Modulation of Allosteric Interactions in Neurophysin Induced by Succinylation of Serine-56 or Cleavage of Residues 1–8†

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Received March 25, 1993; Revised Manuscript Received June 25, 1993®

ABSTRACT: Neurophysin is an allosteric protein in which peptide binding and self-association are positively linked. Reaction of neurophysin with succinic anhydride led to a large decrease in peptide affinity assignable to succinylation of a serine or threonine hydroxyl group. To identify the residue involved, acetimidated protein was reacted with [^{14}C]succinic anhydride and the active and inactive components were separated by affinity chromatography. Performic acid oxidation and tryptic and Asp-N mapping of the two components, followed by automated Edman degradation, allowed identification of the critical residue as Ser-56. This residue is not a direct participant in peptide binding and is distant from the subunit interface of the dimer, but it is immediately adjacent to the site of one of the known mutations associated with familial diabetes insipidus. Examination in solution of the peptide affinity of neurophysin succinylated at Ser-56 indicated a binding affinity $\sim^1/_{20}$th that of the native protein or of protein succinylated at other residues, and a loss of the normal dependence of binding affinity on protein concentration. Under the same buffer conditions, loss of the concentration dependence of binding, in addition to the previously demonstrated loss of binding affinity, also accompanied excision of residues 1–8, an effect attributed to the loss of binding site residue Arg-8. However, in contrast to the effects of succinylation on native neurophysin, only minor effects of succinylation on the binding affinity of the des-1–8 protein were observed. The results indicate that the effects of succinylation and of cleavage of the 1–8 sequence on binding affinity are mediated by a common mechanism that additionally alters self-association under appropriate ionic conditions. Examination of the neurophysin crystal structure indicates a distance of <8 Å between Arg-8 and Ser-56, suggesting that the two residues are part of a common structural locus involved in long-range interactions between the binding site and the subunit interface.

The protein neurophysin specifically binds posterior pituitary peptide hormones and structurally related peptides, and additionally exhibits the properties of dimerization in the unliganded state and a ligand-mediated increase in dimerization constant (Nicolas et al., 1980); the effect of ligand is thermodynamically equivalent to preferential binding to the dimer [reviewed in Breslow and Burman (1990)]. Although the three-dimensional structure of the liganded state has recently been solved by X-ray crystallography (Chen et al., 1991), neither the conformation of the unliganded state nor the basis of the ligand-mediated increase in dimerization constant is known. Early studies in this laboratory of the effects of succinylation, aimed at modifying neurophysin self-association, indicated variable effects on peptide affinity that were not attributable to amino group modification (unpublished results). The present studies represent a detailed investigation of the effect of succinylation on neurophysin binding properties and identify the residue responsible for the observed effects as Ser-56. Although Ser-56 is not a component of the peptide-binding site and is distant from the monomer–monomer interface, the results additionally demonstrate that its succinylation is accompanied not only by a large decrease in binding affinity but also by changes in self-association properties that lead to loss of the normal dependence of binding on protein concentration. Evidence is presented that a mechanism common to the effects of deletion of binding site residue Arg-8 is involved.

MATERIALS AND METHODS

Preparation of Protein Derivatives. Bovine neurophysins I and II were purified as described previously (Breslow et al., 1971; Virmani-Sardana & Breslow, 1983). Des-1–8 bovine neurophysin I was also prepared and purified by affinity chromatography as described earlier (Peyton et al., 1986). For many studies of the effects of succinylation, the native proteins were first treated with ethyl acetimidate to completely block lysine amino groups (α-amino groups are incompletely blocked); this modification is without effect on binding and self-association properties [e.g., Ando et al. (1987)]. The advantage of prior acetimidation is that it does not alter net charge at pH 6, increases the solubility of the succinylated protein at low pH [cf. Lord and Breslow (1979)], and simplifies interpretation of electrophoretic mobilities of the succinylated protein; i.e., with the exception of products succinylated on residual α-amino groups, all species more negatively charged than the starting material represent succinylation at hydroxyl groups. Exhaustive acetimidation was accomplished by a modification of the method of Wofsy and Singer (1963). Protein (15 mg) was dissolved in 2 mL of 0.2 M sodium borate and reacted with 0.2 g of ethyl acetimidate at room temperature; the initial pH was adjusted to 7.8. After 90 min, the pH had increased to 8.9. At this point, three successive additions of 0.15–0.2 g of ethyl acetimidate were added at 60-min intervals; at each step the pH was adjusted to ~9. After overnight reaction following the last addition, the acetimidated protein was first purified by gel filtration followed by lyophilization and then by affinity chromatography (Rabbani et al., 1982) to remove inactive side products.

† Supported by Grant GM-17528 from NIH.
® Abstract published in *Advance ACS Abstracts,* September 15, 1993.

