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Investigation of the Interactions of Oxytocin with Neurophysins at Low pH Using Carbon-13 Nuclear Magnetic Resonance and Carbon-13-Labeled Hormones†

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ABSTRACT: The specifically ^{13}C-labeled (90% ^{13}C-enriched) peptide hormone derivatives [1-hemi[2-^{13}C]cystine]oxytocin, [1-hemi[1-^{13}C]cystine]oxytocin, and [2-[2-^{13}C]tyrosine]-oxytocin and the analogue [3-[2-^{13}C]leucine]oxytocin were prepared by total synthesis and used to study the interactions of the neurohypophyseal hormones with the bovine neurophysins as a function of pH and temperature. Under all conditions, whether high or low pH, the chemical shifts of the labeled carbon atoms of the bound hormones are the same, but they are shifted significantly from their positions in the free hormone. These results indicate that interactions of the side chain and disulfide moieties of the hormone with the neurophysins do not change as a function of pH. At neutral pH and 20–35 °C, the labeled atoms of the hormone are in slow exchange (1–5 s^{-1}) with the neurophysins for the above hormone derivatives, but at low pH they are in intermediate or fast exchange depending upon the pH and temperature. At low pH, the dissociation rate constant (k_{off}) is about 100-fold greater than the value at neutral pH, and this increase appears to be due exclusively to the breaking of the salt bridge involving the N-terminal amino group of oxytocin and a side-chain carboxyl group of neurophysin. Since the dissociation constant (K_d) also increases by about 100-fold in going from neutral to low pH, the association rate constant is deduced to be the same at neutral and low pH. In contrast to the low pH results, an increase in pH (from 6.6 to 10.5) leads to a continual decrease in the binding constant but to no apparent change in the dissociation rate constant. The bound hormone is always in slow exchange at high pH, even when the binding constant has been reduced by 2 or 3 orders of magnitude. At high pH, the decrease in binding affinity is due solely to the deprotonation of the α-amino group of the free hormone. Thus, at high pH the apparent association rate constant decreases, while the dissociation rate constant remains unchanged.

The interaction of the neurohypophyseal hormone oxytocin with a group of proteins known as neurophysins provides a unique system for investigating peptide hormone–macromolecular interactions, and a large number of studies on this system have been carried out (for reviews, see Breslow, 1974; Walter, 1975). Most of these studies have been conducted at a pH which was close to neutrality though some studies at other pH values have been done. By measuring binding constants as function of pH, it has been found that two groups, one with a pK_a of about 6.5 and the other with a pK_a of 4.5, are important for binding (Camier et al., 1973). From studies with oxytocin analogues, it was deduced that the group with a pK_a of 6.5 probably is the N-terminal group of free oxytocin

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(Stouffer et al., 1963) and this was directly confirmed by ^{13}C NMR[1] studies (Blumenstein et al., 1977). The group of pK_a = 4.5 is believed to be a carboxylic acid side chain of free neurophysin (NP), which in the NP–oxytocin complex forms a salt bridge with the amino group of oxytocin. At low pH (below pH 3) binding appears to become pH independent, and apparently occurs without participation of this salt bridge (Breslow & Gargiulo, 1977). The latter conclusion was based largely on experiments using neurophysin which was nitrated (or dinitrated) at tyrosine-49. It is believed that the hormone binding properties of this protein are very similar to that of native neurophysin, at least for the "strong" hormone binding site (Nicolas et al., 1978). While the pH-independent nature of the low pH binding was clearly demonstrated by the binding of oxytocin analogues to nitrated NP, there was some indication that this phenomenon also characterized the binding of oxytocin to native NP (Breslow & Gargiulo, 1977). Also, NMR studies (Balaram et al., 1973; Deslauriers et al., 1979) have indicated that the dissociation rate constant for oxytocin (and the hormone of similar structure, lysine-vasopressin) from NP increases at low pH.

