Acknowledgment. We thank the Central Instrumentation Facility, Department of Chemistry, Wayne State University (Director, Dr. Robin J. Hood) and, particularly, Dr. M. Ksebati, Dr. O. Mols, and M. Kempf for NMR and mass spectra. The work described herein was supported in part by U.S. Public Health Service Research Grant CA 32779 from the National Cancer Institute, Bethesda, MD, and in part by an institutional grant to the Michigan Cancer Foundation from the United Way of Southeastern Michigan.

Registry No. **4a**, 114987-18-7; **6a**, 117011-71-9; **8a**, 30955-07-8; **13a**, 104715-56-2; **14a**, 135878-73-8; **15a**, 135878-71-6; **16b**, 131489-76-4; **17b**, 135878-74-9; **18**, 135878-72-7; **18**·2Na, 139347-51-6; **19a**, 114987-15-4; **19b**, 121653-93-8; **20a**, 2715-68-6; **20b**, 130584-29-1; **25a**, 139347-53-8; **25b**, 135878-75-0; **30a**, 139347-52-7; **33a**, 139347-54-9; **35a**, 139347-55-0; **36a**, 139347-56-1; **37a**, 139347-57-2; **37a**·2Na, 139347-58-3; **39a**, 114978-79-9.

Characterization of the Macroscopic and Microscopic Acid–Base Chemistry of the Native Disulfide and Reduced Dithiol Forms of Oxytocin, Arginine-Vasopressin, and Related Peptides

Béla Noszál,† Wei Guo, and Dallas L. Rabenstein*

Department of Chemistry, University of California, Riverside, California 92521

Received September 30, 1991 (Revised Manuscript Received January 21, 1992)

Acid–base properties of the native disulfide and reduced dithiol forms of oxytocin, arginine-vasopressin, tocinoic acid, pressinoic acid, and tocinamide were studied by 500-MHz ^{1}H NMR–pH titrations. Cysteine methyl ester and cysteinylglycine served as model compounds for the N-terminal cysteine-1 residue of the reduced oligopeptides and were studied by UV, ^{1}H NMR, and pHmetry. The resonances for ^{1}H nuclei in the various peptides and the model compounds undergo the expected downfield shift upon protonation of adjacent basic sites, with the exception of the resonances for the δ CH_2 protons of the proline-7 residues of oxytocin and arginine-vasopressin, which are shifted significantly upfield, presumably reflecting conformational changes. The interacting basicities of the amino and thiolate groups of the cysteine-1 residues of the reduced dithiol forms of the five peptides and the two model compounds are characterized in terms of protonation microconstants. All other basic groups of the peptides (tyrosine phenolate, cysteine-6 thiolate of the reduced peptides, cysteine-6 carboxylate of the reduced and disulfide forms of tocinoic acid and pressinoic acid, and cysteine-1 amino of the disulfide forms of the peptides) bind protons in isolated pH ranges or are separated by several bonds from other basic groups, and their basicities are characterized in terms of group constants. Analogous groups in the various peptides show somewhat different basicities, depending on the adjacent residues. However, the basicity of the cysteine-6 thiolate of the reduced peptides covers a remarkably wide range. Specifically, the protonation constant of the cysteine-6 thiolate of the reduced forms of tocinoic acid and pressinoic acid is about 1.8 log K units larger than that of the reduced forms of oxytocin and arginine-vasopressin, due to the significantly different electron-withdrawing effects of the adjacent carboxylate and peptide groups, while the protonation constant of the cysteine-6 thiolate group of the reduced form of tocinamide is 0.6 log K units larger than that of oxytocin, even though the covalent environments are the same up to four bonds removed. Using the microscopic and group protonation constants, the probabilities of both thiol groups being in the ionized form were calculated as a function of pD for the dithiol forms of the five peptides. The results show that, at physiological pH, intramolecular disulfide bond formation via thiolate anions is predicted to be more favorable for the nonapeptides oxytocin and arginine-vasopressin than for tocinamide, which in turn is more favorable than for tocinoic acid and pressinoic acid.

Introduction

The neurohypophyseal peptide hormones oxytocin (OXT) and arginine-vasopressin (AVP) have in common disulfide bonds between cysteine residues at positions 1 and 6 which are essential for their biological activity.[1]

Cys-Tyr-X-Gln-Asn-Cys-Y

oxytocin	X = Ile	Y = Pro-Leu-Gly-NH_2
arginine-vasopressin	X = Phe	Y = Pro-Arg-Gly-NH_2
tocinoic acid	X = Ile	Y = OH
pressinoic acid	X = Phe	Y = OH
tocinamide	X = Ile	Y = NH_2

Although the mechanism of formation of the disulfide bond in the biosynthesis of OXT and AVP is not known, it presumably takes place under conditions where the two thiol groups are aligned to favor intramolecular rather than intermolecular disulfide bond formation. However, it has been found that oxidation of the two thiol groups by ferricyanide in the chemical synthesis of neurohypophyseal peptide hormones also results predominantly, but not exclusively, in the formation of intramolecular disulfide bonds,[2] even though formation of the intramolecular disulfide bond requires closure of a 20-membered ring. This is not the case for the related hexapeptides, tocinamide and pressinamide, which comprise the first six amino acids of OXT and AVP, but lack the acyclic tripeptide tail. Oxidation of the dithiol forms of these hexapeptides results predominantly in the formation of dimers and higher polymers.[2]

† Permanent address: Institute of Inorganic and Analytical Chemistry, L. Eötvös University, Budapest, Hungary.

(1) Jost, K. In *CRC Handbook of Neurohypophyseal Hormone Analogs*; Jost, K., Lebl, M., Brtnik, F., Eds.; CRC Press: Boca Raton, FL, 1987; Vol. 1, Part 2, pp 144–155.

(2) Moore, G. *Biochem. J.* 1978, *173*, 403–409.

0022-3263/92/1957-2327$03.00/0

The different tendencies of the reduced dithiol forms of the nonapeptide hormones and their hexapeptide analogues to form intra- and intermolecular disulfide bonds have been interpreted to indicate that the acyclic tripeptide tail of the dithiol forms of the nonapeptides induces a conformation in the preceding hexapeptide which favors the formation of intramolecular disulfide bonds.[2] Another possibility, however, is that the differences are simply due to differences in the redox properties of the thiol groups of the reduced peptide hormones and their hexapeptide analogs.

Direct measurement of oxidation–reduction potentials of thiols by voltammetric methods is difficult due to the formation of stable metal–thiolate complexes at the electrode surface and the concomitant electrochemical irreversibility.[3] Thus, it has been necessary to determine redox potentials of thiols by indirect methods. From such a study, we have recently found a relationship between the basicity and the oxidizability of thiolate groups for a variety of biological thiols.[4] This relationship provides the possibility of predicting the redox properties of the thiol groups of reduced neurohypophyseal peptide hormones and related peptides on the basis of thiolate basicities.