0006-2960/93/0432-10743$04.00/0

Succinylation of active native, acetimidated, or mononitrated (see below) neurophysin was accomplished by dissolving ~11 mg of protein in 2 mL of 0.1 M sodium borate, pH 8.0, and gradually adding 20 mg of succinic anhydride (dissolved in 150 μL of *N*,*N*-dimethylformamide) over a 20-min period. The pH was maintained at 8 by addition of 1.5 M NaOH during a total reaction period of 65 min. The succinylated protein was first purified by gel filtration in 0.2 M acetic acid or in 0.1 M ammonium acetate, pH 6.7, and lyophilized. It was then separated into active and inactive (low binding constant) components by affinity chromatography on a peptide-linked affinity support (Rabbani et al., 1982). For succinylation with radioactive succinic anhydride, the same procedure was used, substituting [1,4-^{14}C]succinic anhydride at a specific activity of 66 dpm/nmol. Succinylated inactive protein could be converted to active protein by desuccinylation at pH 10 for 1 h at room temperature with 1 M NH_2OH.

The above succinylation procedure yielded a mixture of active and inactive components, a comparison of which is presented in Results. Complete inactivation of the native protein by succinylation was initially achieved using more severe succinylation conditions (80 mg of succinic anhydride added to 20 mg of neurophysin in 1 mL of 0.5 M $NaHCO_3$, at pH 8.0 and allowed to react for 90 min). These conditions were used initially for succinylation of the des-1–8 protein after nitration (see below for nitration procedure). Since they had only a minor effect on the binding affinity of the des-1–8 protein (Results), the concentration of succinic anhydride used was increased to 112 mg/mL and the reaction time was extended to 2 h, again with no reduction in binding affinity (Results).

Gel Electrophoresis Studies. The extent of succinylation could be followed by polyacrylamide gel electrophoresis using 15% gels at a running pH of 9.5. Since neurophysin is a small protein, species differing by single charges are readily resolvable under these conditions and the degree of charge change is calculable [e.g., see Rabbani et al. (1982)].

Determination of Peptide-Binding Constants of Nitrated Derivatives by Circular Dichroism. Peptide-binding constants in solution were determined by CD[1] as described earlier (Breslow et al., 1973; Breslow & Gargiulo, 1977). For these studies, the single neurophysin tyrosine, Tyr-49, is first mononitrated. Procedures for the nitration of native neurophysin and purification of the active mononitrated product have been described earlier (Sardana et al., 1987) and were applied here in unmodified form to the nitration of the native protein. The purification procedure in part involves affinity chromatography to remove small quantities of a nonbinding oxidized side product of the nitration reaction. Nitration of the des-1–8 protein was accomplished under similar reaction conditions. However, because this protein exhibits little or no binding to an affinity column prior to nitration, the affinity step was typically omitted. This leaves the small amount of the oxidized side product, which should not in principle affect measurement of the weak binding constants characteristic of the des-1–8 protein.[2] Surprisingly, gel-filtration studies of the des-1–8 protein after nitration indicated the absence of the tyrosine–tyrosine cross-linked side product of the reaction seen on nitration of the native protein (Sardana et al., 1987). This is consistent with a different environment of Tyr-49 in native and des-1–8 proteins as found in NMR studies (Breslow et al., 1992).

Performic Acid Oxidation. Performic acid was prepared by mixing 9 mL of 99% formic acid and 1 mL of 30% H_2O_2 and allowing the mixture to stand at room temperature for 2 h, followed by 10 min on ice immediately before use. For oxidation of protein disulfides, 0.25 mL of freshly prepared performic acid was mixed with 1 mg of protein; the mixture was allowed to stand on ice for 3 h and then diluted with 10 mL of cold water and lyophilized. Amino acid analysis indicated that 97–99% of the cystine was converted to cysteic acid. The specific radioactivity of protein succinylated with [^{14}C]succinic anhydride was found to be unaffected by performic acid oxidation, indicating that the succinyl esters formed were stable to the oxidation conditions.

Mapping of Tryptic Peptides. Acetimidated bovine neurophysin I that had been reacted with ^{14}C-succinic anhydride was fractionated by affinity chromatography into active and inactive components. These were oxidized with performic acid and individually digested with diphenylcarbamyl chloride-treated trypsin (25:1 w/w) in 0.1 M sodium borate, pH 8.0, at 37 °C for 5 h. The digestion mixture was lyophilized and aliquots were dissolved in buffer A (0.1% TFA in H_2O) and fractionated by reverse-phase HPLC on a Zorbax CN column (4.6 × 250 mm) using a gradient of 95% buffer A/5% buffer B (0.1% TFA in acetonitrile) to 65% buffer A/35% buffer B between 0 and 50 min. Assignment of the peaks was by amino acid analysis and comparison with the known sequence. The tryptic fragments were collected, lyophilized, and dissolved in a known volume of water, half of which was added to 10 mL of scintillation fluid and counted and the other half of which was quantitated by amino acid analysis using norleucine as an internal standard. In order to determine whether the succinyl ester bond was stable to the pH and temperature conditions used for digestion with trypsin, we separately incubated a sample of [^{14}C]succinylated neurophysin at pH 8, 37 °C for 5 h in the absence of trypsin and passed it through a Sephadex G-50 column to remove any low molecular weight radioactivity that might have been released. The specific activity of the G-50 purified protein was compared with that of the starting material, the results indicating that 99% of the ester bond was conserved under these conditions.