We have been studying oxytocin–NP interactions by preparing derivatives of oxytocin specifically enriched to 90% ^{13}C in various positions by total synthesis and then using ^{13}C NMR techniques to investigate the nature of these interactions. For some positions in the hormone, we have observed large chemical shift differences between free and bound hormone and have also been able to deduce information on stoichiometry and kinetics of binding (Blumenstein & Hruby, 1977; Blumenstein et al., 1977, 1978). Whereas our previous studies were all conducted near or above neutral pH, we here report on work done at very low pH (2.5 and below), as well as some work at very high pH (above 9.5). By comparing the results we obtain at the various pH values, we are able to deduce some changes that occur at low pH and striking similarities which are invariant with pH.

Experimental Section

Materials. The bovine neurophysins, NPI and NPII, were purified as in our previous work (Blumenstein & Hruby, 1976), by using a slight modification of the procedures of Breslow et al. (1971). N^{α}-Boc-amino acids were purchased from Vega-Fox Biochemicals or Biosynthetica and were checked for purity by thin-layer chromatography (TLC) in three solvent systems and by melting point determination. The polystyrene resin 1% cross-linked with divinylbenzene and chloromethylated to an extent of 1.07 mmol/g of resin was purchased from Lab Systems Inc., San Mateo, CA. Solvents used for partition chromatography purification of peptide hormone derivatives were purified as previously described (Hruby & Groginsky, 1971). The syntheses of the ^{13}C-labeled amino acid derivatives N^{α}-Boc-DL-[2-^{13}C]cystine (Blumenstein & Hruby, 1977) and N^{α}-Boc-DL-[2-^{13}C]tyrosine (Blumenstein et al., 1978) were done as reported.

Analytical Methods. TLC was performed on silica gel G plates by using the solvent systems: (a) 1-butanol–acetic acid–pyridine–water (15:3:10:12); (b) 1-butanol–acetic acid–water (4:1:5, upper phase only); (c) 1-pentanol–pyridine–water (7:7:6); (d) ethyl acetate–pyridine–acetic acid–water (5:5:1:3). Amino acid and peptide spots were detected by ninhydrin, UV light, iodine, and fluorescamine. Purification of the peptide hormone derivatives was accomplished by partition chromatography on Sephadex G-25 as previously reported (Yamashiro et al., 1966; Hruby et al., 1976), followed by gel filtration on Sephadex G-25 (Porath & Flodin, 1959). Amino acid analyses were obtained by the method of Spackman et al. (1958) on a Beckman 120C amino acid analyzer after hydrolysis in 6 N HCl for 24 h. Optical rotation values were measured at the mercury green line (547 nm), by using a Zeiss Old 4 polarimeter. Reverse phase high pressure liquid chromatography (LC) was performed as previously reported in our laboratory for oxytocin derivatives under conditions where diastereoisomers, if present, would be separated from the native hormone and detected (Larsen et al., 1978, 1979).

[1-Hemi[2-^{13}C]cystine]oxytocin. The compound was prepared by the solid phase method of peptide synthesis and purified as previously reported (Blumenstein & Hruby, 1977). Purity was assessed as before, and in addition LC showed that the compound was not contaminated by any of the 1-hemi-D-[2-^{13}C]cystine diastereoisomer.

[2-[2-^{13}C]Tyrosine]oxytocin. The title compound was prepared and purified as previously reported (Blumenstein et al., 1978) by using the solid phase method of peptide synthesis. The purity of the compound was assessed as previously reported and was uncontaminated by the [2-D-[2-^{13}C]tyrosine]oxytocin diastereoisomer as shown by LC.

[1-Hemi[1-^{13}C]cystine]oxytocin. The compound was prepared from the appropriate constituent amino acids by using the solid phase method of peptide synthesis. The title compound was purified by partition chromatography on Sephadex G-25 followed by gel filtration. Details of the synthesis and properties of the peptide will be reported elsewhere (V. J. Hruby, V. Viswanatha, and Y. C. S. Yang, unpublished results). Purity of the compound was assessed by TLC in three solvent systems, reversed phase LC amino acid analysis, optical rotation, bioassay using the milk ejecting assay (Hruby & Hadley, 1975), and ^{13}C NMR spectroscopy (Hruby et al., 1979).