In this paper, we report the results of ^{1}H NMR studies of the acid–base chemistry of the thiolate groups of the reduced dithiol forms of OXT, AVP, tocinoic acid, pressinoic acid, and tocinamide and all other basic sites of these peptides in both their native disulfide and reduced dithiol forms. We have also characterized the acid–base properties of amino and thiolate groups of cysteine methyl ester and cysteinyl glycine as model compounds for the cysteine-1 residue of the reduced form of each of the peptides. NMR–pH titrations were carried out by 500-MHz ^{1}H NMR spectroscopy for all 12 compounds, and UV–pH titrations were used to determine the protonation microconstants of cysteine methyl ester and cysteinylglycine. Basicity values are reported here in terms of protonation microconstants for the amino and thiolate groups of the Cys[1] moieties of the reduced peptides and the model compounds and in terms of group constants for all other phenolate, amino, thiolate, and carboxylate groups.

Theory

Native OXT and AVP in their most basic forms contain N-terminal amino and tyrosine phenolate groups that can be protonated. Other groups such as the arginine guanidinium do not undergo any protonation or deprotonation reactions in the pH 1–13 range[5] studied here. The disulfide forms of tocinoic acid and pressinoic acid contain an additional carboxylate protonation site. The successive phenolate, amino, and carboxylate protonation steps take place in sufficiently separated pH ranges to characterize them in terms of independent group constants.[6,7] The extent of protonation of the phenolate, amino, and carboxylate sites was determined as a function of pD by ^{1}H NMR spectroscopy by monitoring the chemical shifts of carbon-bound protons near the protonation sites.

The reduced forms of all five peptides contain, in addition, thiolate groups at the cysteine-1 and cysteine-6 residues. Protonation of the two thiolate groups and the N-terminal amino group takes place over highly overlapping pH ranges. However, the cysteine-6 thiolate is separated by 18 and 19 bonds from the cysteine-1 amino and thiolate groups, respectively, and thus its proton-binding equilibria can be treated independently of the protonation states of the N-terminal amino and thiolate groups. On the other hand, the amino and thiolate groups of cysteine-1 are separated by only three bonds, and the protonation state of one group strongly influences the basicity of the other. The protonation equilibria of the cysteine-1 amino and thiolate groups can be characterized at the molecular level in terms of microconstants.

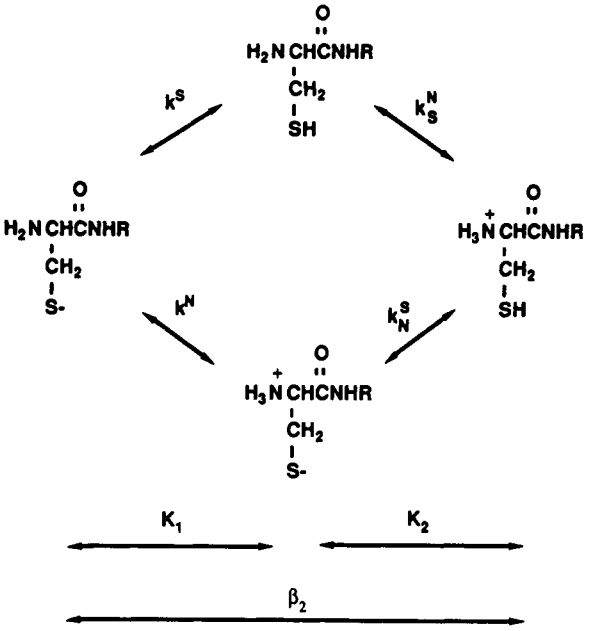


Figure 1. Protonation scheme of the cysteine-1 residue of reduced oxytocin, arginine-vasopressin, and related compounds.

Determination of Microconstants. The microscopic and macroscopic protonation equilibria of the amino and thiolate groups of the cysteine-1 residue of reduced OXT, AVP, tocinoic acid, pressinoic acid, and tocinamide and of cysteine methyl ester and cysteinyl glycine are shown in Figure 1. The S and N abbreviations stand for the thiolate and amino groups, respectively. Microconstants k^S, k^N, k^S_N, and k^N_S can be expressed in terms of microspecies and hydrogen ion concentrations, as follows:

$$k^S = \frac{[SH, NH_2]}{[S^-, NH_2][H^+]} \quad (1)$$

$$k^N = \frac{[S^-, NH_3^+]}{[S^-, NH_2][H^+]} \quad (2)$$

$$k^S_N = \frac{[SH, NH_3^+]}{[S^-, NH_3^+][H^+]} \quad (3)$$

$$k^N_S = \frac{[SH, NH_3^+]}{[SH, NH_2][H^+]} \quad (4)$$

The macroconstants K_1 and K_2 are defined in terms of the microspecies as follows:

$$K_1 = \frac{[SH, NH_2] + [S^-, NH_3^+]}{[S^-, NH_2][H^+]} \quad (5)$$

$$K_2 = \frac{[SH, NH_3^+]}{([SH, NH_2] + [S^-, NH_3^+])[H^+]} \quad (6)$$

The relationships between macroconstants K_1, K_2, and β_2

(3) Jocelyn, P. C. *Biochemistry of the SH Group*; Academic Press: New York, 1972; p 55.
(4) Keire, D. A.; Strauss, E.; Guo, W.; Noszál, B.; Rabenstein, D. L. *J. Org. Chem.* **1992**, *57*, 123–127.
(5) Noszál, B.; Kassai-Tánczos, R. *Talanta*, in press.
(6) Noszál, B. *J. Phys. Chem.* **1986**, *90*, 4104–4110.
(7) Noszál, B. Acid–Base Properties of Bioligands. In *Biocoordination Chemistry*; Burger, K., Ed.; Horwood: New York, 1990; pp 29–32.

and the microconstants are given in eqs 7 and 8.

$$K_1 = k^S + k^N \tag{7}$$

$$\beta_2 = K_1K_2 = k^Sk^N_S = k^Nk^S_N \tag{8}$$

Microscopic protonation constants can be determined if the extent of protonation of at least one of the individual interacting protonation sites can be measured as a function of pH. Microconstants have been determined for several amino thiols by combining information from two experimental techniques, one of which is always pH-metry.[8-20] The second technique has been UV,[8-14] Raman,[15] or NMR[16] spectroscopy, calorimetry,[17,18] or chemical modification of the parent compound.[19-22] UV and Raman spectroscopy, calorimetry, and chemical modification are applicable when there is no more than one thiolate group in the molecule. NMR spectroscopy can be used to monitor protonation of individual groups when there is at least one NMR nucleus, whose chemical shift is influenced exclusively by the protonation state of a single protonation site, for each of the overlapping protonation equilibria.[23] In the case of the reduced forms of the peptides studied here, the coexistence of two thiolate groups and the proximity of the cysteine-1 thiolate and amino sites prevents the direct use of any of the above methods.

