reductase from trimethoprim-resistant strains.

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Studies on Prolactin. Selective Reduction of the Disulfide Bonds of the Ovine Hormone†

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ABSTRACT: Methods for selective reduction of the disulfide bonds in ovine prolactin are reported. Cleavage of all three disulfide bonds abolishes biological activity and denatures the hormone. Reduction–carbamidomethylation of one or two of the disulfide bridges does not diminish the biological activities in the pigeon crop-sac and mouse mammary gland bioassays. When compared to the native hormone, monomers of these two partially reduced–carbamidomethylated derivatives also show only modest changes in properties measured by exclusion chromatography, circular dichroism, and immunological cross-reactivities. However, cleavage of cystine-4–11 and cystine-191–199, followed by carbamidomethylation, destroys the biological activity of this derivative in a teleost fish bioassay (*Gillichthys* urinary bladder). In contrast, reduction of cystine-4–11 actually increased the teleost potency of this derivative compared to the intact hormone. Since teleost prolactin appears to lack a homologue to the cystine-4–11 disulfide bond in the amino-terminal loop of the ovine hormone, selective reduction of this bond in ovine prolactin may produce a derivative whose properties more closely resemble the fish hormone.

Mammalian pituitary prolactins and somatotropins are single-chain proteins which possess homologous amino acid residues at 55–60% of their sequence positions (Bewley & Li, 1969; Niall et al., 1971; Bewley et al., 1972). Similarities in the sequences of prolactins and somatotropins indicate a separate evolution of these proteins from a common ancestral peptide.

Despite sequence homology, certain conserved structural features distinguish prolactins from somatotropins (and from their closely allied placental homologues, the chorionic somatomammotropins; Bewley et al., 1972). With regard to primary structure, prolactins possess three disulfide bonds (Figure 1) (Li et al., 1970; Li, 1976). In contrast, mammalian growth hormones have two disulfide bonds (Li & Dixon, 1971;

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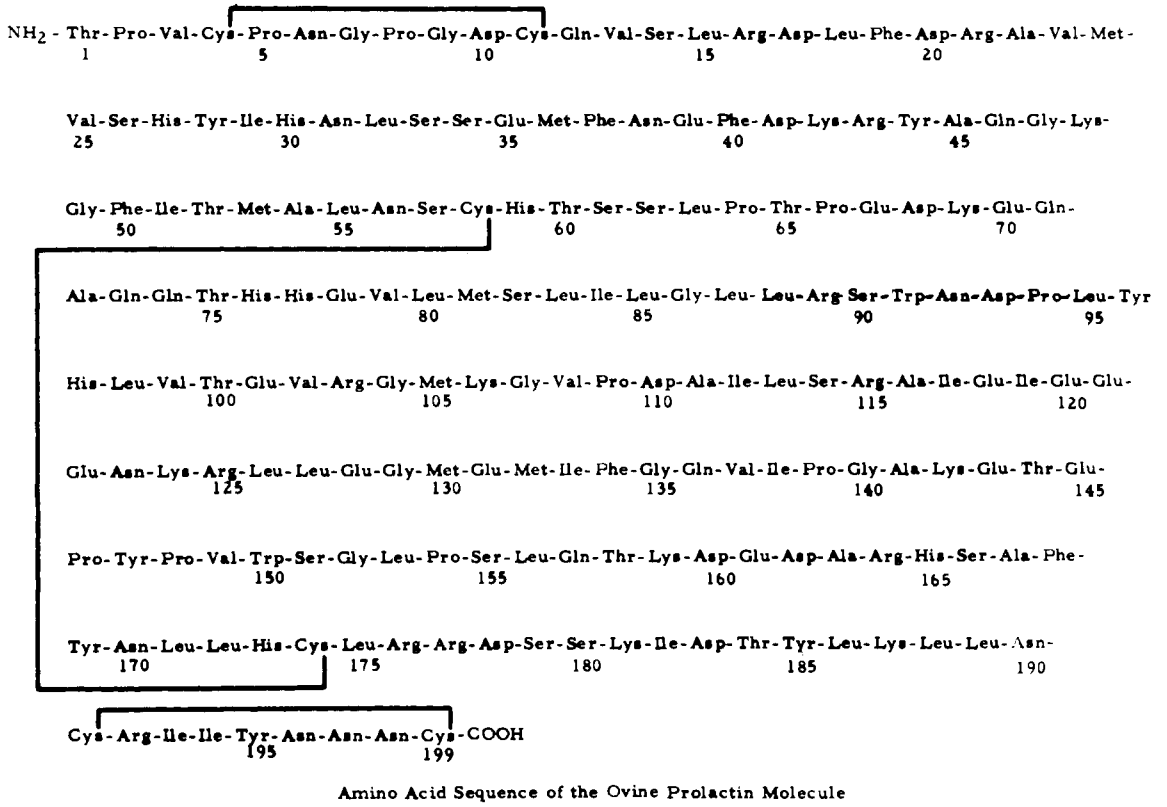

FIGURE 1: Amino acid sequence of ovine prolactin.

Li et al., 1972). The three disulfide bonds of ovine prolactin are formed by cystine-4–11 in the "amino-terminal loop", by cystine-58–174, the "central disulfide bridge", and by cystine-191–199 which forms the "carboxyl-terminal loop" (Figure 1). The disulfide bonds of somatotropins (Cys-53–165 and Cys-182–189 in hGH, bGH, and oGH) are homologous to the central (Cys-58–174) and to carboxyl-terminal (Cys-191–199) disulfide bonds of prolactins, respectively. Somatotropins lack a homologue corresponding to the disulfide bond forming the amino-terminal loop (Cys-4–11) in mammalian prolactins.

Prolactins and somatotropins are phylogenetically ancient as both hormones have been identified in all vertebrate classes more advanced than jawless fishes (Hayashida, 1975; Wallis, 1975). When somatotropins of the various vertebrate classes are compared, they show an overall structural similarity as measured by immunological cross-reactivity (Hayashida, 1975) or as expressed by shared biological activities (Farmer et al., 1976a,b). In contrast, there appear to be greater differences among vertebrate prolactins. The greater structural variety in prolactins from the various classes may be associated with the more diverse physiological roles of prolactins compared to somatotropins and with the greater species specificity observed in the biological actions of prolactins. Biological evidence for the structural diversity of prolactins is seen in the widely different activities of pituitary extracts and purified prolactins derived from several vertebrate classes when tested in certain standard bioassays (Bern & Nicoll, 1968; Doneen, 1976; Farmer et al., 1977). A precise examination of the evolved differences among prolactins requires more complete information on the structure of the hormones of lower vertebrates.

In *Tilapia mossambica* prolactin there are two disulfide bonds (Farmer et al., 1977). Examination of the amino-terminal tetrapeptide portion of teleost prolactin showed the absence of a half-cystine residue. *Tilapia* prolactin, like somatotropins, appears to differ in part from mammalian prolactins by the absence of the disulfide bond forming the amino-terminal loop. Lewis et al. (1972) have described shark prolactin which contained only two disulfide bonds; four 1/2-Cys residues were observed in salmon prolactin (Idler et al., 1978). Therefore, fish prolactins in general appear to lack the third disulfide bridge found in mammalian prolactins.

The presence or absence of particular homologous disulfide bonds appears to be associated with the evolution of the structural and biological differences observed in the prolactin–somatotropin family of pituitary hormones. It is of interest to understand the role of these disulfide bonds in the structures and phylogenetically diverse biological actions of prolactins. We report here methods for the selective reduction and alkylation of one, two, and three disulfide bonds in ovine prolactin. The identity and reactivity of each of these bonds are established. Some physical, structural, and biological effects of reduction–alkylation are described.