[3-[2-^{13}C]Leucine]oxytocin. The title compound was prepared by the solid phase method of peptide synthesis and then purified by preparative LC. Details of the synthesis, purification, and properties of the peptide are given elsewhere (Viswanatha et al., 1979). Purity was assessed by TLC in three solvent systems, LC amino acid analysis, optical rotation, and bioassay using the milk ejecting assay (Hruby & Hadley, 1975).

NMR Methods. Samples for NMR studies were prepared as described previously (Blumenstein & Hruby, 1976, 1977). For many of the samples run at low pH, phosphate was not used in the buffer solution. The presence or absence of phosphate had no measurable effect on the spectra. Spectra of protein–hormone were obtained on a Bruker HX-270 NMR spectrometer as previously described (Blumenstein & Hruby, 1977) by using quadrature phase detection and a spectral width of ±8 kHz.

Results

In Figure 1 are shown spectra of [1-hemi[2-^{13}C]cystine]-oxytocin and [2-[2-^{13}C]tyrosine]oxytocin in the presence of NPI (1:1 mol ratio), pH 2.5. For comparison, we also include spectra of solutions of identical NP and hormone concentrations at pH 6.6. The spectra at low pH contain a relatively

[1] Standard abbreviations and nomenclature for amino acids, peptides, and peptides derivatives are used throughout. Amino acids are of the L configuration unless otherwise stated. Other abbreviations used: NMR, nuclear magnetic resonance; NP, bovine neurophysin; NPI, bovine neurophysin I; NPII, bovine neurophysin II; TLC, thin-layer chromatography; Boc, *tert*-butyloxycarbonyl; EDTA, ethylenediaminetetraacetic acid.

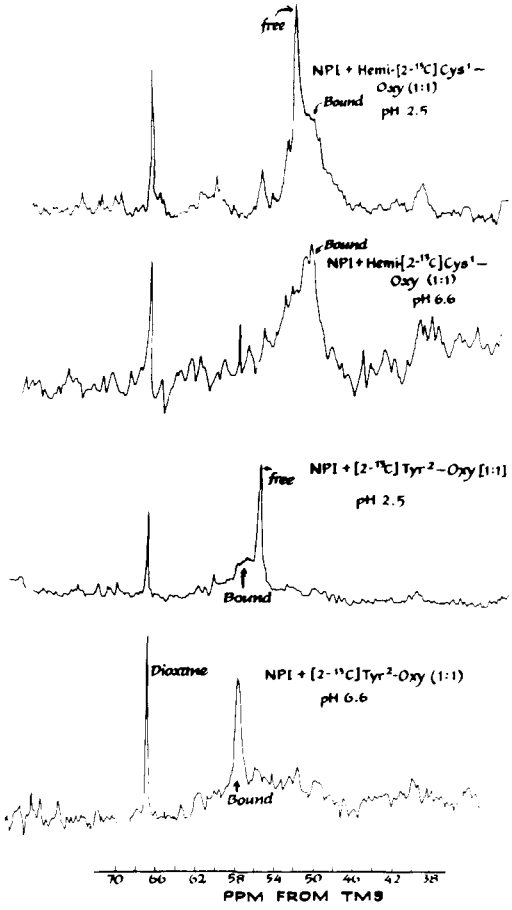

FIGURE 1: ^{13}C NMR spectra of (top) neurophysin I (NPI) + [1-hemi[2-^{13}C]cystine]oxytocin at pH 2.5; (second) NPI + [1-hemi-[2-^{13}C]cystine]oxytocin at pH 6.6; (third) NPI + [2-[2-^{13}C]tyrosine]oxytocin at pH 2.5; and (bottom) NPI + [2-[2-^{13}C]tyrosine]-oxytocin at pH 6.6. For all spectra, the NP concentration was about 25 mg/mL, and the protein:hormone ratio was 1:1. The bottom spectrum was taken at 37 °C; all others were at 22 °C. Spectra were measured at 67.9 MHz on a Bruker HX-270 spectrometer. The total accumulation times, using a 1-s repetition rate and 90° pulses for the spectra, were 3–6 h. The sharp peak at 66.5 ppm is due to dioxane internal standard (3 μL/mL).

sharp resonance due to free hormone as well as a broad resonance due to bound hormone.