Endoproteinase Asp-N Treatment. Tryptic fragments representing residues 44–66, derived from active and inactive [^{14}C]succinylated protein, were separately treated with endoproteinase Asp-N (Calbiochem). As recommended by the supplier, protein samples (~125 μg) were dissolved in 100 μL of 25 mM sodium phosphate, pH 8.0, and added to vials containing 2 μg of lyophilized enzyme. Samples were incubated for 3 h at 37 °C and then fractionated by reverse-phase HPLC, using the Zorbax CN column system described above but with a slightly modified gradient of 0–30% acetonitrile in 0.1% TFA over 50 min. The major peaks were collected and lyophilized, and specific radioactivity was determined as above.

Automated Gas-Phase Edman Sequencing. Selected radioactive peaks obtained from endoproteinase Asp-N treatment (see Results) were lyophilized and subjected to automated gas-phase Edman degradation using an Applied Biosystems gas-phase sequencer (Model 470A) equipped with a Model 120A PTH analyzer. The radioactivity released at each cycle

[1] Abbreviations: CD, circular dichroism; NP, neurophysin; Mes, 2-(*N*-morpholino)ethanesulfonic acid; NMR, nuclear magnetic resonance; HPLC, high-pressure liquid chromatography; TFA, trifluoroacetic acid; PTH, phenylthiohydantoin; $\bar{\nu}$, moles of peptide bound per polypeptide chain.

[2] The side product will not contribute to the CD changes at 350 nm that are used to follow the binding reaction. Moreover, because binding is very weak for this derivative, the exact concentration of active protein does not contribute significantly in binding constant calculations [cf. Breslow and Gargiulo (1977)].

was compared with the quantity of released amino acid, allowing determination of the specific radioactivity of each released amino acid as described elsewhere (Huang & Breslow, 1992).

Procedures for the Specific Study of Ser-25 and Thr-38. Enzymatic digestion and purification procedures to isolate the peptide representing residues 22–25 were performed as described earlier (Burman et al., 1987). The molecular weight of this peptide was determined by mass spectrometry, also as previously described. For initial determination of the degree of succinylation of Thr-38, succinylated protein was treated with trypsin to cleave at Arg-43 (Breslow et al., 1982), followed by overnight carboxypeptidase B digestion (pH 7.6, protein: protease weight ratio = 15). Residual protein was precipitated with sulfosalicylic acid and the amino acid content of the supernatant was directly analyzed; the chromatographic conditions used were demonstrated to separate Thr from O-succinylated Thr, which was synthesized as a control.

Other Procedures. Amino acid analyses were performed using a Durrum D-500 analyzer. For analysis of fragments from trypsin and endoproteinase Asp-N digestion, peptide fractions were first subjected to a 24-h digestion in 6 N HCl at 110 °C.

RESULTS

Affinity Chromatography of Succinylated Neurophysin. Succinylation of either native or nitrated bovine neurophysin I or II, or of neurophysin in which the amino groups have been blocked by acetimidation (Materials and Methods) leads to a similar loss of binding activity. Upon affinity chromatography, protein succinylated under typical reaction conditions (Materials and Methods) gives two fractions in approximately 1:1 ratio, one eluting in the void volume at pH 6.2 (inactive protein) and one eluting only at pH 2 (active protein). Electrophoresis of the two fractions obtained upon succinylation of acetimidated protein indicated that the binding fraction represented a mixture of the original acetimidated protein and species with one and two additional negative charges per chain. The nonbinding fraction principally represented protein having one and two additional negative charges per chain and contained none of the original acetimidated protein. Treatment of the nonbinding fraction with 1 M hydroxylamine at pH 10 for 1 h restored binding activity to 90% of the protein, as measured by affinity chromatography, and reversed the principal succinylation-induced effects on electrophoretic mobility (data not shown). The same restoration of activity was obtained by hydroxylamine treatment of the inactive fraction derived from succinylation of non-acetimidated neurophysin. The desuccinylation conditions necessary for restoration of activity are those leading to the cleavage of aliphatic esters (Glazer et al., 1975). Milder conditions sufficient for deesterification of tyrosine hydroxyl groups (hydroxylamine treatment at neutral pH) have no effect on binding or electrophoretic mobility. Identical results were obtained with bovine neurophysins I and II, indicating that succinylation of a serine or threonine common to both neurophysins is responsible for the decrease in binding activity. This limits the responsible residue to Ser-25, -52, -56, or -63 or to Thr-38 (Figure 1).

Localization of the Site of Succinylation Responsible for the Loss of Binding Activity to Ser-52, -56, or -63. Preliminary results, confirmed below, argued against either Ser-25 or Thr-38 as the source of the inactivation. A tetrapeptide representing the 22–25 sequence was isolated in good yield from the nonbinding succinylated fraction and was demonstrated

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                    10                  20                  30
NP-I  AVLDLDVRTCLPCGPGGKGRCFGPSICCGDELGCF
NP-II ---M S------E L---Q----------------------------------------

                    40                  50                  60                  70
NP-I  VGIAEALRCQEENYLPSPCQSGQKPCGSGGRCAAA
NP-II ----------------------------------------------------------

                    80                  90
NP-I  GICCSPDGCHEDPACDPEAAFS
NP-II ----------N D E S----V T E----E---R E GIVG---P R R V
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FIGURE 1: Amino acid sequences of bovine neurophysins I and II [e.g., Breslow and Burman (1990)]. The complete sequence of neurophysin I is shown, with the exception of residue 93, which is absent in our preparations. The sequence of neurophysin II is identical to that of neurophysin I, except where indicated. Serine and threonine residues common to both proteins are indicated in boldface and underlined.