Materials and Methods

Prolactin was isolated from fresh sheep pituitary glands by procedures previously described (Sluyser & Li, 1964). The protein concentration was determined by ultraviolet spectra, using the relation $E_{1cm,277nm}^{0.1\%} = 0.909$ (Bewley & Li, 1972). Dithiothreitol and α-iodoacetamide were purchased from Calbiochem. [1-^{14}C]-α-Iodoacetamide was obtained from New England Nuclear. TPCK–trypsin (217 units/mg) was a product of Worthington Biochemicals. All other chemicals were of the highest purity commercially available and were used without further purification.

6-Cam-PRL[1] was prepared by modification of the method

[1] Abbreviations used: GH, growth hormone, somatotropin; oGH, ovine GH; hGH, human GH; bGH, bovine GH; PRL, ovine prolactin; DTT, dithiothreitol; 6-Cam-PRL, Cys(Cam)^{4,11,58,174,191,199}-PRL; 4-Cam-PRL, Cys(Cam)^{4,11,191,199}-PRL; 2-Cam-PRL, Cys(Cam)^{4,11}-PRL; CD, circular dichroism.

described for disulfide bond reduction in hGH by Bewley et al. (1969). PRL (10 mg) was dissolved in 12.5 mL of nitrogen-gassed 0.1 M NH_4HCO_3 (pH 8.5). A 20 M excess of DTT over PRL disulfide bond content was added in 25 μL of buffer. Reductions were performed at either 22 or 2 °C at pH 8.5. After 90 min, a 40 M excess of α-iodoacetamide over DTT concentration was added in nitrogen-gassed 0.5 mL of buffer and allowed to react for 10 min. Considerable protein precipitated during both reduction and alkylation, though precipitation was minimized by performing the reactions at 2 °C. Following alkylation, the solution was centrifuged at 1000g for 10 min to remove precipitated protein. The supernatant was desalted on Sephadex G-25 (0.1 M NH_4HCO_3, pH 8.5). Desalted protein was concentrated by ultrafiltration (Diaflo, PM-10 membrane) and applied to a Sephadex G-100 column (2.4 × 8.4 cm, 0.1 M NH_4HCO_3, pH 8.5), previously calibrated with Blue dextran (>2 × 10^6 daltons), myoglobin (19 890 daltons), bovine serum albumin fraction V (65 000 daltons), and PRL (22 700 daltons). Only protein eluting at V_e/V_0 = 2.06 corresponding to PRL monomer was collected and frozen (−20 °C) for subsequent analyses. By amino acid analysis, ≥96% of this derivative had all disulfide bonds totally reduced–alkylated (see Results).

The DTT concentration and time of treatment required to selectively reduce one and two disulfide bonds were determined empirically in a number of trials using 5–10 mg of PRL. The extent of reduction–carbamidomethylation was quantitatively evaluated by amino acid analysis following methanesulfonic acid hydrolysis (Simpson et al., 1976). Protocols eventually developed to obtain larger amounts of 4-Cam- and 2-Cam-PRL are as follows. 4-Cam-PRL was prepared by reduction of 50 mg of PRL (0.9 mg/mL in nitrogen-gassed buffer) using 3 M excess DTT over PRL disulfide bond content for 1 h. Reduction was followed by carbamidomethylation (40 M iodoacetamide in excess of DTT for 10 min). The products were desalted, concentrated, and applied to a calibrated Sephadex G-100 column (2.4 × 84 cm, 0.1 M NH_4HCO_3, pH 8.5). Thirty-one milligrams of a fraction corresponding to monomer (V_e/V_0 = 2.06) was obtained, pooled, and frozen for subsequent characterization. Monomeric 2-Cam-PRL (41 mg) was prepared from 50 mg of PRL as described for the 4-Cam derivative except that DTT concentration was equimolar with PRL disulfide bond content and the reduction time was shortened to 30 min.

In order to know which disulfide bonds were reduced–carbamidomethylated by each treatment, we determined Cys(Cam) residue positions. 2-Cam-PRL (5 mg) was prepared as described above, but carbamidomethylation was performed with [1-^{14}C]-α-iodoacetamide (1.12 mCi/mmol). Monomeric [^{14}C]-2-Cam-PRL (3 mg) (having one reduced–alkylated disulfide bond) was digested with trypsin [enzyme/substrate, 1:50 (w/w)] in 2 mL of NH_4HCO_3 buffer (pH 8.5) for 16 h at 37 °C. The digest was lyophilized, dissolved in 50 μL of water, and applied to Whatman 3MM paper. Two-dimensional chromatography–electrophoresis was then performed at 22 °C [chromatography in butanol–glacial acetic acid–water (60:15:17 v/v) and electrophoresis in formic acid–acetic acid, pH 2.1, at 2000 V for 45 min]. After drying, we taped the paper to X-ray film (Kodak RP-X-omatic) for 4 days and subsequently developed the film with a Kodak RP-X-omatic processor. The single major radioactive area was cut from the paper and eluted for 24 h in 0.1 M NH_4HCO_3 buffer, and the eluate was lyophilized. A portion of the labeled peptide was hydrolyzed in 6 N HCl at 110 °C for 16 h for amino acid analysis. Another portion of the labeled tryptic peptide was subjected to end-group analysis by dansylation (Woods & Wang, 1967). (A second tryptic peptide contained a very small amount of radioactivity and was subsequently identified; see following and Results.) The second reduced–carbamidomethylated disulfide bond was located by investigation of tryptic peptides from [^{14}C]-4-Cam-PRL essentially as described above. The starting material for this reduction and alkylation, however, was 2-Cam-PRL which had been previously reduced with equimolar DTT and carbamidomethylated with unlabeled α-iodoacetamide. A second reduction of 5 mg of the unlabeled derivative using 3 M excess DTT over disulfide bond content, followed by alkylation with [1-^{14}C]-α-iodoacetamide (1.07 mCi/mmol), was then performed. ^{14}C-Labeled tryptic peptides were identified by amino acid analyses and by dansylation as described above.

Amino acid analyses were used to quantitate the extent of reduction–carbamidomethylation obtained in each preparation. Triplicate samples of derivatives and native hormone were hydrolyzed in 4 N methanesulfonic acid for 22 h at 110 °C (Simpson et al., 1976). Analysis was done in a Beckman 120B amino acid analyzer according to the procedure of Spackman et al. (1958). Hydrolysis of tryptic peptides eluted from paper was carried out in 6 N HCl for 16 h at 110 °C. Preliminary experiments showed that tryptic peptides could not be reliably hydrolyzed by using methanesulfonic acid. Acid hydrolysates of tryptic peptides converted *S*-Cam-cysteine to some unknown and more acidic derivative. The analytical constant for this compound was estimated as the mean constant of four amino acids, Asp, Thr, Ser, and Glu. This constant may be in some error, however, as the yield of the *S*-Cam-cysteine derivative was consistently lower than expected in all digests done on peptides eluted from paper (Table III). For discussion of the possible identity of the derivative of *S*-Cam-cysteine, see Houghten & Li (1978). The completeness of reduction–cambamidomethylation was also evaluated by measuring radioactivities of ^{14}C-labeled proteins and their tryptic peptides. Specific radioactivities of the labeled proteins were estimated by liquid scintillation counting (Packard Tri-Carb Model 3320; counting efficiency 79%) of aliquots of Sephadex G-100 fractions with known protein concentrations. In the case of tryptic peptides, specific radioactivities were first obtained as the ratio of radioactivity (disintegrations per minute) to amounts of peptides eluted from the paper chromatograms as estimated by amino acid analysis. These values were converted to the ratio of nanomoles of S-carbamidomethylated cysteine per nanomole of peptide.

CD spectra were obtained by using the Cary Model 60 spectropolarimeter equipped with a Model 6002 CD attachment. All spectra were taken at 27 ± 2 °C in 0.1 M NH_4HCO_3 (pH 8.5) in fused quartz cells with protein concentrations of 0.21–0.53 mg/mL (205–250 nm) or 1.20–1.86 mg/mL (250–315 nm). Mean residue molecular ellipticities, $[\Theta]_{mrw}$, were calculated by using 115.6 as the mean residue weight. Helical contents were computed as described previously (Bewley et al., 1969).