In our previous studies at neutral pH, the bound line widths for these derivatives were found to be highly temperature dependent, and we could get significantly narrower lines by raising the temperature from 22 to 37 °C. This line narrowing was not achieved at low pH for reasons which will be discussed later. The presence of both free and bound hormone even when excess hormone has not been added is due to the fairly weak oxytocin–NP binding interaction at low pH. The dissociation constant for the complex is about 4×10^{-4} M (Breslow & Gargiulo, 1977) at this pH, so at the concentrations used here, 2.5×10^{-3} M, NP will not be saturated and some oxytocin will be free. Due to the fact that (1) the free resonance is much narrower than the bound resonance and (2) the free resonance should have a nuclear Overhauser enhancement of about 2, as compared with 1.1 for the bound resonance (Doddrell et al., 1972), the concentration of free hormone at first glance appears to be much greater than it really is. When all these factors are considered, we can estimate that 20–25% of the hormone is free, which indicates a dissociation constant of between 1×10^{-4} and 3×10^{-4} M.

The width of the bound resonances at 22 °C makes determination of their chemical shifts difficult, but it appears

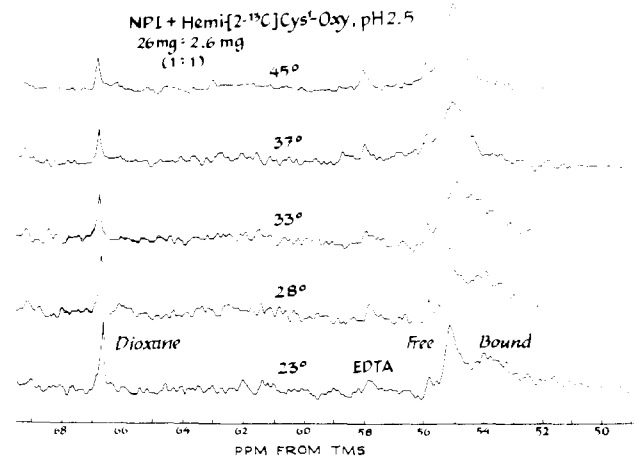

FIGURE 2: ^{13}C NMR spectra of NPI + [1-hemi[2-^{13}C]cystine]oxytocin at pH 2.5 (protein to hormone ratio is 1:1) as a function of temperature. Spectral conditions are as in Figure 1. Accumulation times were 3–6 h.

that, for both the labeled 1-hemicystine and 2-tyrosine compounds, the shift of the bound resonance at low pH is very similar to the shift at neutral pH (Blumenstein & Hruby, 1977; Blumenstein et al., 1978). The chemical shift difference between free and bound [1-hemi-[2-^{13}C]cystine]oxytocin at low pH, 1.5 ppm, is much smaller than that at neutral pH, 2.7 ppm, due to the pH dependence of the shift of the free hormone.

We have also examined several other labeled derivatives at low pH. Based on our previous work (Blumenstein et al., 1977), the free and bound resonances for [1-hemi[1-^{13}C]-cystine]oxytocin would be expected to have very similar shifts at low pH, and indeed at pH 2.5 we observe only one resonance, slightly broadened due to a small chemical shift difference (<0.25 ppm) between free and bound forms, as well as exchange between them. In [3-[2-^{13}C]leucine]oxytocin, an oxytocin analogue which binds to NP with an affinity 70–80% as great as that of oxytocin, a downfield shift of 4.5 ppm is seen on binding at neutral pH (M. Blumenstein, V. J. Hruby, and V. Viswanatha, manuscript in preparation; our NMR results with this derivative are in substantial disagreement with results of others (Griffin et al., 1977) and the reasons for this disagreement will be published elsewhere). At low pH, this 4.5 ppm shift is also observed, as well as the peak due to free hormone.