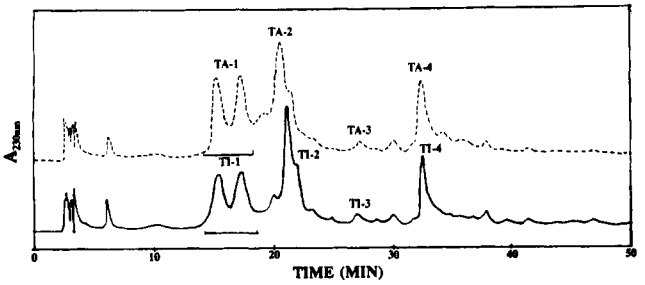

FIGURE 2: HPLC patterns of the tryptic peptides obtained from active (- - -) and inactive (—) components of succinylated acetimidated neurophysin-I following performic acid oxidation. TA-1 (from active protein) and TI-1 (from inactive protein) each represent a mixture of the two tryptic peptides (residues 9–20 and 67–92) that were not resolved into their individual components. TA-2 and TI-2 represent residues 44–66; TA-3 and TI-3 represent residues 1–8; TA-4 and TI-4 represent residues 21–43.

to be nonsuccinylated by mass spectrometry (Materials and Methods). Similarly, Thr-38, released from the protein by partial enzymatic digestion (Materials and Methods) was found not to be significantly succinylated in the inactive protein.

To more specifically identify the residue involved, acetimidated bovine neurophysin I was succinylated with [^{14}C]-succinic anhydride and fractionated into its active and inactive components. Following demonstration that the succinyl esters were stable to the conditions of performic acid oxidation (Materials and Methods), the labeled protein fractions were oxidized with performic acid to cleave the disulfide bridges and subjected to tryptic mapping (Figure 2); identities of the isolated peaks were assigned by amino acid analysis. As monitored chromatographically, the sole difference between active and inactive fractions was a small difference in the mobility of tryptic peptide 2, representing the sequence 44–66 (Figure 2). This reflects a large difference between active and inactive components in the degree of succinylation of this peptide as measured by specific radioactivity (Figure 3); no significant difference between active and inactive components in the degree of succinylation of other peptides was seen. The results indicate that the inactive fraction differs from the active fraction in the succinylation of Ser-52, -56, and/or -63.

Demonstration That the Residue Responsible for Inactivation Is Ser-56. The tryptic peptides representing the 44–66 sequence were treated with endoproteinase Asp-N, which cleaves peptide bonds on the amino-terminal side of Asp and of cysteic acid residues (Drapeau, 1980). Under the conditions used, this enzyme failed to cleave at Cys-61 but cleaved at Cys-54, allowing HPLC isolation of the Asp-N-generated peptides 44–53 and 54–66. Each of these peptides gave two

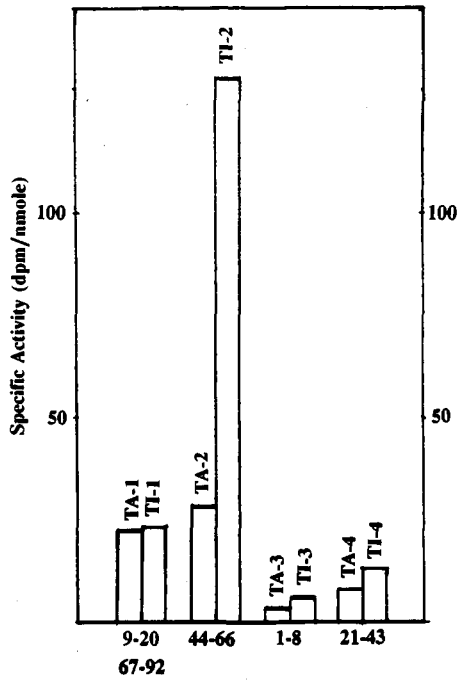

FIGURE 3: Specific activity of the tryptic peptide fractions isolated in Figure 2.

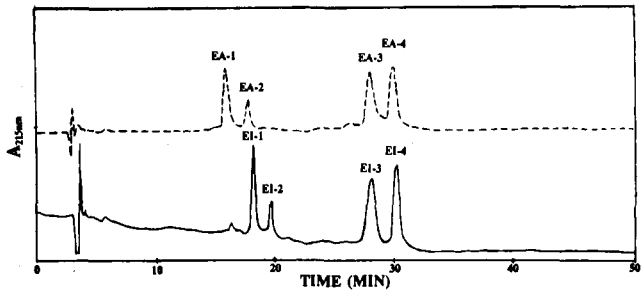

FIGURE 4: HPLC patterns of the tryptic peptides TA-2 and TI-2 (Figure 3) following digestion with endoproteinase Asp-N. The series EA (- - -) is derived from TA-2; the series EI (—) is derived from TI-2. The identities of the individual peptides are given in the text and in Table I.