We have characterized the microscopic protonation equilibria of the cysteine-1 amino and thiolate groups by an experimental approach involving the measurement of NMR chemical shift–pH titration data for the dithiol forms of the peptides and NMR chemical shift–UV–pH titration data for the model compounds. Protonation of the cysteine-1 amino and thiolate groups results in composite protonation shifts at adjacent NMR-active nuclei. The relationship between the mole fractions of the cysteine-1 thiolate and amino groups which are protonated (α_S and α_N, respectively) and the observed protonation shift at a given pH ($\Delta\delta_{pH}$) can be written as[24]

$$\Delta\delta_{(pH)} = \alpha_S\kappa_S + \alpha_N\kappa_N \tag{9}$$

where κ_S and κ_N are the protonation shift coefficients. Implicit in this equation is that κ_S and κ_N are independent of the protonation states of the cysteine-1 amino and thiolate groups, respectively. α_S and α_N can be expressed in terms of the concentrations of the various microspecies:

$$\alpha_S = \frac{[SH, NH_2] + [SH, NH_3^+]}{[S^-, NH_2] + [S^-, NH_3^+] + [SH, NH_2] + [SH, NH_3^+]} \tag{10}$$

$$\alpha_N = \frac{[S^-, NH_3^+] + [SH, NH_3^+]}{[S^-, NH_2] + [S^-, NH_3^+] + [SH, NH_2] + [SH, NH_3^+]} \tag{11}$$

Introducing eqs 1–5, 7, and 8 into (10) and (11) yields:

$$\alpha_S = \frac{k^S[H^+] + \beta_2[H^+]^2}{1 + K_1[H^+] + \beta_2[H^+]^2} \tag{12}$$

$$\alpha_N = \frac{k^N[H^+] + \beta_2[H^+]^2}{1 + K_1[H^+] + \beta_2[H^+]^2} \tag{13}$$

Substitution of eqs 12 and 13 into eq 9 yields:

$$\Delta\delta_{(pH)} = \frac{k^S[H^+] + \beta_2[H^+]^2}{1 + K_1[H^+] + \beta_2[H^+]^2}\kappa_S + \frac{k^N[H^+] + \beta_2[H^+]^2}{1 + K_1[H^+] + \beta_2[H^+]^2}\kappa_N \tag{14}$$

Macroconstants K_1, K_2, and β_2 can be determined directly by pH-metry or from NMR chemical shift–pH titration data.[25] Thus, one of the microconstants can be eliminated by using eq 7:

$$k^N = K_1 - k^S \tag{15}$$

Similarily, one of the protonation shift coefficients can be eliminated using eq 16 where $\Delta\delta_{max}$ is the observed change in chemical shift upon complete protonation of the thiolate and amino groups. After these substitutions are made,

$$\kappa_N = \Delta\delta_{max} - \kappa_S \tag{16}$$

the number of unknown parameters in eq 14 is two. However, even though two or more equations can be set up by measuring $\Delta\delta_{(pH)}$ at two or more pH values over the pH range where the cysteine-1 amino and thiolate groups are protonated, it is not possible to determine the two unknown parameters simultaneously from the chemical shift–pD data because they are codependent, highly correlated parameters. Thus, either microconstant can be determined if the protonation shifts are known or the protonation shifts can be determined if the microconstants are known. We therefore used cysteinylglycine as a model compound to determine the protonation shifts. Cysteinylglycine contains amino and thiolate groups, analogous to the N-terminal end of the reduced peptide hormones and their analogs, but it lacks the perturbing UV-active side chains, e.g., the second thiol group of the peptides. Thus, microconstants for cysteinylglycine were determined by the Benesch method, which combines data from UV spectrometry and pH-metry.[8] κ_S and κ_N values were then calculated using the NMR chemical shift–pH titration data and the macroconstants and microconstants for cysteinylglycine. These values were used as described below for determination of the microconstants of the amino and thiolate groups of the cysteine-1 residue of the reduced peptides. The similarity of the κ_S and κ_N parameters of the model compound and the peptides was verified by comparison of $\Delta\delta_{max}$ values and NMR–pH profiles of the model compound and the parent compounds.

(8) Benesch, R. E.; Benesch, R. *J. Am. Chem. Soc.* **1955**, *77*, 5877–5881.
(9) Coates, E.; Marsden, C. G.; Rigg, B. *J. Chem. Soc., Faraday Trans.* **1969**, *65*, 3032–3036.
(10) Clement, G. E.; Hartz, T. P. *J. Chem. Educ.* **1971**, *48*, 395–397.
(11) Reuben, D. M. E.; Bruice, T. C. *J. Am. Chem. Soc.* **1976**, *98*, 114–121.
(12) Tanaka, H.; Sakurai, H.; Yokoyama, A. *Chem. Pharm. Bull.* **1970**, *18*, 1015–1020.
(13) Wilson, E. W.; Martin, R. B. *Arch. Biochem. Biophys.* **1971**, *142*, 445–454.
(14) Noszál, B.; Guo, W.; Rabenstein, D. L. *J. Phys. Chem.* **1991**, *95*, 9609–9614.
(15) Elson, E. L.; Edsall, J. T. *Biochemistry* **1962**, *1*, 1–7.
(16) Rabenstein, D. L. *J. Am. Chem. Soc.* **1973**, *95*, 2797–2803.
(17) Wrathall, D. P.; Izatt, R. M.; Christensen, J. J. *J. Am. Chem. Soc.* **1964**, *86*, 4779–4783.
(18) Jagt, D. L. W.; Hansen, L. D.; Lewis, E. A.; Han, L. B. *Arch. Biochem. Biophys.* **1972**, *153*, 55–61.
(19) Ryklan, L. R.; Schmidt, C. L. A. *Arch. Biochem.* **1944**, *5*, 89–98.
(20) Grafius, M. A.; Neilands, J. B. *J. Am. Chem. Soc.* **1955**, *77*, 3389–3390.
(21) Martin, R. B.; Edsall, J. T. *Bull. Soc. Chim. Biol.* **1958**, *40*, 1763–1771.
(22) Walters, D. C.; Leyden, D. E. *Anal. Chim. Acta* **1974**, *72*, 275–283.
(23) Rabenstein, D. L.; Sayer, T. L. *Anal. Chem.* **1976**, *48*, 1141–1146.
(24) Sudmeier, J. L.; Reilley, C. N. *Anal. Chem.* **1964**, *36*, 1698–1706.
(25) Rabenstein, D. L.; Greenberg, M. S.; Evans, C. A. *Biochemistry* **1977**, *16*, 977–981.