A study of the binding of [1-hemi[2-^{13}C]cystine]oxytocin to NPI as a function of temperature is shown in Figure 2. At low temperature, both free and bound forms are observed as described above. As the temperature is raised these two peaks coalesce into a single very broad peak, and further increases in temperature lead to a sharpening of this peak. Thus raising the temperature leads to an increased exchange rate and causes a transition from slow to intermediate, and finally to fast exchange. This same trend is seen when other labeled oxytocin derivatives such as [2-[2-^{13}C]tyrosine]oxytocin and [3-[2-^{13}C]leucine]oxytocin are examined at low pH.

Our efforts at quantitation of the exchange rate were hindered by the following factors: (1) the difficulty in accurately measuring line widths of the bound peaks; (2) the fact that as the temperature is varied there are large line width changes due to effects other than exchange (Blumenstein & Hruby, 1977; Blumenstein et al., 1978); (3) the weak binding at low pH and the significant temperature dependence of the oxytocin–NP binding constant (Glasel et al., 1976; Camier et al., 1973). This means that, even in the presence of excess

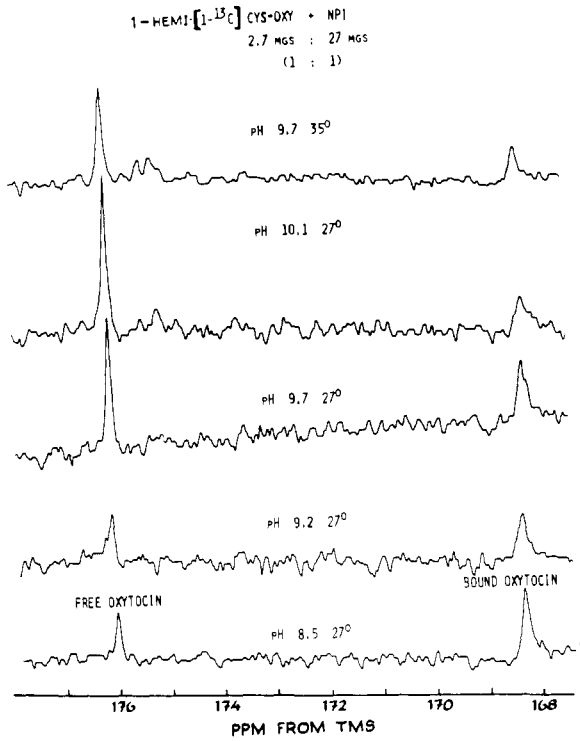

FIGURE 3: ^{13}C NMR spectra of [1-hemi[1-^{13}C]cystine]oxytocin + NPI as a function of pH (in the high pH range) and of temperature (compare upper and third spectra). In all cases, the hormone to protein molar ratio was 1:1. Repetition rate (90° pulse) was 2.4 s and accumulation times were 1–2 h.

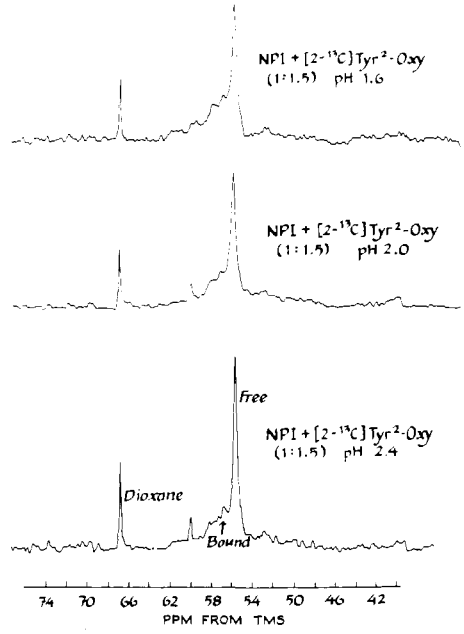

FIGURE 4: ^{13}C NMR spectra of NPI + [2-[2-^{13}C]tyrosine]oxytocin (molar ratio of protein to hormone 1:1.5 in all cases) as a function of pH at low pH. Spectral conditions as in Figure 1. Accumulation times were 2–4 h.

hormone, the protein is not fully saturated and that the percent saturation changes as a function temperature.