Table I: Specific Activity and Identity of the Fragments Resulting from Endoproteinase Asp-N Treatment of Tryptic Peptides TA-2 and TI-2[a]

fragment	residues represented	specific activity (dpm/nmol)
EA-1	54–66 (nonsuccinylated)	2
EA-2	54–66 (monosuccinylated)	62
EA-3	44–53 (nonsuccinylated)	11
EA-4	44–53 (monosuccinylated)	60
EI-1	54–66 (monosuccinylated)	92[b]
EI-2	54–66 (disuccinylated)	123
EI-3	44–53 (nonsuccinylated)	11
EI-4	44–53 (monosuccinylated)	49[b]

[a] The identity of the purified peptides was determined by amino acid analysis. [b] The theoretical specific activity of a monosuccinylated peptide is 66 dpm/nmol, based on the specific activity of the initial anhydride (Materials and Methods). Significant deviations from this value are attributed to combined inaccuracies in peptide quantitation and counting. In the case of EI-1, for example, its identity as a monosuccinylated peptide is confirmed by sequencing (see text and Figure 5).

principal HPLC peaks, demonstrated from their radioactivity to represent different degrees of succinylation (Figures 4, Table I). The identities of the individual peaks, as demonstrated by amino acid analysis and specific radioactivity, are given in Table I. No difference between active and inactive proteins

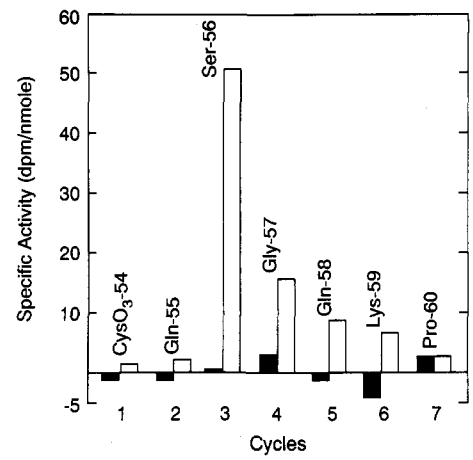

FIGURE 5: Radioactivity of amino acids released upon Edman degradation of the peptides EA-2 (solid bars) and EI-1 (open bars). The sequencer was run so that half of the released amino acid at each cycle was used for amino acid identification and quantitation and half was used for radioactivity measurement, allowing the specific activity to be calculated. EI-1 was sequenced through Gly-64, but no radioactivity was released after Pro-60. EA-2 was sequenced only through Pro-60.

in the degree of succinylation of the sequence 44–53 is seen. This sequence yields an unsuccinylated peptide and a monosuccinylated peptide in similar relative proportions in both proteins (peptides EA-3,4 and EI-3,4 in Figure 4 and Table I), indicating that succinylation of Ser-52 is without significant effect on activity. By contrast, peptides representing the sequence 54–66 are different in the active and inactive proteins. The active protein yields the unsuccinylated peptide (EA-1) and monosuccinylated peptide (EA-2) in approximately 2:1 ratio. The inactive protein yields a monosuccinylated peptide (EI-1) and a disuccinylated peptide (EI-2) in approximately 3:1 ratio.

The mobilities of the monosuccinylated peptides (EA-2 and EI-1) derived from active and inactive fractions, respectively, are nonidentical (Figure 4), indicating that the two are succinylated at different positions. To identify the positions involved in each case, EA-2 and EI-1 were each subjected to Edman degradation and the radioactivity liberated at each cycle determined (Figure 5). With EI-1, which was sequenced through position 64, radioactivity was liberated only from Ser-56. With EA-2, which was sequenced only through Pro-60, no radioactivity was associated with Ser-56, and the residual radioactivity found to still be associated with the uncleaved peptide remained on the filter after the Pro-60 cycle. Accordingly, EA-2 appears to represent succinylation on Ser-63. The results indicate that succinylation of Ser-56 is uniquely associated with inactivation.

Binding and Conformational Properties of Succinylation-Inactivated Neurophysin. To determine the degree to which the peptide affinity of neurophysin was reduced by succinylation of Ser-56, bovine neurophysin II, mononitrated at Tyr-49 and purified to remove inactive side products, was succinylated. The succinylated nitrated protein was separated into active and inactive components by affinity chromatography, and the binding properties of each were determined by CD at protein concentrations of 0.2 and 2.0 mg/mL using the ligand peptide Phe-TyrNH_2. The weight fraction of dimer in the unliganded native protein increases from ~15% to 50% over this concentration range and the observed peptide-binding constant increases by a factor of ~2.4 (Breslow et al., 1991). As shown in Table II, the binding isotherms of the active succinylated component show the same dependence on protein

Table II: Binding of Phe-TyrNH$_2$ to Native and Modified Neurophysins[a]

protein	binding constant[b] (M^{-1}) 2 mg/mL	0.2 mg/mL	concn factor[c]
nitrated NP-II	19000	8000	2.4
active nitrated succinylated NP-II[d]	15000	6300	2.4
inactive nitrated succinylated NP-II	700[d]	750	0.9[e]
nitrated NP-I	14000	6000	2.3
nitrated des-1–8 NP-I	1200	1600	0.75
succinylated nitrated des-1–8 NP-I	700[f]		
desuccinylated nitated des-1–8 NP-I	900		

