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Circular Dichroism of a Hypocalcemic Protein in Bovine Parotid Gland[1]

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Circular dichroism (CD) of the hypocalcemic protein purified from bovine parotid gland was studied under various conditions. The CD spectra and hypocalcemic activity were measured after incubation of the sample in 6.6 M urea or 6 M guanidine hydrochloride (Gdn-HCl) and subsequent removal of the denaturants by dialysis. The sample dialyzed after incubation in 6.6 M urea retained the hypocalcemic activity and its CD spectra were very similar to that of the native protein. The sample treated in the same way with 6 M Gdn-HCl had no activity, though its CD pattern was similar to that of the native protein. The pattern of thermal transition at $[\theta]_{222}$ of the urea-treated sample resembled that of the native protein but that of the Gdn-HCl-treated sample was slightly different from that of the native protein. The CD pattern of the sample reduced with mercaptoethanol showed a slightly low α-helix content and the hypocalcemic activity was also low compared to the native protein. Scission of the peptide bond was not found from the results of polyacrylamide gel disc electrophoresis of these samples.

Keywords——parotin; parotid gland; hypocalcemic protein; denaturation of protein; circular dichroism

It has previously been reported that the contents of the α-helix, β-structure, and random form in the hypocalcemic protein purified from bovine parotid gland are 54%, 26%, and 20%, respectively, through the measurements of their circular dichroism (CD) and optical rotatory dispersion spectra.[3] The high content of α-helix in the hypocalcemic protein is similar to that in proteins such as myoglobin and muscle myosin. In studies on denaturation with guanidine hydrochloride (Gdn-HCl) or urea using a partially purified hypocalcemic protein, incubation of the protein in 6M Gdn-HCl followed by dialysis of the incubated solution brought about the disappearance of the hypocalcemic activity in contrast to the incubation in 6.6 M urea, where the activity was retained.[4] The hypocalcemic activity of the partially purified sample reduced with cysteine-HCl, of the sample incubated at 60° for 5hr, and of the sample incubated in acidic solution (pH 1.4) or basic solution (pH 12.6) has also been reported.[5] We report in this paper the CD studies on the hypocalcemic protein treated by these procedures.

Materials and Methods

Materials——The hypocalcemic protein from bovine parotid gland was prepared by isoelectric precipitation at pH 5.4, fractional precipitation with ammonium sulfate, chromatography on diethylaminoethyl-(DEAE)-cellulose, gel filtration on Sepharose 6B, and preparative polyacrylamide gel electrophoresis according to the procedures described in our previous paper.[6] Two pure samples (Lot No. 1 and No. 2) were used for the experiments. Lot No. 1 was used throughout all experiments and No. 2 was used for treatment with

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3) A. Mizutani, T. Mizutani, and P.-F. Kuo, *Chem. Pharm. Bull.* (Tokyo), **24**, 2733 (1976).
4) A. Mizutani, *Yakugaku Zasshi*, **72**, 1507 (1952).
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urea or Gdn-HCl. Lot No. 1 was slightly weaker than Lot No. 2 in the usual range of hypocalcemic activity. Urea and Gdn-HCl were purchased from Tokyo Kasei, and guaranteed grade mercaptoethanol was obtained from Katayama Chemicals, Osaka.

Measurements of CD Spectra——All CD measurements were made in 0.2 M NaCl–0.01 M phosphate buffer, pH 7.38, at 21° in an attempt to normalize salt effects, using a JASCO model J-20 automatic recording spectro-polarimeter. α-Helix in the protein molecule was determined by the method of Greenfield and Fasman or of Chen and Yang.[7] The amount of protein in the solution used for the CD measurements was determined by spectrophotometry using an experimentally determined extinction of $E^{1\%}_{1\,cm}=8.6$ at 280 nm and this value was also ascertained by the calculation from amino acid composition.[6] Mean residue ellipticity, $[\theta]$, is expressed in units of deg cm^2/dmol using a mean residue molecular weight of 116 for the hypocalcemic protein.[6] Thermal melting transition was measured at 222 nm as follows: The cuvette containing a sample solution was warmed to an appropriate temperature and the samples were used for the CD measurement with a pen period of 1 sec on the JASCO J-20 spectro-polarimeter. The temperatures were corrected in the ranges of $\pm 1°$ by control experiments.