We estimate that the temperature at which the bound and free peaks of [1-hemi[2-^{13}C]cystine]oxytocin coalesce is 35 °C and that at this temperature the concentrations of free and bound species are equal if protein and hormone are mixed in a 1:1 ratio. The exchange rate at this temperature is then equal to $\sqrt{2}\pi x$ (chemical shift$_{bound}$ − chemical shift$_{free}$) or 450 s^{-1}. At lower temperatures, the bound hormone is in excess and the exchange rate is equal to the exchange broadening of the free peak multiplied by π. We estimate an exchange rate of 50 s^{-1} at 25 °C. A deuterium NMR study (Glasel et al., 1973) performed at pH 2.5, 34 °C, 0.45 M KCl, led to a stated estimate for k_{off} of >300 s^{-1}, and if all the labeled hormone analogues were in fast exchange, a limit of >1000 s^{-1} could be inferred.

This exchange behavior at low pH differs from that previously observed at neutral pH (Blumenstein & Hruby, 1977; Blumenstein et al., 1977, 1978) where slow exchange and an absence of exchange broadening was seen under all conditions. Furthermore, this behavior differs from that observed at high pH. In Figure 3 are shown spectra of [1-hemi[1-^{13}C]-cystine]oxytocin at high pH values and different temperatures. As the pH and temperature are raised the intensity of the bound resonance decreases as expected due to a decrease in the binding constant of the oxytocin–NP complex. Neither free nor bound resonance shows significant broadening, indicating that the exchange rate is still very slow. Additional studies at a variety of pH values (pH 7.5 to 10.5) and temperatures (23–40 °C) gave similar results, as did high pH studies with [1-hemi[2-^{13}C]cystine]oxytocin.

Recently, Breslow & Gargiulo (1977) have found that tripeptides binding to nitrated on Tyr-49 show a binding which becomes pH independent at low pH. We have now performed experiments to test whether this same phenomenon is observed with oxytocin. In Figure 4 are shown spectra of [2-[2-^{13}C]tyrosine]oxytocin–NPI at pH 2.5, 2.0, and 1.6. Again, exact quantitation of the relative amounts of free and bound form is difficult. However, the data do show that the binding constant changes very little from pH 2.5 to pH 1.6, at most by a factor or two. These results suggest that, at this pH, the hormones and tripeptides have similar binding interactions, and furthermore native NP displays the same binding characteristics as nitrated NP.

Discussion

There are two major observations to be made on comparing oxytocin–NP interaction at low pH with that at neutral pH and above: (1) the chemical shifts of several carbon atoms of oxytocin are very similar in the low pH and neutral pH complexes; (2) the k_{off} at low pH is about two orders of magnitude greater than it is at neutral pH. The large chemical shifts observed in the α carbons of residues 1 and 2 of oxytocin as well as residue 3 of [3-leucine]oxytocin are very distinct indicators of the electronic environments of these atoms. In particular, we believe these shifts of from 1 to 5 ppm are dominated by the precise side chain orientation which exists when oxytocin binds to NP (manuscript in preparation). Since these shifts are invariant with pH, this indicates that the interactions involving the side chains of residues do not change when the pH is lowered. This also implies that the disulfide conformation remains the same.

The increased off rate at low pH appears to be exclusively due to the breaking of the salt bridge between the amino group of oxytocin and a side chain carboxyl of NP. A priori one might expect that such a change would lead to a significant chemical shift of the carbonyl carbon of residue 1. We note, however, that, at neutral pH, the shift of this carbon is identical with the low pH shift observed in free oxytocin (Blumenstein et al., 1977). Thus, the shift of this carbon, as opposed to the shifts of the above mentioned α carbons, appears to mainly reflect the ionization state of the α-amino group, and since this group is fully protonated in both the low pH and neutral pH forms of the complex, the carbonyl shift does not change.

