observed here. Ring-opened monomeric oxytocin has a binding constant ~1/200 that of the intact hormone (Breslow & Burman, 1990). Binding constants of disulfide-linked dimeric and oligomeric oxytocins, which are also potential products of reaction with GSH, are similarly likely to be low. These effects are almost certain contributors to the relatively inefficient folding of NP in the presence of oxytocin in the redox system (Table 2), manifested by values of K^o/K' that are significantly higher than those of other peptides if the alterations in oxytocin structure are not taken into account. While the reduced value might, in principle, be due to the depletion of GSH via its interactions with the oxytocin disulfide, the same results were obtained when the concentration of redox buffer was increased to 5 mM each component (Table 2). In any event, under both sets of conditions the results indicate that the initial rate of folding in the presence of oxytocin (Table 2) is the same as that obtained with other peptides (Table 1).

How Scrambled Is the Scrambled State? Relatively little information on the nature of improperly folded (scrambled) NP is available, except that it is less compact than the native state, as judged by gel electrophoretic behavior, and exhibits an altered CD spectrum in the far UV (Menendez-Botet & Breslow, 1975 and unpublished results). Earlier studies of thiol distribution at the penultimate stage of NP folding suggested the possibility that the carboxyl domain might be self-folding in the absence of peptide and that disulfide scrambling in improperly folded NP might be confined to the amino domain; in the latter, evidence of interchange among at least three of its four bridges was obtained (Huang & Breslow, 1992). Preliminary attempts to directly analyze disulfide pairs in the carboxyl domain of improperly folded NP yielded ambiguous results. Accordingly, we examined the folding properties of an isolated peptide representing the carboxyl domain.

The neurophysin carboxyl domain is minimally composed of residues 61−85, as this segment contains its three disulfides and all other residues of this domain known to be important to neurophysin function (Chen et al., 1991). We prepared an extended carboxyl-domain peptide composed of residues 49−90 of BNP-II and containing a carboxymethyl group on Cys_{54}, the sole half-Cys residue from the amino domain. The peptide was isolated under conditions considered not to perturb carboxyl-domain disulfides (Materials and Methods). The refolding properties of this fragment were followed in part by using CD spectroscopy. Figure 9 shows the spectrum of the fragment, as originally isolated. As with native NP (e.g., Menendez-Botet & Breslow, 1975), the negative long wavelength band is assigned to disulfides. The 275 nm location of this band compares with a 280 nm minimum in the native protein (Figure 7) and the ~260 nm location of the long wavelength disulfide band of simple open chain disulfides (e.g., Beychok, 1966). This and its relative intensity (>2000 deg·cm^2/dmol per disulfide) are consistent with a degree of neurophysin-like character. The positive 250 nm band is also seen in NP (Figure 7) and is assigned to disulfides, but the band is much weaker in the isolated carboxyl-domain peptide. Since the three disulfides of the NP carboxyl domain are structurally homologous to three of the four disulfides of the amino domain, both spectral

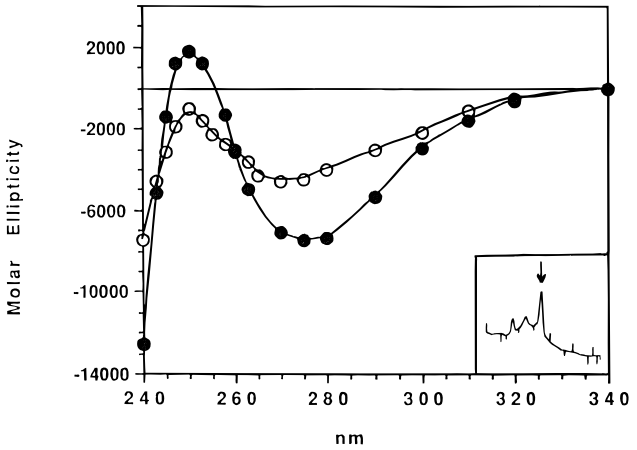

FIGURE 9: CD spectra of the peptide representing the neurophysin carboxyl domain as originally isolated (●) and after complete reduction by DTT and refoleding (○). Refolding was performed by the mercaptoethanol procedure of Huang and Breslow (1992). The inset shows peptide behavior during HPLC on a C18 column, following reduction and subsequent reoxidation of 0.06 mM peptide in a redox buffer containing 0.4 mM GSH and 0.8 mM GSSG. The arrow points to the location of the fragment prior to reduction. The sharp peak seen at this location in the reoxidized peptide is the same as that seen in the fragment prior to reduction. The other components shown are unique to the reoxidized state.

similarities and nonidentities between the peptide and intact protein are within expectation.