Immunoreactivity of each derivative (100 μg) was compared to that of the native hormone in the Ouchterlony test (Ouchterlony, 1953) using undiluted guinea pig antiserum raised against PRL.

Lactogenic activities of the derivative were compared to PRL in the pigeon crop-sac assay (Nicoll, 1967) and in a modification of the in vitro mouse mammary gland assay (Doneen, 1976). Teleost osmoregulatory activity was measured in the *Gillichthys* urinary bladder bioassay (Doneen,

Table I: Amino Acid Compositions[a] of PRL and of 6-Cam-, 4-Cam-, and 2-Cam-PRL

	PRL[b] (theoretical)	PRL (obsd)	6-Cam-PRL	4-Cam-PRL	2-Cam-PRL
Trp	2	1.71 ± 0.23	1.80 ± 0.21	1.74 ± 0.13	1.87 ± 0.31
Lys	9	8.96 ± 0.83	8.17 ± 0.37	9.04 ± 0.23	8.71 ± 0.04
His	8	7.19 ± 0.51	7.63 ± 0.42	6.58 ± 0.56	7.18 ± 0.19
Arg	11	10.01 ± 0.52	10.03 ± 0.41	9.19 ± 1.36	10.48 ± 0.30
S-CM-Cys	0	0	6.20 ± 0.17	3.88 ± 0.13	1.89 ± 0.13
Asp	22	22.84 ± 1.25	23.15 ± 0.94	23.64 ± 0.78	23.42 ± 0.83
Thr	9	8.63 ± 0.10	8.82 ± 0.17	9.37 ± 0.15	9.07 ± 0.13
Ser	15	14.46 ± 0.54	14.27 ± 0.65	14.69 ± 0.53	14.47 ± 0.74
Glu	22	23.22 ± 0.79	22.81 ± 1.10	22.13 ± 1.90	25.27 ± 0.97
Pro	11	11.13 ± 0.55	11.08 ± 0.73	10.56 ± 0.62	10.67 ± 0.83
Gly	11	12.98 ± 0.94	11.26 ± 0.92	12.67 ± 0.86	12.72 ± 0.77
Ala	9	10.29 ± 0.61	9.18 ± 0.48	10.16 ± 0.97	9.92 ± 0.13
$^1/_2$-Cys	6	5.58 ± 0.16	0	1.94 ± 0.07	3.95 ± 0.22
Val	10	8.92 ± 0.18[c]	9.43 ± 0.43	8.77 ± 0.52	9.38 ± 0.39
Met	7	6.66 ± 0.21	6.20 ± 0.38	7.33 ± 0.21	6.68 ± 0.28
Ile	11	7.12 ± 0.47[c]	8.69 ± 0.41	8.21 ± 0.31	8.43 ± 0.24
Leu	22	21.43 ± 0.66	23.47 ± 0.68	22.37 ± 0.44	23.10 ± 0.75
Tyr	7	6.89 ± 0.06	6.52 ± 0.13	6.18 ± 0.09	6.96 ± 0.22
Phe	6	5.91 ± 0.07	5.64 ± 0.17	5.65 ± 0.26	6.01 ± 0.24

[a] Nanomoles per nmole of protein analyses performed after hydrolysis in 4 N methanesulfonic acid for 22 h, at 110 °C. Values are mean ± standard deviation ($n = 3$) and were not corrected for residue destruction. [b] From the amino acid sequence (see Figure 1). [c] These are -Ile-Ile- (positions 193 and 194) and -Val-Ile-Pro- (positions 137, 138, and 139) linkages in the PRL molecule (see Figure 1). It is known that these linkages are resistant to hydrolysis.

1976). Statistical significance of hormone and derivative responses was compared to control values by using the Student's *t* test (two tailed).

Results

Amino Acid Analyses of Reduced–Carbamidomethylated Derivatives. Table I presents the amino acid compositions of prolactin derivatives produced by reduction with three different DTT concentrations. Reduction with a 20 M excess of DTT over disulfide bond content appeared to quantitatively cleave the three disulfide bonds of prolactin as shown by the absence of half-cystine and by recovery of 6.20 ± 0.17 ($n = 3$) *S*-(carboxymethyl)cysteines. This product was therefore labeled 6-Cam-PRL. Reduction with a 3 M excess of DTT produced a derivative with 1.94 ± 0.07 half-cystines and 3.88 ± 0.13 *S*-(carboxymethyl)cysteines. This derivative was labeled 4-Cam-PRL. Reduction using equimolar DTT gave a product having 3.95 ± 0.22 half-cystines and 1.89 ± 0.13 *S*-(carboxymethyl)cysteines and which was designated 2-Cam-PRL. In all three derivatives (within the precision of amino acid analysis and in the absence of corrections for residue destruction), the contents of other residues appeared to be identical with that of native PRL, hydrolyzed and analyzed in the same way (Table I).

Residue Positions of Selectively Reduced Disulfide Bonds. The tryptic map of ^{14}C-labeled 2-Cam-PRL is shown in Figure 2(i). A single major radioactive peptide was observed (peptide 1). A second tryptic peptide showed minor labeling as indicated by faint darkening of the overlying X-ray film. (The faintly labeled peptide appeared to correspond to peptide 2 derived from the C-terminal disulfide loop as described below.) The amino acid composition of the major labeled fragment, peptide 1, and its NH_2-terminal residue (Thr) are presented in Table II. This peptide corresponded in composition to carbamidomethylated PRL-(1–16). Figure 2(ii) is the peptide map of ^{14}C-labeled 4-Cam-PRL and shows three major radioactive peptides, numbered in descending order of apparent radioactivity as revealed by intensity of the spots in the overlying X-ray film. Amino acid and end-group analyses of these peptides are shown in Table III. Peptide 1 corresponded to PRL-(1–16) previously observed in ^{14}C-labeled 2-Cam-PRL (Table II). Labeled peptide 2 was also present (but with much lower radioactivity) in the previous experiment. However, in the digest of ^{14}C-labeled 4-Cam-PRL, peptide 2 was highly labeled. Labeled peptide 3 was unique in the tryptic map of

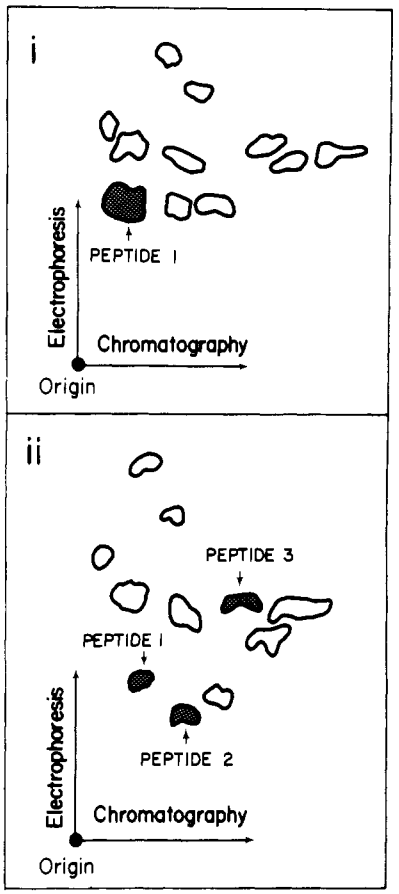

FIGURE 2: Tryptic maps of reduced–[^{14}C]carbamidomethylated derivatives of PRL. (i) Tryptic map of ^{14}C-labeled 2-Cam-PRL. (ii) Tryptic map of ^{14}C-labeled 4-Cam-PRL. Each protein (3 mg) was digested with 0.06 mg of TPCK-trypsin for 16 h, at 37 °C. The lyophilized digest was applied to Whatman 3MM paper for chromatography. First dimension, upper phase butanol–glacial acetic acid–water (60:15:75); second-dimension electrophoresis in formic–acetic acids, pH 2.1, 2000 V, 45 min. Crosshatched areas represent the major ^{14}C-labeled peptides as detected by autoradiography.