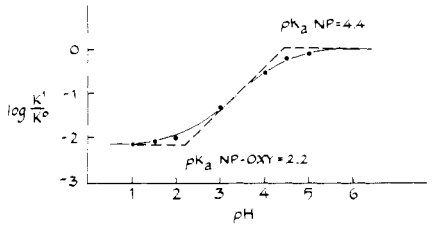

FIGURE 5: Dixon plot of data of Breslow & Gargiulo (1977) for binding of an oxytocin related peptide (Phe-Tyr-NH_2) to nitrated NP at low pH.

The k_{off} at low pH, 0.5–5 × 10^2 s^{-1}, is about two orders of magnitude greater than the value of 1–5 s^{-1} which we estimate at neutral pH (Blumenstein et al., 1977, Blumenstein & Hruby, 1977). Breslow & Gargiulo (1977) have found that the dissociation constant of tripeptide derivatives (similar to the N-terminal tripeptide of oxytocin) binding to NP increases by the same amount in going from pH 6.2 to pH 2. If the major (or only) change in interaction involves breaking of the carboxylate–amino salt bridge, we would expect the same relative change in the binding of oxytocin to NP. Our estimate of the oxytocin–NP dissociation constant at low pH of about 3 × 10^{-4} M is consistent with this expectation. Thus, the change in equilibrium constant is roughly the same as the change in the dissociation rate constant, k_{off}. Since K_{eq} = k_{on}/k_{off}, this implies that k_{on} is virtually unchanged for the two different forms of complex. The association rate, k_{on}, often reflects the conformation changes which occur in protein and/or hormone in going from the free to the bound state. The constant value of k_{on} suggests that any rate limiting conformation changes which occur at low and neutral pH and which are associated with the binding interaction are very similar.

In addition to performing experiments at pH 2.5, we have also observed spectra at lower pH values (2.1 and 1.6) and we find, as did Breslow & Garguilo using tripeptide ligands, that the change in binding constant below pH 2.5 is rather slight. The binding data of Breslow & Gargiulo (1977) may be replotted according to Dixon (1953) as shown in Figure 5 to show a group which in free NP has a pK_a of 4.4, which is lowered to 2.2 in the peptide hormone–NP complex.

We believe our data presented above shows that this carboxyl group must be the group involved in the salt linkage (at neutral pH and above) to oxytocin. Breslow & Gargiulo (1977), while favoring this interpretation, raised the possibility that this group might rather trigger a conformation change of neurophysin. Our results show that (1) the conformation of the NP–hormone complex (as monitored by side chain interactions) is unchanged at low pH and (2) conformational differences between NP and the NP–hormone complex (as monitored by k_{on}) are unchanged. These observations taken together imply that the conformation of unliganded neurophysin (at least those parts of the molecule which affect hormone binding) also does not change as the pH becomes very low, thus eliminating the possibility of the carboxyl group being crucial for this conformation change. With this possibility eliminated, the group whose pK_a goes from 4.4 to 2.2 on ligand binding must be involved in a salt bridge to the N-terminal α-amino group of oxytocin.

In contrast to the increased k_{off} which is observed at low pH, experiments at high pH lead to a continual decrease in binding constant, but no apparent change in dissociation rate constant. Due to the gradual decomposition of free oxytocin which begins to occur during runs of several hours above pH 9, our experiments at high pH were rather limited. Nonetheless, the above observations are consistent with a complex which is identical with that found at neutral pH, with the decreased binding affinity due solely to the deprotonation of the α-amino group of free oxytocin. This results in a decreased concentration of protonated oxytocin, which is the species which binds to NP. Binding at high pH is therefore characterized by an apparent decreased k_{on}, and an unchanged k_{off}, in contrast to low pH binding, which displays an unchanged k_{on}, and an increased k_{off}.