[a] At pH 6.2 and 25 °C in 0.1 mM ammonium acetate/2 mM Mes buffer. [b] Because of the cooperativity of some binding isotherms, binding constants are calculated from the slope at half-saturation as previously described, rather than from the linear least-squares fit [e.g., Breslow and Gargiulo (1977) and Breslow et al. (1991)]. [c] Calculated as the ratio of the apparent binding constant at 2 mg/mL to that at 0.2 mg/mL (Breslow et al., 1991). [d] Electrophoretic analysis of this product indicated virtually complete succinylation of ϵ-amino groups and partial succinylation of Ser residues, the latter reversed by hydroxylamine treatment. Therefore, differences in the properties of this derivative and the inactive succinylated product should not reflect differences in the succinylation of ϵ-amino groups. [e] If the binding constant at 2 mg/mL is calculated from the linear least-squares fit in Figure 6, it increases to 800 M^{-1} and the concentration factor increases to 1.1. The apparent binding constant at 0.2 mg/mL is independent of the method of calculation. [f] The sample used for this study was initially succinylated to give a binding constant of 650 M^{-1} (Figure 7). It was then desuccinylated and resuccinylated under more vigorous conditions (text) to give a binding constant of 750 M^{-1}. The value shown is the average of these two determinations.

concentration as unsuccinylated protein, with calculated binding constants only 20% lower than those of unsuccinylated protein. The inactive fraction is shown by the solution data to retain a small but measurable binding affinity, representing $\sim 1/20$th the affinity of the active fraction at a protein concentration of 2 mg/mL (Table II). The loss of binding affinity was similar for both Phe-TyrNH$_2$ and the peptide Gly-TyrNH$_2$, indicating that it did not reflect steric hindrance of the succinyl group and the bound side chain of peptide residue 1; the latter interacts with Pro-53 in the crystal (Chen et al., 1991), which is potentially within reach of a succinyl group on Ser-56. No CD-demonstrable structural differences between the native and inactive succinylated proteins were found, indicating that the low binding activity of the inactive protein was not the result of a global structural rearrangement.

Of particular significance is that the binding isotherms of the inactive succinylated protein showed no dependence of binding on protein concentration over the concentration range studied (Table II). This is illustrated in Figure 6, where the binding isotherms of the inactive succinylated protein at two protein concentrations are compared with that of unsuccinylated protein. The results indicate that succinylation of Ser-56 alters monomer–dimer equilibria in the liganded and/or unliganded states (Discussion), although Ser-56 is distant from the monomer–monomer interface (Chen et al., 1991).

Effect of Cleavage of the 1–8 Sequence on the Preferential Binding by Dimer. To determine whether the loss of the concentration dependence of binding was uniquely associated with succinylation of Ser-56, we investigated another modification known to reduce binding affinity, tryptic excision of the 1–8 sequence. This decreases peptide affinity (Abercrombie et al., 1982; Breslow et al., 1982; Sardana & Breslow, 1984) because of the loss of Arg-8, a residue shown by the crystal structure to be located at the binding site (Chen et al., 1991). Under the conditions in these studies, the nitrated des-1–8 protein exhibited $1/10$ the binding affinity of the native

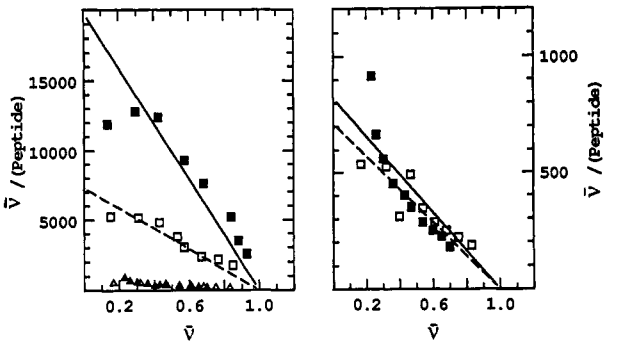

FIGURE 6: Scatchard plots showing the effects of succinylation of bovine neurophysin II on the binding of the peptide Phe-TyrNH$_2$ as determined in 0.1 M ammonium acetate/2 mM Mes buffer, pH 6.2 at 25° C. Left: Binding to nitrated native neurophysin II at protein concentrations of 2 mg/mL (■) and 0.2 mg/mL (□). Also shown is binding to the inactive fraction of the succinylated nitrated protein at concentrations of 2 mg/mL (▲) and 0.2 mg/mL (△). Lines serve principally to delineate the data; note weak cooperativity in the native protein (Breslow et al., 1991). Right: Expanded view of binding to the inactive fraction of the succinylated nitrated protein concentrations of 2 mg/mL (■) and 0.2 mg/mL (□); lines are linear least-squares fits of the data, weighting all points equally.

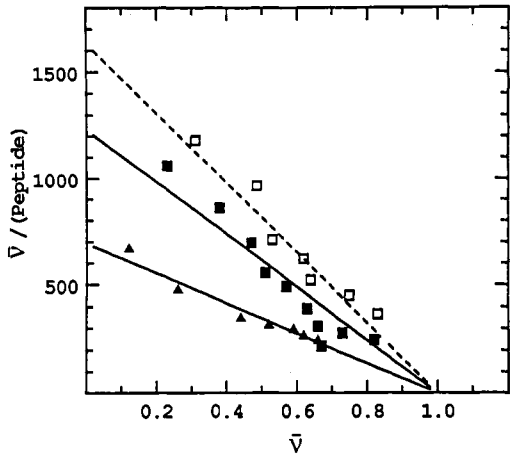

FIGURE 7: Scatchard plots of the binding of Phe-TyrNH$_2$ to nitrated des-1–8 neurophysin I and its succinylated product. (■) Binding to nitrated des-1–8 neurophysin at a protein concentration of 2 mg/mL; (□) binding to the same protein at a concentration of 0.2 mg/mL; (▲) binding to the same preparation of the nitrated des-1–8 protein following vigorous succinylation and using a protein concentration of 2 mg/mL. Lines are least-squares fits of the data.