Table II: Amino Acid Composition, Amino-Terminal End Group, and Specific Activity of Peptide 1 from the Tryptic Digest of ^{14}C-Labeled 2-Cam-PRL[a]

residue	yield (nmol)	mole ratio obsd	mole ratio oPRL-(1-16)
Arg	2.24	0.75	1
S-CM-Cys[b]	2.85	0.95[d]	2 ($^1/_2$-Cys)
Asp	5.63	1.87	2[c]
Thr	2.27	0.76	1
Ser	4.44	1.48	1
Glu	4.45	1.48	1
Pro	6.97	2.32	3
Gly	5.90	1.96	2
Val	5.89	1.96	2
Leu	2.72	0.91	1

NH_2-terminal residue: threonine

[a] Radioactivity of peptide 1 [see Figure 2(i)] indicated 8.62 nmol of ^{14}C-labeled cysteine per 6.12 nmol of peptide. The extent of reduction–carbamidomethylation of one disulfide bond was, therefore, 70.4%. The yield of peptide 1 (6.12 ± 1.95 nmol) was calculated as the mean yield of all residues (amino acid analysis) except the derivatives of *S*-Cam-cysteine. [b] Detected as a derivative of *S*-Cam-cysteine. The analytical constant used was the mean constant for Asp, Thr, Ser, and Glu. [c] One Asp plus one Asn. [d] Acid hydrolysis of *S*-CM-Cys in proteins led to its partial destruction and to an increase of the Ser and Glu peaks in the chromatogram.

^{14}C-labeled 4-Cam-PRL. Amino acid composition of peptide 2 appeared to most closely resemble the derivatized carboxyl-terminal heptapeptide of PRL (sequence positions 193–199), having isoleucine as NH_2-terminal residue. Peptide 3 had an amino acid composition which paralleled most closely PRL residue positions 188–192 (Leu as major end group). Whereas the correspondence in amino acid compositions of PRL-(193–199) with peptide 2 and of PRL-(188–192) with peptide 3 is clearly not as good as that observed before between PRL-(1–16) and peptide 1, peptides 2 and 3, each containing a derivatized cysteine, corresponded most closely to tryptic peptides of the C-terminal PRL disulfide loop (191–199; Figure 1).

*Radioactivity of ^{14}C-Labeled 2-Cam-PRL and 4-Cam-*PRL. Based on the calculated specific activity of the [^{14}C]iodoacetamide used for alkylation (1.12 mCi/mmol), ^{14}C-labeled 2-Cam-PRL had 1.37 ± 0.11 (mean ± standard deviation, n = 3) nmol of *S*-(carbamidomethyl)cysteine per nmol of prolactin (Table II). As the complete reduction and alkylation of a single disulfide bridge would be expected to produce 2 nmol of the labeled cysteine derivative per nmol of hormone, it is apparent that ≃70% of a single bond was reduced in 2-Cam-PRL. The labeling of peptide 1 from the tryptic map of ^{14}C-labeled 2-Cam-PRL is also shown in Table II. This peptide, identified as PRL-(1–16) from its amino acid composition, should have displayed 12.24 nmol of ^{14}C-labeled (carbamidomethyl)cysteine if fully reduced and alkylated. Its specific activity, however, was 8.62 nmol of ^{14}C-labeled (carbamidomethyl)cysteine (carbamidomethylation ≃70%). The presence of a second minor labeled peptide (peptide 2) suggested that cleavage at a second disulfide bond began before reduction at the N-terminal bond was completed. Radioactivity of ^{14}C-labeled 4-Cam-PRL indicated 1.75 nmol of (carbamidomethyl)cysteine per nmol of hormone. This corresponded to ≃90% reduction–carbamidomethylation, assuming cleavage at one disulfide bond. However, the peptide map indicated reduction at two separate disulfide bonds [Figure 2(ii)]. Radioactivities of the three major ^{14}C-labeled peptides derived from labeled 4-Cam-PRL are shown in Table III: 10.45 ± 1.18 nmol of peptide 1 PRL-(1–16) was eluted

Table III: Amino Acid Compositions, Amino-Terminal End Groups, and Radioactivities of Peptides 1-3 from the Tryptic Digests of [^{14}C]-4-Cam-PRL

(a) Peptide 1[a,b]

residue	yield (nmol)	mole ratio obsd	mole ratio oPRL-(1-16)
Arg	4.16	0.90	1
S-CM-Cys[c]	6.75	1.46	2 ($^1/_2$-Cys)
Asp	10.68	2.31	2 (1 Asp + 1 Asn)
Thr	4.47	0.96	1
Ser	5.17	1.12	1
Glu	4.98	1.08	1
Pro	12.33	2.67	3
Gly	10.53	2.28	2
Val	9.16	1.99	2
Leu	4.66	1.01	1

NH_2-terminal residue: Thr

(b) Peptide 2[d,b]

residue	yield (nmol)	mole ratio obsd	mole ratio oPRL-(193-199)
Asp	6.21	4.12	3
Ile	1.44	0.96	2
Tyr	1.39	0.92	1
S-CM-Cys[c]	1.09	0.64	1 ($^1/_2$-Cys)

NH_2-terminal residue: Ile

(c) Peptide 3[e,b]

residue	yield (nmol)	mole ratio obsd	mole ratio oPRL-(188-192)
Arg	1.90	1.09	1
Asp	2.40	1.39	1 (Asn)
Leu	3.61	2.09	2
S-CM-Cys[c]	0.77	0.45	1 ($^1/_2$-Cys)

NH_2-terminal residues: Leu, Ala, and Asp

[a] Radioactivity of peptide 1 [see Figure 2(ii)] labeled *S*-CM-Cys in 10.45 nmol of peptide 1. The extent of [^{14}C] carbamidomethylation of the disulfide bond in peptide 1 was 22.3%. [b] Yields of peptide 1 (10.45 ± 1.8 nmol), peptide 2 (3.06 ± 1.49 nmol), and peptide 3 (4.48 ± 0.70 nmol) were calculated as the mean yield of all residues (by amino acid analysis) except the acidic derivatives of *S*-Cam-cysteine. [c] Detected as an acidic derivative of *S*-Cam-cysteine. The analytical constant used was the mean constant for Asp, Thr, Ser, and Glu. [d] Specific activity of peptide 2 [see Figure 2(ii)] was 4.33 nmol of [^{14}C]-Cam-cysteine in 3.06 nmol of peptide 2 (141.0%). [e] Specific activity of peptide 3 [see Figure 2(ii)] was 3.26 nmol of [^{14}C]-*S*-Cam-cysteine in 4.48 nmol of peptide 3 (72.7%).

from the tryptic map [Table III(a)]. This peptide contained 6.7 nmol of ^{14}C-labeled *S*-(carbamidomethyl)cysteine. As peptide 1 contains two cysteine residues (cystine 4–11), it appears that ≃20% of this bond was alkylated during the second reaction with [^{14}C]iodoacetamide, while ≃80% of the bond had been carbamidomethylated in the previous reduction–alkylation using equimolar DTT and unlabeled iodoacetamide. Peptide 2 (3.06 ± 1.49 nmol) [PRL-(193–199)] was also obtained from the map [(Table III(b)]; its radioactivity indicated 4.33 nmol of *S*-(carbamidomethyl)cysteine per nmol of peptide. Therefore, its *S*-(carbamidomethyl)-cysteine content, judged by radioactivity, appeared to exceed its peptide content by ≃40%. Peptide 3 appears to most closely resemble PRL-(188–192), also a fragment in the C-terminal disulfide loop. The end groups identified in this eluate consisted of three residues, of which one was Leu (possibly Leu-188). The reason for failing to obtain equivalent yields of the various labeled peptides from the tryptic maps is not known. Two possibilities can be suggested. First, after reduction–carbamidomethylation, the susceptibility of particular basic residues to tryptic digestion may have been