Table I. Macroscopic or Group Protonation Constants of the Native Disulfide and Reduced Dithiol Forms of Neurohypophyseal Peptide Hormones and Related Compounds[a-c]

residue	protonation constant	Cys O Me	Cys Gly	oxytocin		AVP		tocinoic acid		pressinoic acid		tocinoic amide	
				disulfide	dithiol	disulfide	dithiol	disulfide	dithiol	disulfide	dithiol	disulfide	dithiol
Cys[1]	log K_1	9.45	9.85	6.95	9.47	6.92	9.30	7.21	9.54	7.17	9.38	7.17	9.46
	log K_2	7.18	7.58		7.16		7.02		7.13		7.04		7.06
Tyr[2]	log K (phenolate)			10.40	10.69	10.47	10.70	10.58	10.74	10.56	10.75	10.49	10.79
Cys[6]	log K (thiolate)				8.59		8.65		10.42		10.35		9.26
	log K (carboxylate)							3.39	3.44	3.33	3.45		
Gly[2]	log K (carboxylate)		3.64										

[a] Abbreviations: Cys O Me, cysteine methyl ester; Cys Gly, cysteinyl glycine; AVP, arginine-vasopressin. [b] D_2O solution, 25 ± 2 °C, and I = 0.2–0.3 M. [c] Uncertainties are estimated to be 0.02–0.05 log K units.

Results

Protonation constants for the various basic sites in the native disulfide and reduced dithiol forms of the five peptides were determined from ^1H chemical shift–pD titration data. ^1H NMR spectra of the native disulfide and reduced dithiol forms of pressinoic acid, tocinoic acid, OXT, and AVP have been assigned previously,[26] and the assignments were confirmed in this work. ^1H NMR spectra of the thiol and disulfide forms of tocinamide were assigned in this work. In most cases, protonation constants for the amino, thiolate, and carboxylate groups of the disulfide and reduced dithiol forms of the peptides were determined using chemical shift data measured as a function of pD for the α-CH protons of the cysteine-1 and cysteine-6 residues.[27] The multiplets for the α-CH protons are the X part of ABX patterns and are generally sufficiently well resolved at 500 MHz that chemical shifts can be accurately measured, as illustrated in Figure 2 by the α-proton regions of the 500-MHz ^1H NMR spectra of the disulfide and dithiol forms of tocinoic acid. The two spectra also show that reduction of the disulfide bond results in significant changes in the chemical shifts of the α-CH resonances, particularly those of cysteine-1 (3.626 ppm to 3.236 ppm) and cysteine-6 (4.350 ppm to 4.023 ppm).

The data used to determine protonation constants are illustrated by the chemical shift–pD titration curves for the dithiol and disulfide forms of tocinoic acid in Figure 3. The chemical shift of the tyrosine 3,5 protons of the disulfide and dithiol forms of tocinoic acid is essentially constant, except over the pD range 12–9 where the phenolate group undergoes protonation. The major change in chemical shift of the cysteine-1 α-CH resonance of the disulfide form is over the pD range 9–5 where the cysteine-1 amino group is protonated, while the major change in chemical shift of the cysteine-6 α-CH resonance is over the pD range 5–1 where the cysteine-6 carboxylate group is protonated. It is interesting to note, however, that the chemical shift of the cysteine-6 α-CH resonance also changes as the cysteine-1 amino group is protonated, and there is a very small change in the chemical shift of the cysteine-1 α-CH resonance upon protonation of the carboxylate group, both presumably due to decreased shielding from electronic effects transmitted through the disulfide bond. Both of these chemical shift changes are absent from the chemical shift titration curves for the reduced peptide.

The cysteine-6 α-CH resonance for the reduced peptide shows two well-separated titration shifts, corresponding to protonation of the cysteine-6 thiolate and carboxylate groups. The chemical shift of the cysteine-1 α-CH resonance shows more complicated behavior, having the appearance of two overlapping titration shifts. This is due to the protonation of either the amino or the thiolate group of the cysteine-1 residue to form a monoprotonated species in the first titration step, followed by protonation of the other group to give the second titration shift. Because the basicity of the cysteine-1 amino and thiolate groups is similar, the two groups protonate over the same pD range (Figure 1), and thus the first and second protonation shifts cannot be assigned to protonation of specific groups. Protonation of one group decreases the basicity of the second group sufficiently that the second protonation takes place over a lower pD range.

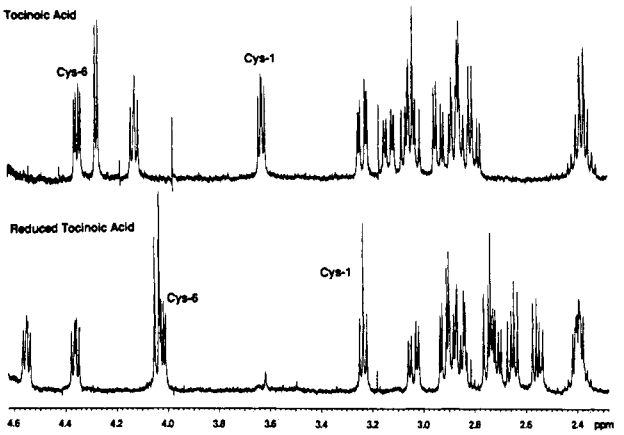


Figure 2. α proton region of the 500-MHz ^1H NMR spectrum of a 1 mM solution of tocinoic acid before and after addition of an excess of DTT-d_{10}. The pD of the solutions was 11.66 and 11.59, respectively.

Specific (group) protonation constants for the tyrosine phenolate and the cysteine-6 carboxylate groups of the disulfide and dithiol forms of tocinoic acid and for the amino group of the disulfide form were obtained directly from the chemical shift–pD titration data in Figure 3. The results are reported in Table I. The chemical shift–pD titration curve for the cysteine-1 α-CH of reduced tocinoic acid was fitted to a diprotic acid model[25] to determine the macroscopic constants K_1, K_2, and β_1 (Figure 1) for the cysteine-1 amino and thiolate groups. These constants are also reported in Table I.

The protonation constants listed in Table I for the disulfide and dithiol forms of the other peptides and the macroscopic constants for cysteine methyl ester and cysteinyl glycine were determined from similar chemical shift titration data, with the following exceptions. The protonation constant for the carboxylate group of the dithiol form of pressinoic acid was determined from the chemical shift titration curve of one of the cysteine-6 β-CH_2 protons because the resonance for the cysteine-6 α-CH proton shifts under the HDO resonance upon protonation of the carboxylate group. Protonation of the cysteine-6 thiolate group of reduced OXT and AVP also causes the resonance

(26) Larive, C. K.; Rabenstein, D. L. unpublished results.
(27) The resonances for the β-CH_2 protons of the cysteine-1 and cysteine-6 residues undergo larger changes in chemical shift upon protonation of the thiolate groups; however, they are more complicated multiplets (the AB part of ABX patterns) and are generally overlapped with our resonances.