Denaturation with Urea or Gdn-HCl, and Renaturation——CD spectra of the hypocalcemic substance were measured at the concentration of 0.3 mg/ml, after incubation in the NaCl–phosphate buffer containing 2, 4, or 6.6 M urea or 2, 4, or 6 M Gdn–HCl, overnight at room temperature. The renaturation of the denatured protein was performed according to the method of Mizutani.[4] After incubation overnight at room temperature in 6.6 M urea or 6 M Gdn–HCl, the solutions were dialyzed against distilled water at 4°, with three renewals of the water over a period of 24 hr. A part of the solution was further dialyzed against 0.2 M NaCl–0.01 M phosphate buffer (pH 7.38) for 2 days at 4°, and then CD spectra were measured. Another part of the dialyzed solution was further dialyzed against saline for 2 days at 4° and was subjected to bioassay for hypocalcemic activity.

Reduction with Mercaptoethanol——The content of disulfide residue in the protein was colorimetrically determined after reduction with mercaptoethanol by the use of Ellman's reagent.[8] The reduction was performed by preventing air oxidation according to the method of Utsumi and Karush,[9] with minor modifications as follows: Ten mg of the sample in 0.2 ml of 6M urea and 1.4M mercaptoethanol was placed in the main chamber of the Thunberg tube, and 0.03 ml of 0.1N HCl was placed in the side-arm of the tube, and then the tube was evacuated by means of a water aspirator for 5 min. After incubation for 2 hr at 30°, pH of the solution in the main chamber was adjusted to about 2 with HCl from the side-arm of the Thunberg tube, and the tube was freed from vacuum. The protein in the solution was separated from urea and mercaptoethanol by gel filtration on Sephadex G-25 (column size: 1.1×50 cm) in 0.01 N HCl–0.005 M ethylenediaminetetraacetic acid (EDTA) in a cold room, and the content of SH in the protein before and after the reduction was quantitated by the use of Ellman's reagent. The sample was reduced for bioassay on the hypocalcemic activity and for CD measurements in 0.15 M NaCl (pH 5.8) containing 1.4 M mercaptoethanol and 6 M urea to prevent denaturation in the acidic condition and air oxidation. After gel filtration on the column of Sephadex G-25 in saline (pH 5.8), the SH content in the protein was determined with Ellman's reagent, and the vessel containing the reduced sample was sealed after replacing air with N_2 to prevent air oxidation. These methods were analogous to that of Sears, *et al.* for IgG.[10] A part of the solution was subjected to the bioassay and another part of the solution was used for the CD measurements, which was performed immediately after addition of the phosphate buffer (pH 7.38). The protein was found to be oxidized when stood in air at neutral pH (7.38) for several hours.

CD Measurements in the Solution at Various pH——CD measurements of the samples were carried out at various pH after adjustment to an appropriate pH with HCl or NaOH. The sample solutions of pH 1.4 or 12.6 were incubated at 30° for 5 hr,[5] and then were applied to CD measurements after pH of the solution was adjusted to 7.38.

Bioassay——Hypocalcemic activity was assayed according to the method of Mizutani, *et al.*[11] Experimental animals were two groups of 6 male rabbits, weighing more than 2 kg after fasting for 24 hr. The sample dissolved in saline was injected into the aural vein in a dose of 0.5 ml/kg body weight. Each aliquot of serum (0.1 ml) separated from the blood, drawn before injection, and 4, 5, and 6 hr after injection, was mixed with 10 ml of Sr solution, and the amount of calcium was determined by the atomic absorption method using a Shimadzu model AA-610 atomic absorption spectrophotometer. The lowering rate of serum calcium after injection to that before injection was calculated, and a mean of the maximum values of the 3 rates of fall was taken as the hypocalcemic rate.

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The difference in the hypocalcemic rate of the experimental group and that of the control group injected with the same dose of saline was examined by the *t*-test and the value which was significant at 5% level of significance was taken as being effective.