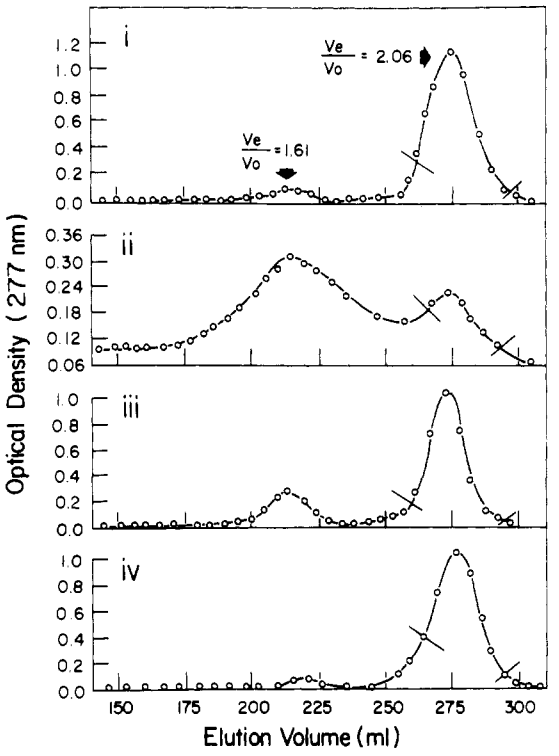

FIGURE 3: Elution patterns on Sephadex G-100 of (i) PRL, (ii) 6-Cam-PRL, (iii) 4-Cam-PRL, and (iv) 2-Cam-PRL. The column was equilibrated and eluted with 0.1 M NH_4HCO_3 (pH 8.5); flow rates were 3.7–4.2 mL/(fraction 30 min). Void volume was 134 mL; dimer V_e/V_0 = 1.61; monomer V_e/V_0 = 2.06. Vertical lines indicate monomer fractions collected and pooled for analyses.

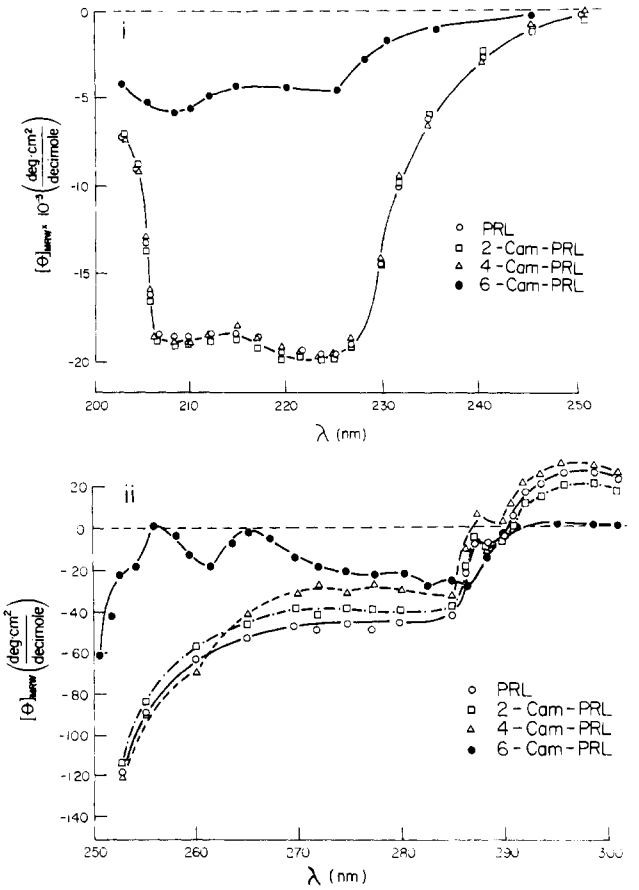

FIGURE 4: Circular dichroism spectra of PRL, 6-Cam-PRL, 4-Cam-PRL, and 2-Cam-PRL. (i) CD spectra in the region of amide bond adsorption, 203–250 nm. (ii) CD spectra in the region of side-chain absorption, 250–302 nm. Points shown are the mean of three spectra obtained with the same protein preparation. (○) oPRL; (●) 6-Cam-PRL; (△) 4-Cam-PRL; (□) 2-Cam-PRL. α-Helix contents calculated at 223 nm are as follows: PRL, 52.8%; 6-Cam-PRL, 20.3%; 4-Cam-PRL, 53.6%; 2-Cam-PRL, 55.2%.

reduced. Second, the derivatized tryptic peptides may have had differing solubilities in water which caused unequal amounts of each to be applied to the map.

Sephadex G-100 Elution Patterns of PRL and of Its Reduced–S-Carbamidomethylated Derivatives. Figure 3(i) shows the elution pattern of PRL in 0.1 M NH_4HCO_3 (pH 8.5) on Sephadex G-100. The monomer (82% of the applied protein) eluted at V_e/V_0 = 2.06. The smaller peak at V_e/V_0 of 1.61 represents dimer and constituted 10.4% of this preparation. Reduction of 10 mg of PRL (0.9 mg/mL) with 20 M excess DTT over the disulfide bond content gave only a small amount of soluble product (6-Cam-PRL) which had the elution pattern shown in Figure 3(ii). When reduction and carbamidomethylation were performed at 2 °C, 34% of the protein applied to the Sephadex G-100 column (4.54 mg) had an elution volume corresponding to PRL monomer. The remaining protein (62%) eluted as dimer and higher order aggregates. After centrifugation, ≃2% of the soluble protein applied to the column appeared in the void volume. When 6-Cam-PRL was prepared at 22 °C, protein precipitation and aggregation were even more pronounced than at 2 °C (74% of the protein precipitated during the reduction–alkylation). At the higher temperature, only 4% of the product was recovered as monomer. Figure 3(iii) illustrates the elution pattern observed for 4-Cam-PRL; the predominant product was monomeric (62%), but some dimer (18%) and higher order aggregates were also observed. Figure 2(iv) shows the elution pattern of 2-Cam-PRL; this derivative displayed limited dimerization (about 10% of the total, comparable to dimer content in the starting material). As in native PRL, 82% of 2-Cam-PRL was obtained as monomer.

Circular Dichroism Spectra of PRL and Its Reduced–S-Carbamidomethylated Derivatives. In Figure 4(i) the far-UV (205–250 nm) circular dichroism spectra of PRL and 2-, 4- and 6-Cam-PRL are presented. PRL displayed a strong negative adsorption band at 223 nm and a slightly weaker band around 209 nm (Bewley & Li, 1972). Far-UV spectra for 2-Cam- and 4-Cam-PRL did not differ detectably from that of native hormone. The α-helical contents as calculated at 223 nm were 53 ± 5% for PRL, 55 ± 5% for 2-Cam-PRL, and 54 ± 5% for 4-Cam-PRL. In contrast, 6-Cam-PRL monomer displayed a greatly altered CD spectrum in the far-UV. Its negative dichroism below 250 nm was greatly diminished (percent helix at 223 nm was 20 ± 5%). Negative minima at 223 and 209 nm appear to have been retained, though relative intensities were greatly attenuated and bandwidths somewhat broadened. CD spectra of native PRL and of its derivatives in the region of side-chain absorption (250–315 nm) are presented in Figure 4(ii). The 2-Cam and the 4-Cam derivatives displayed only modest differences from the pattern of native PRL. 2-Cam-PRL possessed a slightly greater ellipticity with respect to the native molecule in the poorly resolved region at 265–285 nm. Repeated measurements showed these small differences to be reproducible. 4-Cam-PRL showed increased positive ellipticity in the 293–300-nm region compared both to PRL and to 2-Cam-PRL. As in the far-UV, the near-UV CD spectrum of 6-Cam-PRL was markedly different, including the disappearance of the positive band between 293 and 300 nm and an increased positive dichroism throughout the side-chain region from 250 to 285 nm. Moreover, 6-Cam-PRL displayed four positive maxima (277, 272, 265, and 258 nm) not observed in PRL

or in its partially reduced–carbamidomethylated derivatives.