Table II. Macro- and Microscopic Protonation Constants and Amino–Thiolate Interactivity Parameters for the Amino and Thiolate Groups of the Cysteine-1 Residue of Reduced Neurohypophyseal Peptide Hormones and Related Compounds[a-c]

	Cys O Me	Cys Gly	oxytocin dithiol	AVP dithiol	tocinoic acid dithiol	pressinoic acid dithiol	tocinoic amide dithiol
log K_1	9.45	9.85	9.47	9.30	9.54	9.38	9.46
log K_2	7.18	7.58	7.16	7.02	7.13	7.04	7.06
log k^S	9.30	9.72	9.35	9.18	9.41	9.24	9.31
log k^N	8.92	9.26	8.87	8.69	8.97	8.84	8.91
log k^S_N	7.71	8.17	7.76	7.63	7.71	7.58	7.61
log k^N_S	7.33	7.71	7.28	7.14	7.26	7.19	7.21
Δ log k_{N-S}	1.59	1.55	1.59	1.55	1.70	1.66	1.70

[a] Abbreviations: Cys O Me, cysteine methyl ester; Cys Gly, cysteinyl glycine; AVP, arginine-vasopressin. [b] D_2O solution, 25 ± 2 °C, and I = 0.2–0.3 M. [c] Uncertainties are estimated to be 0.04–0.1 log k units.

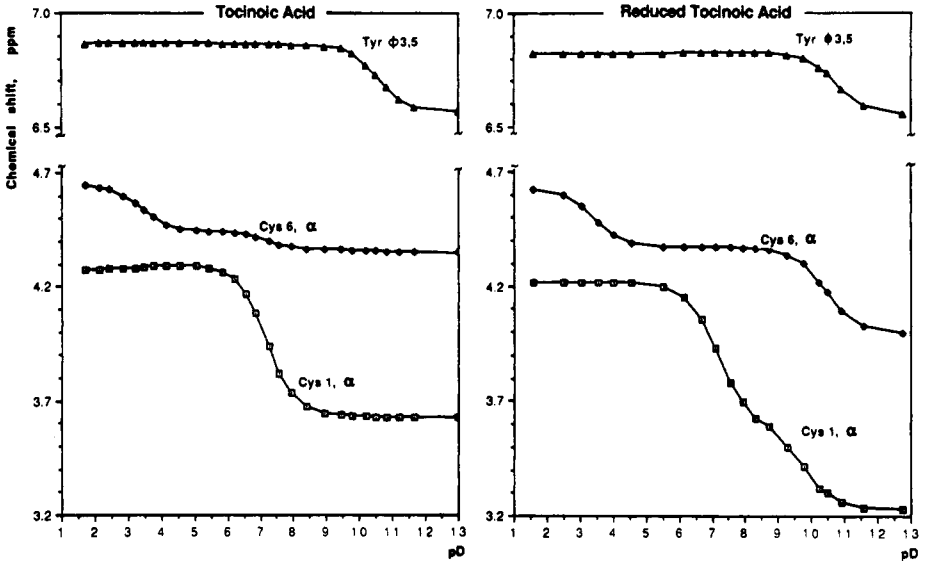


Figure 3. Chemical shifts of the 3,5 protons of the aromatic ring of tyrosine and the cysteine-1 and cysteine-6 α-CH protons of tocinoic acid and reduced tocinoic acid as a function of pD.

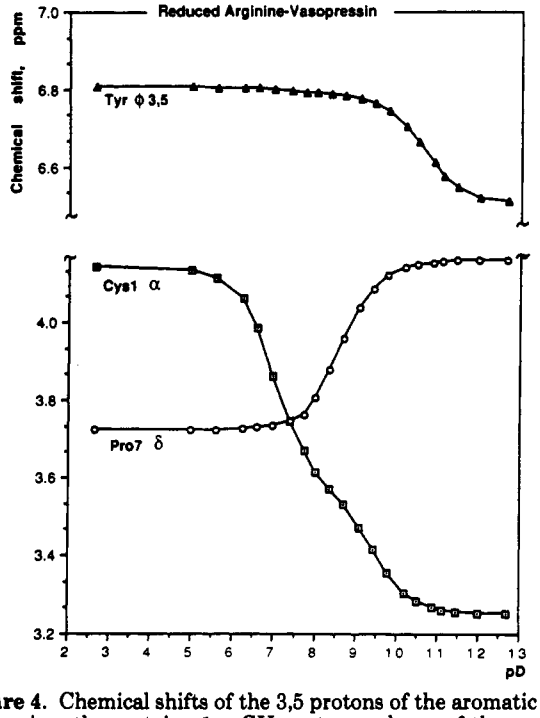


Figure 4. Chemical shifts of the 3,5 protons of the aromatic ring of tyrosine, the cysteine-1 α-CH proton and one of the proline δ-CH_2 protons of reduced AVP as a function of pD.

for the cysteine-6 α-CH to shift under the HDO resonance. Protonation constants for the cysteine-6 thiolate groups of reduced OXT and AVP were determined using the resonance for the proline δ-CH_2 protons, which undergoes an unusual upfield shift upon protonation of the cysteine-6 thiolate group (Figure 3). The occurence and magnitude (~0.45 ppm) of this shift suggests a significant change in the conformation of OXT and AVP when their cysteine-6 thiolate groups are protonated.

Microconstants were determined for the cysteine-1 thiolate and amino groups of the dithiol forms of the five peptides using eqs 14–16. The values used for the protonation shifts κ_S and κ_N were based on the thiolate and amino protonation shifts determined for the cysteine α-CH resonance of cysteinyl glycine.[28,29] Microconstants for the dithiol forms of the five peptides, together with microconstants determined from UV–pD data for cysteinyl glycine and cysteine methyl ester, are reported in Table II.[30]

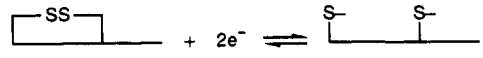
(28) Ester, amide, and peptide bonds influence similarly the amino and side-chain basicities in amino acids and derivatives. Ebert, L. *Z. Physik. Chem.* **1926**, *121*, 385–400. Noszál, B.; Sándor, P. *Anal. Chem.* **1989**, *61*, 2631–2637.

(29) Microscopic protonation constants for the cysteine amino and thiolate groups of cysteinyl glycine were determined in D_2O solution by the Benesch method.[8] Protonation shifts for the thiolate and amino groups were then determined from cysteine α-CH chemical shift data for cysteinyl glycine using these microconstants, the macroconstants determined from the chemical shift data (Table I), and eqs 14–16. The values (κ_S = 0.228 ppm and κ_N = 0.682 ppm) were proportionally adjusted to total the experimental $\Delta\delta_{max}$ for each of the reduced peptides.