The most significant feature of the fragment CD spectrum for present purposes is that it is altered following complete reduction and reoxidation (Figure 9). The changes seen are similar to those associated with scrambling of the disulfides of the native protein (Menendez-Botet & Breslow, 1975), but are less dramatic with the fragment. Refolding following complete reduction was also monitored by HPLC. The inset in Figure 9 shows the behavior of the fragment on a C18 column following reoxidation from the reduced state in a glutathione buffer. The arrow points to the position of the intact fragment, which migrates as a sharp peak prior to reduction and reoxidation (Materials and Methods). The results indicate that approximately half of the fragment refolds to its original state, while the remainder folds to two different states. Similar results were obtained under a broad range of reoxidation conditions.

Preliminary folding studies were also carried out on a synthetic peptide consisting of residues 59−92 of bovine NP-I; the two neurophysins are highly homologous in both structure and function and contain the same disulfides (e.g., Breslow & Burman, 1990). At equilibrium, under a broad range of oxidation conditions, two components in 1:1 ratio were always seen (Deeb, Breslow, and Merrifield, unpublished observations).

The results suggest that the folding efficiency of an isolated carboxyl domain is approximately 50%. This is a *maximum* estimate since it has not been strictly established that the disulfide pairing of the fragment isolated from BNP-II is the same as that in the native protein (Materials and Methods) or that either of the two products of oxidation of the C-terminal peptide from BNP-I represents the native pairing. Efficient folding of the carboxyl domain therefore appears to be dependent on the amino domain—presumably in the correctly folded state—the results suggesting that the scrambled state to which NP is first converted during refolding probably represents significant scrambling of both domains.

DISCUSSION

Evidence suggesting the thermodynamic instability of disulfide pairing in unliganded, native neurophysin was first obtained in early studies under conditions that did not meet the test of thermodynamic reversibility (Menendez-Botet & Breslow, 1975; Chaiken et al., 1975). In more recent work, evidence of a disulfide-scrambled state common to both the reduction of a single disulfide of unliganded neurophysin and oxidation from the completely reduced state was obtained, consistent with the concept of a thermodynamically controlled intermediate (Huang & Breslow, 1992). The present work demonstrates, under conditions that more closely approach true thermodynamic reversibility, the presence of a scrambled state of unliganded NP that is readily accessed from both the completely reduced state and the unliganded, folded state[4] and that is approximately 1 kcal/mol (±0.3 kcal/mol) more stable than the unliganded, folded protein. Folding of neurophysin within the precursor therefore is thermodynamically dependent on the stabilization of the folded state by intramolecular interactions between hormone and NP segments.

The difference in stability between folded and scrambled forms estimated here requires qualification. In an air−mercaptoethanol system, the degree of refolding in the absence of peptide was ∼30%, both in the absence and in the presence of protein disulfide isomerase (Huang & Breslow, 1992). This result allows a difference of only 0.5 kcal/mol between folded and scrambled states. The two refolding systems differ in that both folded and scrambled species are completely oxidized in the air−mercaptoethanol system, while the scrambled state here shows evidence of partial reduction (∼1 SH per mol) under the conditions in which its stability is evaluated. Our general conclusions are not affected by this qualification.

We found no effect of occupancy of the hormone-binding site on the kinetics of folding. The reduced protein folded slowly in the absence of peptide to an extent dictated by the equilibrium between the unliganded, folded state and the scrambled state. Peptides that bind to the hormone-binding site increased folding efficiency, but did not obviously impact on the rate of folding, indicating that they functioned solely to provide a thermodynamic sink for the folded form after folding occurred. This finding extended to oxytocin under conditions where its reduced form was likely to be present, also suggesting that its thiols had no advantage over those of GSH in promoting folding. Nonetheless, the possibility that the hormone segment might modulate the kinetics of folding within the precursor is not excluded. The covalent attachment of hormone to NP within the precursor has the potential (depending on protein conformation) to increase *effective* hormone concentrations by orders of magnitude over true concentrations (e.g., Jencks, 1975), allowing weak interactions of the hormone with partially folded NP species that might serve to lower energy barriers along the folding pathway. For some subtilisin systems, it has already been found that the covalent attachment of propeptide is essential to its role in folding (Strausberg et al., 1993). An increase in effective hormone concentration within the precursor would also help to overcome thermodynamic problems found here that are introduced by the lability of the oxytocin disulfide. Note that the concentrations of GSH and GSSG used here (2 mM each) are similar to those in the endoplasmic reticulum (Ruoppolo & Freedman, 1995), so that the problem potentially is present in vivo as well. Determination of the kinetics of precursor folding awaits the availability of precursor in sufficient quantities.