protein at a protein concentration of 2 mg/mL, and as with the inactive succinylated fraction, binding did not increase with protein concentration over the range studied (Table II, Figure 7). Note that the small apparent inverse concentration dependence in Figure 7 (increased affinity at lower concentration) is not seen with all preparations of the des-1–8 protein and is not viewed as significant.

Although it was not foreseen, inspection of the neurophysin crystal structure (Chen et al., 1991) suggested the potential proximity of Arg-8 and a succinyl group attached to Ser-56 (Figure 8). Accordingly, we asked whether both modifications might affect a common locus or mechanism by looking at the effects of succinylation of the nitrated des-1–8 protein. If completely different mechanisms for the effects of succinylation and excision of the des-1–8 sequence were involved, succinylation of the des-1–8 protein should further reduce its peptide affinity by a factor of ~20. Since there was no convenient method to separate protein modified at Ser-56 from protein not modified at this position (i.e., both the starting des-1–8 protein and its succinylated derivative do not bind well to the affinity column), the nitrated des-1–8 protein was

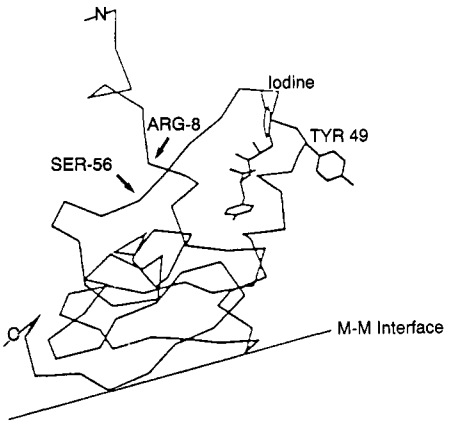

FIGURE 8: α-Carbon map of a subunit of bovine neurophysin II with the bound peptide *p*-iodo-L-Phe-L-TyrNH$_2$ [adapted from the crystal structure of Chen et al. (1991)]. Positions of the α-carbons of Ser-56 and Arg-8 and of the monomer–monomer (M–M) interface are indicated.

succinylated under two sets of conditions giving total inactivation of the native protein (Materials and Methods). The results of both studies (Table II, and Figure 7) indicate that succinylation of the des-1–8 protein reduces the binding affinity by a factor of less than 2, so that the binding affinity of the succinylated des-1–8 protein is essentially that of the succinylation-inactivated native protein. Deesterification of the des-1–8 protein partially reverses the small succinylation effect (Table II).

The loss of the dependence of binding on protein concentration in the des-1–8 protein in this study (conducted in 0.1 M ammonium acetate/2 mM Mes) was at variance with conclusions from NMR studies. Under NMR conditions (representing ~0.05 M NaCl, pH 6), the des-1–8 protein exhibits the same self-association properties as the native protein in the unliganded state (Peyton et al., 1986). Moreover, preliminary results (Peyton et al., 1987), subsequently supported by other studies (unpublished), indicated that the des-1–8 protein dimer bound ligand more strongly than monomer under NMR conditions. Accordingly, we investigated the binding properties of the nitrated des-1–8 protein in 0.05 M NaCl and 0.1 M NaCl, both in the presence of 2 mM Mes. In agreement with the NMR studies, the observed binding constant increased by a factor of 1.9 between 0.2 and 2.0 mg/mL protein in 0.05 M NaCl and by a factor of 2.4 in 0.1 M NaCl. The change in concentration dependence principally represented a reduction in the apparent binding constant at the lower protein concentration. Therefore, the des-1–8 protein has allosteric properties similar to those of the native protein in NaCl, although its absolute binding affinity remains markedly depressed.

DISCUSSION

Given the neurophysin crystal structure, it might have been predicted that the serine responsible for the observed succinylation-induced inactivation would be Ser-52, the carbonyl group of which is a direct participant in binding (Chen et al., 1991). Preliminary modeling studies, using the crystal structure, suggest that the reason succinylation of Ser-52 is not inactivating is that its hydroxyl group is completely exposed, allowing an attached succinyl group to be oriented toward the solvent. By contrast, the observed effects of succinylation of Ser-56 both on binding and on the concentration dependence of binding would probably not have been predicted. Ser-56 is not directly involved in binding and is distant from the subunit interface (Figure 8). Significantly, however, mutation of its neighbor Gly-57 to Ser has been recently implicated as the cause of one form of familial diabetes insipidus (Ito et al., 1991). Although the region in which these residues are located is a loop (Figure 8), it is therefore possible that this region plays an important indirect role in modulating neurophysin properties. Our results most particularly suggest that the effects of succinylation of Ser-56 are mediated via a mechanism similar to or in common with that associated with deletion of the 1–8 sequence. Both modifications of the native protein lead to a similar decrease in binding affinity and to loss of the concentration dependence of binding under the appropriate buffer conditions, and, once the 1–8 sequence has been excised, succinylation fails to produce a significant further decrease in binding affinity.