Polyacrylamide Gel Disc Electrophoresis——In order to examine cleavage of the peptide chain, polyacrylamide gel disc electrophoresis, staining with Amido Black 10B, and destaining were carried out by the Davis method.[12]

Results and Discussion

CD Spectra of the Protein in the Presence of Denaturants

The CD spectra of the hypocalcemic protein (Lot No. 1) in the solution of urea or Gdn-HCl are shown in Fig. 1a. The relations between the concentration of the denaturant and the value of $[\theta]_{222}$ are shown in Fig. 1b. The conformation of the protein in 4 M Gdn-HCl

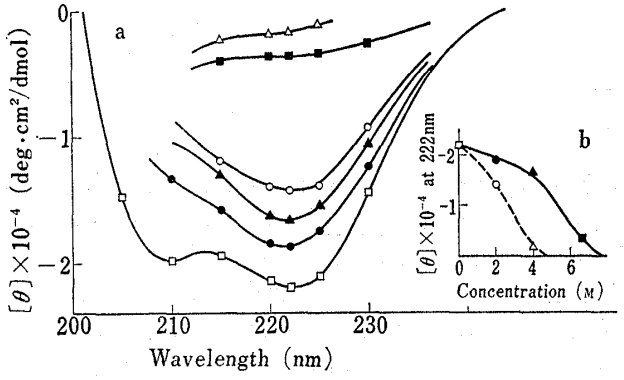

Fig. 1. CD of the Bovine Parotid Hypocalcemic Protein in the Denaturants

a) CD spectra in the denaturants. —□—, native; —●—, 2M urea; —▲—, 4M urea; —■—, 6.6M urea; —○—, 2M Gdn-HCl; —△—, 4M Gdn-HCl.
b) $[\theta]$ at 222 nm as a function of urea (——) and Gdn-HCl (----) molarity.

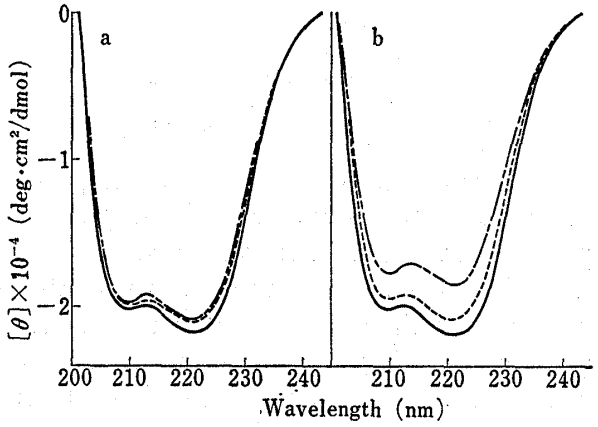

Fig. 2. CD Spectra of the Renatured Protein

a) the samples after removal of urea.
b) the samples after removal of Gdn-HCl. ——, the native protein; ----, the sample of Lot 1; ———, the sample of Lot 2.

was almost disrupted but that in 6.6 M urea was slightly retained. The effect of Gdn-HCl on denaturation of the protein was stronger than that of urea as in the case of other proteins.[13] The dependence of denaturation of this protein on the concentration of the denaturant was higher than that of α-chymotrypsin, and lower than that of lysozyme.[14] Denaturation with denaturants may not always depend on the α-helix content of these proteins. CD spectra of the hypocalcemic protein dialyzed after incubation in 6.6 M urea or 6 M Gdn-HCl are shown in Fig. 2. The α-helix content of the samples (Lot No. 1) dialyzed after incubation in 6.6 M urea and 6 M Gdn-HCl was 53% and 52%, respectively, and was similar to that of 54% for the native hypocalcemic protein. Similar results were also obtained with the sample of Lot No. 2. These observations showed that the conformation of this protein was restored after removal of the denaturants by dialysis. Hypocalcemic activities of these samples (Lot No. 1 and No. 2) which were treated with denaturants and subsequently dialyzed are shown in Table I, and these results were consistent with that obtained by Mizutani,[4] using partially purified hypocalcemic protein. In the case of urea treatment, activity of the sample after removal of urea was almost fully restored to the level of the native protein. The activity was irreversively lost by treatment with Gdn-HCl, although the CD pattern of the Gdn-HCl-treated sample resembled that of the native protein.