A residual question is why the rate of neurophysin folding is so slow. The lack of a strong temperature effect argues against cis−trans proline isomerization as a factor in this

[4] The final GSH/GSSG ratios during folding and unfolding reactions are nonidentical, but there is no evidence that they impact significantly on the products; i.e., the amount of folded protein at equilibrium in the two types of reactions is the same within experimental error in both the presence and absence of peptide (Results). The difference in GSH/GSSG ratios during folding and unfolding is also relatively small under some of the conditions used. In folding studies (Results), the final ratio ranged from 1.5 (initial concentration of 5 mM each GSH and GSSG) to 2.6 (initial concentration of 2 mM each component); the difference in ratio did not materially affect the results. The equilibrium ratio during unfolding studies is 1 and is calculated as follows. Like the folding reactions, the unfolding reactions were initiated at equimolar GSH and GSSG concentrations (2 and 5 mM each). However, in this case, the initial ratio of 1 is unchanged at equilibrium, because any *net* oxidation of GSH by the oxidized protein (0.1 mM) is negligible relative to the total redox buffer concentration; i.e., the *maximum* GSH oxidized will be <0.1 mM, since less than 1 SH per chain is present upon folding at the higher equilibrium GSH/GSSG ratios.

case, and X-ray data to date have not revealed the presence of any *cis*-Pro bonds in neurophysin (J. Rose, personal communication). Moreover, refolding of disulfide-paired NP following guanidine denaturation is rapid (unpublished results). Previous work implicated the unstable 10−54 disulfide bridge of the amino domain as the likely source of the inefficiency of folding in the absence of peptide (Huang & Breslow, 1992). One explanation of the slow rate of folding therefore would lie in a kinetic barrier to the formation of this bridge, with this barrier allowing prior equilibration among different scrambled species, only a subset of which are on the path to the folded form. In this context, it is relevant that the trace thiols detected in scrambled NP were located in large part on Cys_{10}, allowing the possibility that ionization of the Cys_{10} thiol and its attack on incorrect disulfide pairs containing Cys_{54} represent the rate-determining step in folding. While such a model remains generally attractive, the current work also indicates the presence of folding problems within the carboxyl domain. Since the three disulfides of this domain are homologous to the remaining three disulfides of the amino domain, the kinetic problems might arise from multiple barriers.

The lack of an intermolecular kinetic effect of peptides representing the pro region of the NP precursor contrasts with the intermolecular effects of pro region peptides on the folding of subtilisin (Shinde et al., 1993; Strausberg et al., 1993) and α-lytic protease (Baker et al., 1992). The protease pro region peptides are considered to function by reducing a kinetic barrier to folding. However, there are common elements to the effects of pro region peptides in the different proteins. With protease folding, the guanidine-unfolded protein appears trapped in an intermediate state until peptide is added, after which it rapidly converts to the folded peptide complex. Binding of peptide is so tight that it is removed by destroying it enzymatically, a process that leaves the protein in the functional folded form. In the case of neurophysin, the reduced, unfolded protein remains largely unfolded until peptide is added, after which it can fold with 100% efficiency. Removal of the peptide, which in this case is reversibly achieved, also leaves the protein in the functional folded form. How does one differentiate between thermodynamics and kinetics in these two cases? In the case of NP, the processes of folding, unfolding, and peptide dissociation are achieved under reversible conditions and folding kinetics are slow, allowing clear separation of thermodynamic and kinetic effects. The results establish that the functional folded form is a trapped metastable state and that the role of peptide is to provide stabilization during folding. Therefore, in the case of the proteases, an argument *perhaps* might also be made that one role of peptide is to stabilize the folded conformation during folding and that it is the folded state (as opposed to the folding intermediate) that is metastable, but kinetically frozen. This is not the prevailing view. Interestingly, recent studies of the effects of mutant propeptides on subtilisin folding indicated a linear relationship between the binding affinities of the peptides to mature subtilisin and the efficiency with which they induced refolding when compared at constant concentration (Li et al., 1995). These results parallel the effects of different peptides on neurophysin folding. Thus, the role of thermodynamics in peptide-mediated folding might not always be self-evident; potentially hidden thermodynamic contributions may be revealed only by critical analysis.

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