The linkage between the two modifications might in principle be indirect (e.g., both might independently produce the same conformational change), but the crystal structure suggests a direct linkage, indicating a distance of ~7.5 Å between the –OH of Ser-56 and the terminal guanidinium nitrogen atom of Arg-8 [Chen et al. (1991) and unpublished data]. A plausible thesis is that the linkage between Ser-56 and Arg-8 reflects formation of a bridge between the succinyl group on Ser-56 and the Arg-8 guanidinium group; such a bridge would place the negatively charged oxygen of the succinyl group less than 2 Å from the positively charged guanidinium terminus. In this context it is relevant that the crystal structure, which represents the liganded state, does not suggest complete exposure of the Ser-56 hydroxyl group to solvent. Since Ser-56 can be shown to be succinylated in the unliganded state at least as efficiently as Ser-52 or any other neurophysin hydroxyl group,[3] the results suggest that there is a conformational difference in the region around Ser-56 in liganded and unliganded states (consistent with a possible role for this loop region in allosteric modulation) and/or that the positive charge on Arg-8 might facilitate succinylation of Ser-56 by stabilizing the developing negative charge on the succinyl group in the transition state.[4]

In the crystal structure, the guanidinium group of Arg-8 is located at the binding site, where it participates in a salt bridge with the same protein carboxyl group (Glu-47) that interacts with the protonated α-amino group of bound peptide (Chen et al., 1991). Thus, either excision of Arg-8 or its interactions with a proximal succinyl group on Ser-56 would directly destabilize the liganded state and decrease binding affinity. However, the observed effects of both modifications on the concentration dependence of binding (in ammonium acetate) must be mediated by long-range interactions, since both Arg-8 and Ser-56 are on the opposite side of the protein from the monomer–monomer interface (Figure 8). While the nature of these long-range interactions remains to be elucidated, a role for Arg-8 in modulating dimerization properties has been suggested by two other studies. First,

[3] The rate of succinylation of Ser-56 relative to that of other residues can be deduced semiquantitatively from several observations. For example, under our standard succinylation conditions, half of the protein is inactivated (Results), indicating that Ser-56 is 50% succinylated. Under the same conditions (e.g., Figure 4), Ser-52 is also 50% succinylated, indicating comparable rates of succinylation of these two residues. Similarly, electrophoresis studies (text) indicate that Ser-56 is 50% succinylated under conditions representing a low average degree of total succinylation.

[4] Strictly speaking, we have not proven that Ser-56 is completely succinylated in the des-1–8 protein, despite the vigorous succinylation conditions used. However, a decrease in the rate of succinylation of Ser-56 in the des-1–8 protein would only strengthen the argument for a connection between Arg-8 and Ser-56.

NMR studies of the unliganded state (in NaCl) have demonstrated that, while dimerization of the des-1–8 protein is unimpaired under these conditions, dimerization-dependent changes at the binding site are nonidentical in the native and des-1–8 proteins, the differences assigned to the contributions of Arg-8 (Peyton et al., 1986; Breslow et al., 1992). Second, affinity chromatography studies, carried out in an ammonium acetate buffer, indicated that the unliganded des-1–8 protein had very weak dimerization properties relative to the native protein (Abercrombie et al., 1982). While the reason for the difference in dimerization properties of the des-1–8 protein found in the affinity chromatography studies and in NMR studies was initially unclear (Peyton et al., 1986), the present studies suggest that these differences arise from differences in the ionic milieu in the two studies. This is surprising, since the self-association properties of native bovine neurophysin I, in contrast to those of the des-1–8 protein, are not impaired in ammonium acetate [e.g., Abercrombie et al. (1982) and Breslow et al. (1991)]. Nonetheless, it is therefore possible that the effect of cleavage of the 1–8 sequence (and presumably of succinylation of Ser-56) on allosteric properties in ammonium acetate at least in part reflects loss of normal dimerization properties in the unliganded state. Similarly, the normal allosteric properties of the des-1–8 protein in NaCl appear to correlate with normal dimerization properties under these conditions.

The present studies are the first to demonstrate an alteration of neurophysin allosteric properties by chemical modification and identify the region of three-dimensional space that involves Ser-56 and Arg-8 as an important participant in the long-range control of neurophysin self-association. Additional studies of ionic effects on allosteric properties are needed. For example, evaluation of the role of individual cations and anions in altering the dimerization constant and allosteric properties of the des-1–8 protein and correlation with effects on the binding site as monitored by NMR [e.g., Breslow et al. (1992)] should be experimentally feasible and would provide useful insights into the mechanisms by which events at the binding site and at the subunit interface are linked.

ACKNOWLEDGMENT

The early contributions of Paul Chang to this work are particularly recognized; his unpublished studies in this laboratory provided the first evidence that succinylation of an aliphatic hydroxyl group was the basis of the succinylation-induced inactivation of neurophysin. The authors are additionally grateful to Dr. Daniel Wellner for assistance with amino acid sequencing. The mass spectrometric studies reported were performed by the Mass Spectrometry Facility at The Rockefeller University, which is supported by NIH Grants RR00862 and GM-38724.

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