Immunological Studies. The Ouchterlony test was used to measure immunoreactivity of 100 μg each of PRL, 2-Cam-PRL, 4-Cam-PRL, and 6-Cam-PRL using guinea pig antiserum to PRL. Prolactin and 2-Cam-PRL cross-reacted most strongly with the antiserum, each displaying a line of identity (data not shown). Cross-reaction of 4-Cam-PRL with anti-PRL showed spurs indicative of partial antigenic identity with PRL. 6-Cam-PRL had no detectable cross-reactivity with the antiserum.

Biological Activities of PRL and Its Reduced–S-Carbamidomethylated Derivatives. Table IV summarizes data on the bioassay of native PRL and of its derivatives. Table IV(a) shows the pigeon crop-sac bioassay in which 2-Cam- and 4-Cam-PRL had biological activities which, within bioassay precision, appeared identical with that of the intact hormone. In contrast, 6-Cam-PRL was inactive at the single high dose (32 μg) examined. In the mammary gland assay [Table IV(b)], 2-Cam-PRL was also equipotent with intact PRL. 4-Cam-PRL displayed substantial bioactivity in the mammary gland but was less active than either PRL or 2-Cam-PRL. 6-Cam-PRL possessed no activity in the mammary gland. In the teleost (*Gillichthys*) urinary bladder bioassay [Table IV(c)], 4-Cam-PRL and 6-Cam-PRL were inactive at the concentrations tested (highest concentration 125 μg/mL). In contrast, 2-Cam-PRL produced the maximum observed response at the lowest concentration (1 μg/mL) at which it was tested; thus, this derivative exhibited even greater potency than native PRL. A second bioassay also included in Table IV(c) confirmed the increased bioactivity of this derivative.

Discussion

Selective Reduction of PRL Disulfide Bonds. DTT has previously been applied in the absence of denaturant to quantitatively reduce the three disulfide bonds of PRL (Bewley et al., 1969). Results in this paper show that graded concentrations of DTT can be used to selectively reduce one, two, and three disulfide bonds of PRL. Monomers of reduced–carbamidomethylated derivatives of PRL have been isolated and partially characterized with regard to the completeness of reduction and with regard to structural and biological effects of reduction–carbamidomethylation.

Amino acid analyses of the alkylated derivatives of PRL produced by reduction with equimolar, with 3 M, and with 20 M excesses of DTT over disulfide bond content showed that approximately 2 (1.89 ± 0.13), 4 (3.88 ± 0.13), and 6 (6.20 ± 0.17) 1/2-Cys were reduced–carbamidomethylated, respectively (Table I). These derivatives of PRL were named 2-Cam-PRL, 4-Cam-PRL, and 6-Cam-PRL. Cystine contents (indicative of unreduced disulfide bonds) were closely and inversely related to *S*-(carbamidomethyl)cysteine content in each derivative (Table I).

Analysis of the tryptic maps of ^{14}C-labeled 2-Cam- and of 4-Cam-PRL established the order of reducibility of the three disulfide bonds of PRL. The major labeled peptide derived from ^{14}C-labeled 2-Cam-PRL uniquely corresponded in amino acid composition to tryptic fragment PRL-(1–16) (Table II). The end group of peptide 1, threonine (Table II), also corresponded to the amino-terminal residue of PRL-(1–16) (Figure 1). Therefore, the cystine-4–11 bond was selectively reduced by the lowest DTT concentration used. A 3 M excess of DTT over PRL disulfide content was required to reduce a second bond. The tryptic map of ^{14}C-labeled 4-Cam-PRL had two major labeled peptides [Figure 3(ii)]. Peptide 2 appeared to represent reduced–carbamidomethylated PRL-(193–199) (Table III; Figure 1). Composition of peptide

Table IV: Bioassays of PRL, 6-Cam-PRL, 4-Cam-PRL, and and 2-Cam-PRL

preparation	dose (μg)	response[b] (mg dry wt)	significance[c]
(a) Pigeon Crop-Sac Bioassay[a]			
control (saline)		9.6 ± 1.3	
PRL	2	24.6 ± 3.2	<0.01
PRL	8	32.4 ± 2.6	<0.01
2-Cam-PRL	2	26.4 ± 2.6	<0.01
	8	32.5 ± 4.7	<0.01
4-Cam-PRL	2	27.2 ± 4.8	<0.01
	8	35.6 ± 4.7	<0.01
6-Cam-PRL	32	10.2 ± 1.1	NS

preparation	dose (μg/mL)	response (cpm/mg × 10^{-4})	significance
(b) Mammary Gland Bioassay[d]			
control (saline)		3.48 ± 0.26	
PRL	0.03	4.55 ± 0.29	<0.05
	0.09	6.27 ± 0.51	<0.01
2-Cam-PRL	0.03	5.26 ± 0.88	<0.01
	0.09	6.45 ± 0.48	<0.01
4-Cam-PRL	0.27	4.47 ± 0.59	<0.05
	0.81	6.63 ± 0.52	<0.01
6-Cam-PRL	0.81	3.23 ± 0.17	NS

preparation	dose (μg/mL)	response (% wt lost/h)	significance
(c) (*Gillichthys*) Urinary Bladder Bioassay[e]			
Assay 1			
control (saline)		27.0 ± 2.8	
PRL	5	20.9 ± 3.2	<0.05
	25	15.7 ± 2.3	<0.01
2-Cam-PRL	1	14.0 ± 2.3	<0.01
	5	15.3 ± 1.4	<0.01
	25	15.5 ± 1.4	<0.01
4-Cam-PRL	25	25.4 ± 2.8	NS
	125	26.7 ± 4.3	NS
6-Cam-PRL	125	28.3 ± 5.2	NS
Assay 2			
control (saline)		32.3 ± 4.6	
PRL	10	28.7 ± 3.1	<0.05
	40	17.4 ± 2.2	<0.01
2-Cam-PRL	5	15.3 ± 2.8	<0.01
	20	16.8 ± 1.9	<0.01
4-Cam-PRL	10	29.6 ± 3.4	NS
	40	26.2 ± 4.2	NS

[a] Proteins were dissolved in 0.9% NaCl and injected in three divided doses given daily in one side of the crop-sac region of pigeons for 2 days. The dose (micrograms) is the cumulative amount of proteins injected. Responses were measured from the stimulation of the crop-sac mucosa (mg of dry weight; $n = 4$) (Nicoll, 1967). [b] Mean response ± standard error. [c] Student's t test; NS, not significantly different from control. [d] Mammary gland tissue was removed from primaparous midpregnant BALB/c CRGL mice. Minced tissues were cultured in Waymouth's medium containing insulin (5 μg/mL), cortisol (1 μg/mL), and the graded doses of the proteins being assayed. After 3 days of culture, 25 μCi of [^{3}H]-leucine was added to each culture dish for 1 h; tissues were then rinsed and homogenized (an aliquot was saved for protein determination), and total protein was precipitated with 10% trichloroacetic acid after addition of 2 mg of bovine serum albumin fraction V as the carrier. Cl_3AcOH precipitates were rinsed in 5% Cl_3-AcOH and dissolved in glacial acetic acid, and the radioactivity in each was measured by liquid scintillation spectrophotometry. Results are expressed as counts per minute per milligram of protein in the homogenate ($n = 5$) (Doneen, 1976). [e] *Gillichthys* urinary bladders were dissected from fish 1 day after transfer from sea water to tap water. Bladders were cultured in medium 199 for 3 days at 15 °C in the presence of cortisol (1 μg/mL) and graded concentrations of the proteins being bioassayed. Bladders were then rinsed, filled with a diluted *Gillichthys* Ringer solution (20%), and bathed in full strength Ringer solution for 20 min. Bladders were weighed at the beginning and end of the incubation period, and bladder osmotic permeability was estimated from water loss (percent water lost per hour) ($n = 6$) (Doneen, 1976).