(30) The uncertainty in the microconstants is estimated to be 0.04–0.1 log k units. The microconstants for cysteinyl glycine and cysteine methyl ester have the lowest uncertainties because protonation of their thiolate groups could be monitored directly by UV spectroscopy. Although the peptide bond of cysteinyl glycine is UV-active at 234 nm, the thiolate absorption maximum and both cysteinyl glycine and cysteine methyl ester UV peaks show much less pH dependence than cysteine, homocysteine, penicillamine, and other amino thiols with free carboxylate groups.[8-14]

Discussion

A major objective of this research has been to determine if the different tendencies of the reduced peptide hormones and their hexapeptide analogues to form intramolecular vs intermolecular disulfide bonds can be accounted for in terms of intrinsic differences in the redox properties of their thiol groups. As discussed above, redox potentials of thiols cannot be measured directly by voltammetric methods.[3] Thus, our plan was to estimate their redox potentials using a relationship between the redox potential for the reaction $RSSR + 2e^- \rightleftharpoons 2RS^-$ and thiolate basicity.[4] However, we find, as indicated by the results in Tables I and II, that the basicities of the two thiolate groups in each of the reduced peptides are quite different and thus the basicity–redox potential relationship cannot be used to estimate redox potentials for the redox reactions of these peptides:



(17)

We can, however, still draw conclusions about their relative tendencies to form intramolecular disulfide bonds from the results in Tables I and II. Oxidation of thiol groups takes place via thiolate anions,[31,32] and thus the redox properties of thiol groups are strongly pH(pD) dependent.[4] The fractional concentrations of the cysteine-1 and cysteine-6 thiol groups in the reactive thiolate form can be calculated using eqs 18 and 19 and the protonation constants in Tables I and II. The fractions calculated for

$$\alpha_{S^-(Cys1)} = \frac{[S^-, NH_2] + [S^-, NH_3^+]}{[S^-, NH_2] + [S^-, NH_3^+] + [SH, NH_2] + [SH, NH_3^+]} = \frac{1 + k^N[H^+]}{1 + K_1[H^+] + \beta_2[H^+]^2} \quad (18)$$

$$\alpha_{S^-(Cys6)} = \frac{[S^-]}{[S^-] + [SH]} = \frac{1}{1 + K_T[H^+]} \quad (19)$$

reduced oxytocin, reduced tocinamide, and reduced tocinoic acid are plotted as a function of pD in Figure 5. The probability P of both thiol groups being in the reactive thiolate form is also plotted in Figure 5.

The dependence of the fractional concentration of the cysteine-1 thiolate on pD is similar for all three peptides. However, that for the cysteine-6 thiolate is quite different, with the fractional concentration at a given pD increasing in the order reduced oxytocin > reduced tocinamide > reduced tocinoic acid. The probability of both thiol groups being in the reactive thiolate form increases in the same order. Thus, these results suggest that the greater tendency of reduced OXT and AVP, as compared to reduced tocinamide and pressinamide, to form intramolecular vs intermolecular disulfide bonds[2] is due, in part at least, to the higher fractional concentrations of reduced OXT and AVP in the reactive dithiolate form.

It is of interest to also note other interesting aspects of the acid–base chemistry of the peptides studied in this work. The protonation constants in Tables I and II for the native disulfide and reduced dithiol forms of OXT, AVP, tocinoic acid, pressinoic acid, and tocinamide are the first to be reported which characterize the acid–base chemistry of these peptides at the molecular level. With these constants, the concentrations of specific protonated forms can be calculated. For example, the calculated

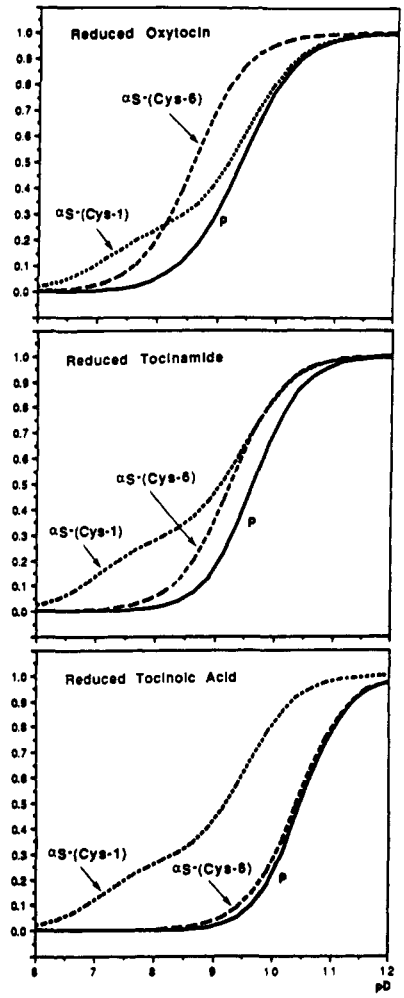


Figure 5. Fraction of the cysteine-1 (α_{S^-Cys1}) and cysteine-6 (α_{S^-Cys6}) thiol groups in the thiolate form and the probability (P) of both thiol groups of reduced oxytocin, tocinamide, and tocinoic acid being in the thiolate form as a function of pD. The analogous diagrams for reduced arginine-vasopressin and reduced pressinoic acid are similar to the diagrams for reduced oxytocin and reduced tocinoic acid, respectively.

fractional concentrations of the various protonated forms of native and reduced OXT are plotted vs pD in Figure 6. The species distribution curves for OXT show that protonation of the tyrosine phenolate is essentially complete before the amino group is protonated, and thus the acid–base chemistry of OXT is quite simple. However, the species distribution curves for the reduced form of OXT show that its acid–base chemistry is somewhat more complex, with nine protonated species possible (Figure 7).

The excellent fit of the pD 8–12 region of the cysteine-6 α-CH chemical shift titration curve for reduced OXT, and for the reduced forms of the other four peptides, to a model involving a single protonation constant indicates that the basicity of the cysteine-6 thiolate group is not affected by the protonation states of the tyrosine phenolate and cysteine-1 amino and thiolate groups. However, this is not the case for the cysteine-1 thiolate and amino groups. The microconstants in Table I indicate that the basicity of the cysteine-1 thiolate group decreases significantly upon protonation of the amino group, and vice versa. The dependence of the basicity of one group on the protonation state of the other group can be expressed quantitatively in terms of the amino–thiol interactivity parameter $\Delta\log k_{N\text{-}S}$.

$$\Delta\log k_{N\text{-}S} = \log k^S - \log k_N^S = \log k^N - \log k_S^N \quad (20)$$

(31) Wallace, C. J.; Schriesheim, A.; Bartole, W. *J. Org. Chem.* **1963**, *28*, 1311–1314.
(32) Wallace, C. J.; Schriesheim, A. *J. Org. Chem.* **1962**, *27*, 1514–1516.