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Mechanism of Action of D-Serine Dehydratase. Identification of a Transient Intermediate†

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ABSTRACT: Static absorbance measurements of D-serine dehydratase from *Escherichia coli* taken at 2 °C show that during the steady-state course of D-serine conversion the absorption maximum of the Schiff base of the cofactor pyridoxal 5′-phosphate (pyridoxal-P) is shifted from 415 to 442 nm. Furthermore, the progress curve of intermediates was monitored by stopped-flow techniques at wavelengths ranging from 320 to 500 nm. A point by point construction of successive spectra from these stopped-flow traces at various time intervals after the start of reaction resulted in a series of consecutive spectra exhibiting two isobestic points at 353 and 419 nm. The half-time of the absorbance changes occurring at 330 and 455 nm was found to be 6.5 ms, suggesting the observation of a single, enzyme-bound intermediate. The spectral data with substrate and inhibitors provide evidence that the intermediate is the Schiff base of α-aminoacrylate and pyridoxal-P. The proposed assignment is strongly supported by experiments of apodehydratase with transient-state analogues which exhibit a similar absorbance shift on binding to apoenzyme. Moreover, these results suggest that the phosphate group of the substrate–pyridoxal-P complex serves as the main anchoring point during catalysis. A reaction mechanism of the D-serine dehydratase is presented.

The de novo synthesis of D-serine dehydratase is induced upon influx of D-serine into cells of *Escherichia coli*. D-Serine is a competitive antagonist of β-alanine which serves as a substrate in the biosynthesis of pantothenate, a precursor of coenzyme A (Maas & Davis, 1950). D-Serine dehydratase (EC 4.2.1.14) from *E. coli* catalyzes the conversion of D-serine to pyruvate and ammonia (Dupourque et al., 1966; Labow & Robinson, 1966). This enzyme is unique among pyridoxal-P[1] utilizing enzymes studied thus far in that it consists of a single polypeptide chain with one active site (Dowhan & Snell, 1970a). Therefore, the dehydratase is particularly well suited for mechanistic studies without the usual problems of multiple binding sites and subunit interactions.

The absorption spectrum for highly purified D-serine dehydratase exhibits a prominent absorbance maximum at 415 nm, which is due to the cofactor, pyridoxal-P, bound to a lysyl residue of the protein via a Schiff base linkage (Dowhan & Snell, 1970a). The cofactor of this dehydratase is embedded in a polar environment as documented by ^{31}P NMR experiments. Furthermore, by use of this technique it could be shown that the phosphate group of pyridoxal-P has to be in its dianionic form to start catalysis. The formation of a salt bridge of the dianion of the cofactor with most probably an arginine residue of the dehydratase is triggered by the addition of substrate. Hence, this salt bridge now serves at least as one if not the main anchoring point of the cofactor–substrate complex (Schnackerz et al., 1979).

D-Serine dehydratase catalyzes an α,β-elimination reaction, but little is known about the intermediates occurring during catalysis. Other pyridoxal-P dependent enzymes catalyzing the same type of reaction have been studied in some detail. Among those studied were tryptophanase (Snell, 1975) and biodegradative L-threonine deaminase (Rabinowitz et al., 1973; Shizuta et al., 1973), both from *E. coli*. Addition of substrate to L-threonine deaminase caused an intermittent shift of the Schiff base absorbance maximum from 415 to 450 nm, but this transiently occurring absorbance band could not unequivocally be assigned to α-aminoacrylate–pyridoxal-P azomethine (Tokushige et al., 1968; Tokushige & Nakazawa, 1972). In the present study a series of spectroscopic, steady-state, and stopped-flow experiments are described which allow the observation of a single intermediate under the conditions applied. The structure of this intermediate is deduced from its spectral properties in conjunction with experiments of transient-state analogues with D-serine apodehydratase.

Experimental Procedures

Materials. DL-2,3-Diaminopropionate was obtained from Fluka (Buchs, Switzerland). DL-Isoserine was a product of ICN Pharmaceutical, Inc. (Cleveland, OH). DL-*O*-Methylserine was purchased from Sigma Chemical Co. (St. Louis, MO). Pyridoxal hydrochloride, pyridoxal-P, and all other amino acids were obtained from Merck (Darmstadt, West Germany). Sodium pyruvate, NADH, and lactate dehydrogenase were products of Boehringer Mannheim Corp.

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[1] Abbreviations used: NMR, nuclear magnetic resonance; pyridoxal-P, pyridoxal 5′-phosphate.

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