3 most closely corresponded to the tryptic pentapeptide containing (carbamidomethyl)cysteine-191 [PRL-(188–192)] (Table III; Figure 1). End-group analyses also aided identification of peptide 2 (end group, isoleucine, sequence position 193) and peptide 3 (end groups were heterogeneous but included leucine, probably from sequence position 188). Therefore, the second disulfide bond reduced by a 3 M excess of DTT was cystine-191–199. The central disulfide bond of PRL (cystine-58–174) was the most resistant to reduction, requiring a DTT concentration in excess of 3 M over disulfide bond content. A 20 M excess was used in these and previous experiments (Table I; Bewley et al., 1969).

An important issue was whether particular disulfide bonds were completely or only partially reduced under the reducing conditions employed. The completeness of reduction–carbamidomethylation reactions in the amino- and carboxyl-terminal disulfide loops was evaluated in two independent ways: by quantitative amino acid analyses and from the specific activities of labeled proteins and their tryptic fragments. Amino acid analysis of 2-Cam-PRL revealed ≃95% reduction–alkylation of the disulfide bridge, provided reduction was actually limited to a single disulfide bond. Cleavage appeared to be confined predominantly to a single bond as shown by a single major labeled peptide in the tryptic digest of ^{14}C-labeled 2-Cam-PRL (Figure 3). However, an additional tryptic peptide appeared to be very slightly labeled. A second disulfide bond therefore must have undergone some limited cleavage coincident with or following reduction at Cys-4–11. The specific activity of labeled, undigested 2-Cam-PRL indicated that ≃70% of cystine-4–11 was reduced–alkylated. Moreover, the specific activity of labeled tryptic peptide 1 derived from this protein also corresponded to ≃70% reduction–alkylation. Therefore, the extent of reduction–carbamidomethylation at the Cys-4–11 bond differed somewhat when measured by amino acid analysis (95%) or by measurement of specific activities of 2-Cam-PRL and its labeled tryptic peptide (≃70%). These separate results indicate that preparations of 2-Cam-PRL contain some proportion (5–30%) of underivatized native PRL. This derivative was probably also contaminated by a considerably smaller amount of hormone cleaved at Cys-191–199.

The specific activity of ^{14}C-labeled 4-Cam-PRL was, as with the ^{14}C-labeled 2-Cam-PRL, somewhat lower than expected from its amino acid analysis (Table I). Tryptic peptides 2 and 3 (each containing one reduced–carbamidomethylated cysteine) derived from the carboxyl-terminal loop of ^{14}C-labeled 4-Cam-PRL had specific activities which corresponded to ≃140 and ≃70% of their respective peptide contents (Table III). These data considered alone make it difficult to judge the completeness of reduction–alkylation of cystine-191–199. Moreover, the radioactively and nonradioactively labeled derivatives may not be strictly comparable. The protein ^{14}C-labeled at Cys-191–199 was obtained by using 2-Cam-PRL previously reduced–carbamidomethylated at Cys-4–11 as the starting material. Prior reduction could have altered the pathway, kinetics, and, therefore, the completeness of reduction at Cys-191–199. However, amino acid analysis (3.88 ± 0.13; Table I) and the labeled tryptic maps together provide evidence that both cysteine-4–11 and cysteine-191–199 were completely, or nearly so, reduced–carbamidomethylated in 4-Cam-PRL. In 6-Cam-PRL, amino acid analysis showed that the three disulfide bonds of PRL, cystine-4–11, cystine-58–174, and cystine-191–199, were completely reduced–carbamidomethylated. We conclude that DTT can be used to selectively reduce the disulfide bridges of PRL. These results also reveal the order in which the disulfide bonds of PRL are reduced: first, cystine-4–11, second, cystine-191–199, third, cystine-58–174. However, our results also indicate that 2-Cam-PRL was contaminated with a small proportion (approximately 5%) of native hormone. It also seems likely that a small portion of the Cys-191–199 bonds may have undergone reduction before every N-terminal bond was completely cleaved.

Structural and Biological Effects of Disulfide Bond Reduction. Several observations showed that reduction and carbamidomethylation of all three disulfide bonds of PRL produced extensive denaturation. Most 6-Cam-PRL aggregated and precipitated. Aggregation probably reflects exposure of internal hydrophobic regions of the reduced protein. Furthermore, the 6-Cam-PRL monomer obtained exhibited important structural changes from the native hormone. Its CD spectra, both in the region of amide bond absorption and in that of side-chain absorption, displayed several features previously observed for PRL in 5 M guanidine hydrochloride (Bewley & Li, 1972) and after titration of PRL to pH 11.0 (Kawauchi et al., 1976). Among these similarities were the loss of 35% of the helical content of prolactin and the loss of the positive band between 290 and 310 nm throughout the side-chain region (Figure 4). Therefore, 6-Cam-PRL displayed a CD pattern consistent with extensive denaturation. Unlike intact PRL in denaturant (Bewley & Li, 1972), but similar to the native protein in highly alkaline solutions (Kawauchi et al., 1973), denaturation as a result of complete reduction–carbamidomethylation of the disulfide bonds of PRL was irreversible. Important structural changes in 6-Cam-PRL were further confirmed by loss of immunological cross-reactivity with antiserum to PRL and by biological inactivity in each of the three bioassays used (Table IV). The central disulfide bond, cystine-58–174, is thus essential for maintenance of biologically important features of the secondary and tertiary structure of PRL.

In contrast to 6-Cam-PRL, 4-Cam- and 2-Cam-PRL displayed only modest changes when compared to the native hormone with regard to chromatographic behavior, CD spectra, immunoreactivity, and biological activity in mouse mammary gland and in pigeon crop-sac assays. Importantly, however, 4-Cam- and 2-Cam-PRL each showed major differences in bioactivity from the native hormone and from one another in the teleost bioassay.

4-Cam-PRL was obtained principally as a monomer, though limited dimerization occurred during reduction–carbamidomethylation [Figure 3(iii)]. Dimerization may have occurred as a result of denaturation or, alternatively, from formation of mixed disulfides. With two of the three disulfide bonds of PRL cleaved (cystine-4–11 and cystine-191–199), monomeric 4-Cam-PRL was stable for long periods at room temperature and after repeated freezing and thawing. Its CD spectrum in the region of amide bond absorption showed insignificant differences from that of the native hormone. The CD of 4-Cam-PRL in the region of side-chain absorption showed modest but reproducible differences from that of PRL [Figure 4(ii)]. Bewley (1977) has recently identified two broad negative bands, one between 250 and 280 nm and the other between 255 and 290 nm, which represent the optical activity of the disulfide bonds of hGH. Thus, the altered near-UV CD spectrum of 4-Cam-PRL may reflect in part the loss of optical activity associated with two disulfide bonds rather than with rearrangements in the local conformations of other side-chain chromophores.

The line of partial identity observed in the reaction with anti-PRL in the Ouchterlony test is further evidence that

4-Cam-PRL, while structurally altered, has retained some native antigenic determinants. The biological activities shown by 4-Cam-PRL in the pigeon crop-sac and in the mammary gland [Table IV(a),(b)] likewise suggest that structural features required for function in these systems were preserved. In noted contrast, 4-Cam-PRL showed no biological activity in the teleost urinary bladder [Table IV(c)]. Inasmuch as reduction of cystine-4–11 actually increased the teleost activity of 2-Cam-PRL, integrity of the 191–199 disulfide bond appears specifically essential for the biological activity of PRL in teleosts but dispensible for its activities in the mammary gland and crop-sac assays.