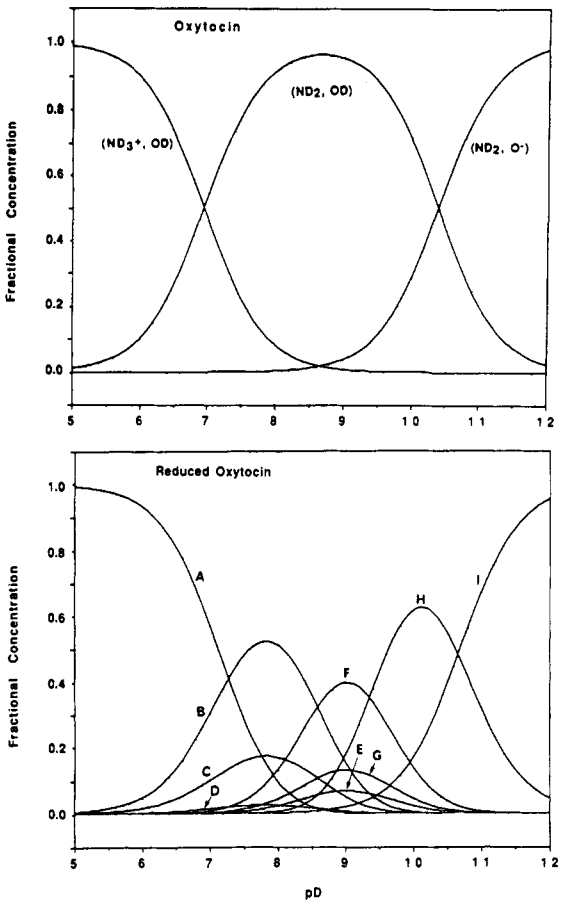


Figure 6. Fractional concentrations of the various protonated forms of oxytocin and reduced oxytocin as a function of pD. The protonated forms of reduced oxytocin are identified in Figure 7. The fractional concentrations were calculated using the protonation constants in Tables I and II.

The interactivity parameter is similar for the seven compounds studied in this work (Table II), and thus the effect of protonation of the amino group on the basicity of the cysteine-1 thiolate group is essentially the same even though the basicities of their cysteine-1 thiolate groups are different. Also, for the seven compounds studied here, the cysteine-1 thiolate group is more basic than the amino group (i.e., log k^S > log k^N and log k^S_N > log k^N_S). This is not the case, however, for the amino thiols cysteine, homocysteine, cysteamine, and penicillamine,[7-22] which indicates that electronic effects associated with formation of a peptide (or ester) bond are much greater at the amino group than at the thiol group of N-terminal cysteine residues.

The effect of peptide bond formation on thiolate basicity is also evident in the protonation constants for the cysteine-6 thiolate group. For OXT and AVP, where the peptide chain continues with a tripeptide tail, the log K of the cysteine-6 thiolate group is near 8.6. For tocinoic and pressinoic acids, where cysteine-6 is the C-terminal amino acid and a carboxylate group is adjacent to the thiolate group, the basicity of the cysteine-6 thiolate group is some 1.8 log K units higher due to the significantly different electron-withdrawing effect of the carboxylate group. Because of similarity in the immediate covalent environment, log K for the cysteine-6 thiolate of reduced tocinamide is expected to be close to those for OXT and AVP. Remarkably, however, log K for the cysteine-6 thiolate of reduced tocinamide is ~0.65 units larger, indicating that the tripeptide tail in reduced OXT and AVP is a significant modifying factor of the cysteine-6 thiolate basicity.

The species distribution diagram in Figure 6 for reduced OXT reveals several other interesting features about the acid–base chemistry of OXT at the molecular level. For example, the basicity (Tables I and II) of the three possible protonation sites of monoprotonated species H is in the order cysteine-1 thiolate > cysteine-1 amino > cysteine-6 thiolate, and thus the relative populations of the diprotonated species are in the order F > G > E. However, because of the interactivity between cysteine-1 amino and thiolate groups, the relative basicities of the basic sites in the diprotonated species E–G are significantly different (cysteine-6 thiolate > cysteine-1 thiolate > cysteine-1 amino) and thus the relative populations of the triprotonated species are B > C > D. Species distributions of the type shown in Figure 6 for reduced OXT will be of use in studies of their solution chemistry, e.g., in studies to elucidate the mechanism of oxidation of reduced neurohypophyseal peptide hormones by thiol/disulfide interchange reactions.

Experimental Section

Oxytocin, arginine-vasopressin, tocinoic acid, and pressinoic acid were obtained in their native disulfide forms from Bachem

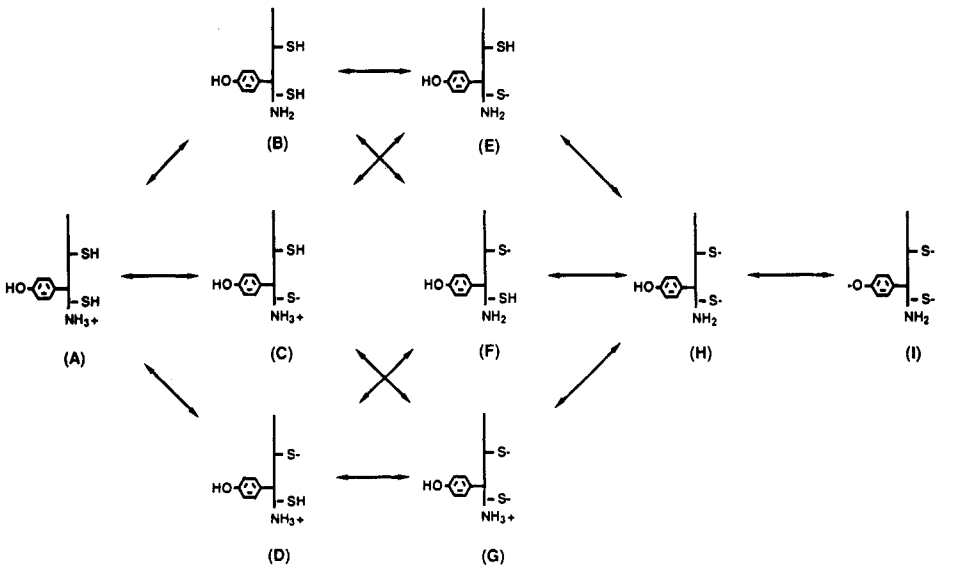


Figure 7. Protonated forms of reduced oxytocin.

Inc. The disulfide form of tocinamide was supplied by Coast Scientific Products. The reduced dithiol forms of the peptides were produced in situ by reduction of the disulfides with D,L-1,4-dithiothreitol-d_{10} (DTT-d_{10}) (MSD Isotopes). Typically, reduction was complete within 10 min after addition of DTT-d_{10} to a pD 12–13.2 solution of disulfide. Because of their susceptibility to air oxidation, the dithiols were prepared and the pD of their solutions was adjusted for NMR measurement in the oxygen-free atmosphere of an air-tight glovebox. NMR samples were capped and sealed with Parafilm before removing them from the glovebox. L-cysteinylglycine and L-cysteine methyl ester were products of Research Plus Inc. and Fluka, respectively. D_2O was obtained from ICON Services Inc.; DCl and KOD were supplied by Sigma Chemical Co. All chemicals were used without further purification.

^1H NMR spectra were measured at 500 MHz. The residual HDO resonance was suppressed by presaturation with the decoupler channel. Probe temperature was 25 °C, and chemical shifts were measured relative to internal standard *tert*-butyl alcohol (1.2366 ppm). The thiolate protonation equilibria of cysteine methyl ester and cysteinylglycine were characterized by measuring their absorbance at 254 nm as a function of pD.

All protonation constants were determined in D_2O, at an ionic strength of 0.2–0.3 M (KCl) and 25 ± 2 °C. The peptide concentration was 1–3 mmol dm^{-3}. pD was adjusted by addition of DCl or KOD titrant solution; the titrant solution concentrations were between 0.1 and 0.3 mol dm^{-3}. pH electrodes were calibrated with Fisher pH = 4.000 and pH = 9.000 H_2O standard solutions. pH meter readings for D_2O solutions were converted to pD using the relationship pD = pH meter reading +0.40.[33]

Acknowledgment. This research was supported by National Institutes of Health Grant GM 37000. The NMR instrumentation was supported in part by BRSG 2 S07 RR07010-20 awarded by Biomedical Research Resources, National Institutes of Health.

(33) Glasoe, P. K.; Long, F. A. *J. Phys. Chem.* **1960**, *64*, 188–190.

Synthesis of Acylhydrazido-Substituted Cephems. Design of Cephalosporin–Vinca Alkaloid Prodrugs: Substrates for an Antibody-Targeted Enzyme

Louis N. Jungheim,* Timothy A. Shepherd, and Damon L. Meyer†

Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, Indiana 46285, and Therapeutics Division, Hybritech Inc., San Diego, California 92121

Received November 15, 1991

Cephalosporin **20** substituted at the C-3′ position with the potent oncolytic agent desacetylvinblastine hydrazide (**3**) was synthesized as a potential prodrug for the treatment of solid tumors. The design of this novel prodrug was based on the knowledge that hydrolysis of a cephalosporin's β-lactam bond can result in the expulsion of the C-3′ substituent. Proper selection of the linkage used to join the cephem to the vinca, e.g., **8** vs **20**, provided a releasable form of the drug as well as a chemically stable prodrug. We envisioned the conversion of prodrug to free vinca to be mediated by an immunoconjugate, consisting of a β-lactamase enzyme covalently attached to a monoclonal antibody, which has been prelocalized at the tumor. Treatment of candidate prodrugs with the P99 β-lactamase enzyme isolated from *Enterobacter cloacae* 265A efficiently catalyzed their conversion to the free drug form. A study of model compounds **11** and **18** indicated that cephem 1-β-sulfoxide **18** was a better substrate for the enzyme than its sulfide counterpart **11**. This finding prompted the synthesis of cephem sulfoxide **20** which was efficiently accomplished via condensation of desacetylvinblastine hydrazide with the cephalothin derived cephem 3′-*p*-nitrophenyl carbonate **15**.

Introduction

The targeting of oncolytic agents to tumor cells using monoclonal antibody (MoAb)-drug conjugates has received considerable attention in recent years.[1] Utilizing the ability of the MoAb to recognize and bind to specific tumor associated antigens, a cytotoxic agent covalently bound to the MoAb may exhibit both antitumor activity and decreased toxicity to nontargeted tissues. An alternative two-step approach has been reported in which radiopharmaceuticals, cytotoxic agents, or hapten-modified cytotoxic agents are localized at tumor targets by prelocalization of a bifunctional antibody (a MoAb with affinity for both a tumor antigen and a small molecule) followed by administration of the small molecule.[2] We have reported[3] another two-step approach[4] which was based upon the insight gained from work on bifunctional antibodies as well as covalent MoAb-cytotoxic agent constructs. Our system employs an enzyme covalently bound to a MoAb which localizes on the targeted tumor cell surface. Subsequent administration of a prodrug (which is a substrate of the enzyme) allows for the specific enzyme-catalyzed release of the cytotoxic agent at the tumor site, as depicted

† Hybritech Inc.

(1) Koppel, G. A. *Bioconj. Chem.* **1990**, *1*, 13.

(2) Stickney, D. R.; Slater, J. B.; Kirk, G. A.; Ahlem, C.; Chang, C.-H.; Frinke, J. M. *Antibody, Immunoconjugates, Radiopharm.* **1989**, *2*, 1. Corvalan, J. R. F.; Smith, W.; Gore, V. A.; Brandon, D. R. *Cancer Immunol. Immunother.* **1987**, *24*, 133.

(3) (a) Shepherd, T. A.; Jungheim, L. N.; Meyer, D. L.; Starling, J. J. *Biomed. Chem. Lett.* **1991**, *1*, 21. (b) A preliminary account of this work has been disclosed: Jungheim, L. N.; Meyer, D. L.; Shepherd, T. A.; Starling, J. J.; Hinson, N. A.; Mikolajczyk, S. D.; Parr, T. R. Jr. Proceedings of the 200th National Meeting of the American Chemical Society, Washington, DC, Aug, 1990; Med. Chem. Abstr. 141.

(4) In the course of this work several publications have appeared which also describe antibody–enzyme/prodrug approaches to drug targeting: Bagshawe, K. D. *Br. J. Cancer* **1989**, *60*, 275 and references cited therein. Springer, C. J.; Antoniw, P.; Bagshawe, K. D.; Searle, F.; Bisset, G. M. F.; Jarman, M. *J. Med. Chem.* **1990**, *33*, 677. Senter, P. D. *FASEB* **1990**, *4*, 188. Senter, P. D.; Schreiber, G. J.; Hirschberg, D. L.; Ashe, S. A.; Hellstrom, K. E.; Hellstrom, I. *Cancer Res.* **1989**, *49*, 5789 and references cited therein. Keufner, U.; Lohrmann, U.; Montejano, Y. D.; Vitols, K. S.; Huennekens, F. M. *Biochemistry* **1989**, *28*, 2288. Senter, P. D.; Saulnier, M. G.; Schreiber, G. J.; Hirschberg, D. L.; Brown, J. P.; Hellstrom, I.; Hellstrom, K. E. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 4842. For an alternative cephalosporin based approach to ADC see: Alexander, R. P.; Beeley, N. R. A.; O'Driscoll, M.; O'Neill, F. P.; Millican, T. A.; Pratt, A. J.; Willenbrock, F. W. *Tetrahedron Lett.* **1991**, *32*, 3269.

0022-3263/92/1957-2334$03.00/0