2-Cam-PRL was obtained as a stable monomer [Figure 3(iv)]. Preparation of 2-Cam-PRL was not accompanied by further dimerization beyond that observed in the starting material. As with 4-Cam-PRL, this derivative displayed a far-UV CD spectra identical with that of PRL, indicative of unchanged secondary structure [Figure 4(i)]. Again, like the 4-Cam derivative, 2-Cam-PRL showed a slightly different CD from PRL in the region of side-chain absorption [Figure 4(ii)]. As its CD between 250 and 290 nm was intermediate between that at PRL and the 4-Cam derivative, decreased negative ellipticity in this region may be associated with the loss of the optical activity of a single disulfide bond (Bewley, 1977).

In the Ouchterlony test 2-Cam-PRL displayed a reaction of identity with antibodies raised against PRL. It also shared full biological potency in the mammary gland and crop-sac assays with native hormone [Table IV(a),(b)]. Importantly, 2-Cam-PRL consistently demonstrated *increased* potency when compared to unreduced hormone in the teleost bioassay. As it elicited the maximum bladder response at one-eighth to one-twenty-fifth the PRL concentration required for a comparable effect, this derivative appeared to be considerably more potent than the native hormone [Table IV(c)]. Failure to observe a dose–response curve precluded calculation of the relative potency of this derivative. Because the 2-Cam derivative was altered specifically at the amino-terminal disulfide bond for which the teleost (*Tilapia*) hormone lacks a homologue, 2-Cam-PRL may structurally resemble the fish hormone to a greater extent than does intact PRL. Whereas PRL has activity in the urinary bladder (Doneen, 1976) and in teleosts generally, the mammalian hormone has only one-eighth to one-fiftieth the potency of purified *Tilapia* prolactin, depending on the bioassay used (Doneen, 1976; Farmer et al., 1977). We hypothesize that 2-Cam-PRL may have lost some structural impediment which in native ovine PRL weakens interaction between hormone and target receptors of the teleost bladder. The increased biological activity of 2-Cam-PRL in the fish bladder clearly distinguishes this derivative from the intact hormone; i.e., increased potency of 2-Cam-PRL cannot be attributed to contamination by native hormone.

Bern & Nicoll (1968) showed that extracts of fish pituitary glands cannot stimulate mouse mammary gland milk secretion. The inability of fish prolactins to act on the mammalian target organ has been confirmed by using purified teleost hormone (Doneen, 1976). In contrast, mammalian prolactins at relatively high doses are biologically active in teleosts. From the species specificity of prolactin–mammary gland interactions, Bern & Nicoll (1968) proposed that lactogenic actions (crop-sac and mammary gland) require structural features in the hormones of higher vertebrates which are not found in fish prolactins. Conversely, the interaction of mammalian hormones with teleost target organs obviously requires structural features shared with the fish hormone. Apparently, these teleostean features have been retained in the evolution of the prolactins of higher vertebrates. Structure–function relationships of PRL and of its reduced–carbamidomethylated derivatives appear to support the hypothesis of Bern & Nicoll (1968). With 2-Cam- and 4-Cam-PRL, it has been possible to separate some biological actions of prolactin. Carbamidomethylation of cystine-4–11 and cystine-191–199 in 4-Cam-PRL destroyed teleost bladder activity. Loss of teleost function cannot be due to reduction–carbamidomethylation of cystine-4–11 as 2-Cam-PRL cleaved at this band showed increased fish bladder potency. The integrity of the carboxyl-terminal disulfide loop of PRL thus appears essential for teleostean but not for avian or mammalian activities. Finally, integrity of Cys-4–11 in PRL is not an absolute requirement for bioactivity in vertebrates generally and actually forms an impediment to activity in fish.

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Iron–Sulfur Clusters in the Molybdenum–Iron Protein Component of Nitrogenase. Electron Paramagnetic Resonance of the Carbon Monoxide Inhibited State†

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ABSTRACT: Carbon monoxide inhibits reduction of dinitrogen (N_2) by purified nitrogenase from *Azotobacter vinelandii* and *Clostridium pasteurianum* in a noncompetitive manner (K_{ii} and K_{is} = 1.4×10^{-4} and 4.5×10^{-4} and 7×10^{-4} atm and 14×10^{-4} atm for the two enzymes, respectively). The onset of inhibition is within the turnover time of the enzyme, and CO does not affect the electron flux to the H_2-evolving site. The kinetics of CO inhibition of N_2 reduction are simple, but CO inhibition of acetylene reduction is complicated by substrate inhibition effects. When low-temperature (~13 K) electron paramagnetic resonance (EPR) spectra of CO-inhibited nitrogenase are examined, it is found that low concentrations of CO ([CO] = [enzyme]) induce the appearance of a signal with *g* values near 2.1, 1.98, and 1.92 with $t_{1/2} \approx$ 4 s, while higher concentrations of CO lead to the appearance of a signal with *g* values near 2.17, 2.1, and 2.05 with a similar time course. The MoFe proteins from *Rhizobium japonicum* and *Rhodospirillum rubrum*, reduced with *Azotobacter* Fe protein in the presence of CO, give similar results. Under conditions which promote the accumulation of H_2 in the absence of CO, an additional EPR signal with *g* values near 2.1, 2.0, and 1.98 is observed. The use of *Azotobacter* nitrogenase components enriched selectively with ^{57}Fe or ^{95}Mo, as well as the use of ^{13}CO, permitted the assignment of the center(s) responsible for the induced signals. Only ^{57}Fe, when present in the MoFe protein, yielded broadened EPR signals. It is suggested that the MoFe protein of nitrogenase contains one or more iron–sulfur clusters of the type found in the simple ferredoxins. It is further proposed that the CO-induced signals arise from states of the MoFe protein in which CO inhibits electron flow to the N_2-reducing site so that the iron–sulfur cluster achieves steady-state net charges of −1 (high CO complex) and −3 (low CO complex) in analogy to the normal paramagnetic states of high-potential iron–sulfur proteins and ferredoxins, respectively. The "no-CO" signal may be either an additional center or the N_2-reducing site with H_2 bound competitively.

Of the iron–sulfur enzymes (Orme-Johnson, 1973), surely nitrogenase is among the least understood with regard to structural features of the prosthetic groups. Enzymatic activity—the MgATP-dependent reduction of N_2, H^+, or acetylene—requires both an Fe protein and a MoFe protein as well as MgATP and a low-potential reductant (Burris & Orme-Johnson, 1974).

The Fe protein binds MgATP and appears to be the proximal reductant of the MoFe protein. The Fe protein in most organisms appears to contain four iron and four labile sulfur atoms per molecule of about 6×10^4 molecular weight. The electronic (Ljones, 1973) and EPR[1] (Orme-Johnson et al., 1972; Eady et al., 1972; Mortenson et al., 1973) spectroscopic properties suggest that the protein possesses a ferredoxin-like iron–sulfur cluster. However, the key property, *MgATP-dependent* electron transfer or "reductive dephosphorylation", remains unexplained.

The structures present in the MoFe protein are less clearly defined. The metal composition is still uncertain [see Eady et al. (1972), Zumft & Mortenson (1975), and Orme-Johnson & Davis (1977) for discussions of the data]. The limits of recently reported compositions seem to be 1 to 2 Mo atoms and 15 to 33 iron atoms per molecule of 2×10^5 to 2.5×10^5 molecular weight. In addition to possible differences between organisms, it has been suggested that partially metal-depleted (or deficient) protein species may accompany the MoFe protein during purification (Zumft & Mortenson, 1973). For example,

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[1] Abbreviations used: EPR, electron paramagnetic resonance; Cp1, MoFe protein from *C. pasteurianum*; Cp2, Fe protein from *C. pasteurianum*; Av1 and Av2 similarly from *A. vinelandii*; nomenclature of Eady et al. (1972).

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