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Table I. Effect of Various Treatments on Hypocalcemic Activity of the Hypocalcemic Protein from Parotid Gland

Sample	Lot No.	Dose (mg/kg)	Mean±S.E. ($n=6$) Decrease of serum calcium (%)
Native	1	0.26	8.90±2.06[a]
		0.10	6.44±0.96[a]
		0.04	2.61±1.92[b]
	2	0.03	5.82±1.61[c]
6.6M Urea-dialysis	1	0.12	5.56±1.36[c]
	2	0.03	5.81±0.50[a]
6M Guanidine-dialysis	1	0.12	1.71±0.93[b]
	2	0.03	3.64±1.06[b]
Reduced	1	0.22	6.05±1.27[a]
		0.10	3.17±0.73[b]
Control			1.57±0.46

a) Significantly different from control ($p<0.01$).
b) Not active at $p>0.05$.
c) Significantly different from control ($p<0.05$).

Thermal transition of these samples (Lot No. 1) was then examined to elucidate the difference of refolding. Relationship between temperature and $[\theta]_{222}$ is shown in Fig. 3. The pattern of the Gdn-HCl-treated sample was slightly different from that of the native protein. The difference between the patterns of the active urea-treated sample and the native protein was smaller than that between the patterns of the Gdn-HCl-treated sample and the native protein. From these observation, it was considered that the conformation of the active urea-treated sample was restored to the native state after dialysis of the treated solution, because the protein in 6.6 M urea preserved approximately α-helix of 9%. Meanwhile, failure of the activity to be restored in the sample treated with Gdn-HCl appears to be alteration of the conformation to the active state, as shown in the pattern of the thermal transition (Fig. 3), even though the CD spectra of the sample after removal of Gdn-HCl resembled that of

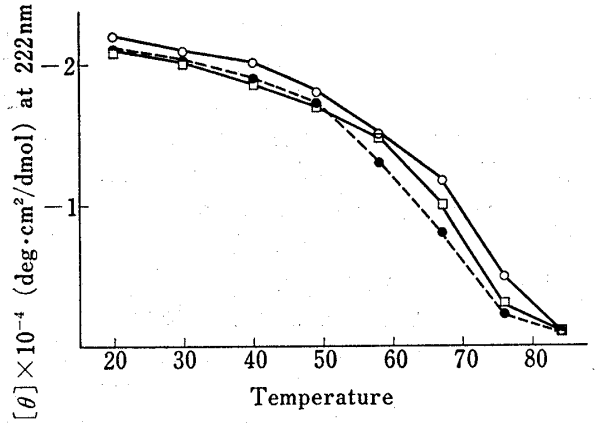

Fig. 3. Thermal Transition of the Proteins at 222 nm

—○—: native protein,
—□—: sample after removal of urea,
—●—: sample after removal of Gdn-HCl.

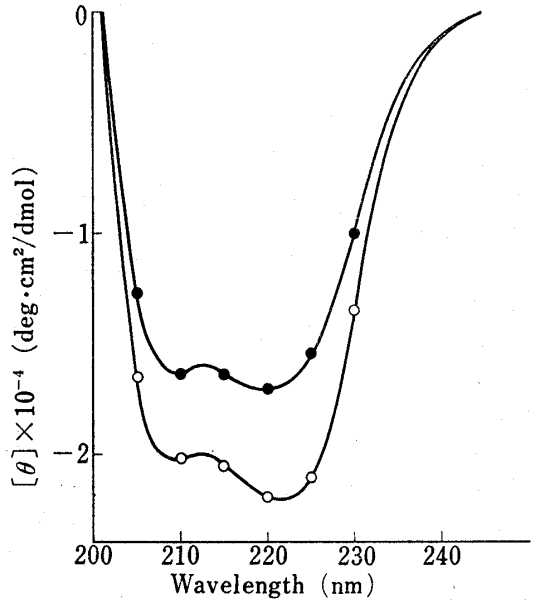

Fig. 4. CD Spectra of the Native and Reduced Proteins

—○—: native protein,
—●—: reduced protein.

native protein, as shown in Fig. 2. From the observations of the thermal transition of the native hypocalcemic protein, conformation of the protein was almost nascent at 60°, and this is consistent with the behavior of the partially purified hypocalcemic protein at 60° for 5 hr; the activity was detected in the sample so treated.[5a)]

Wetlaufer[15)] reported that the nucleus of 8–18 amino acid residues in the protein was necessary for the refolding, and Chou and Fasman indicated that leucine might possibly be the amino acid with the highest propensity for forming the α-helix and played a major role as nucleation centers in the folding and evolution of large protein molecules.[16)] The small substructure of leucine might be kept fixed or rigid.[17)] The content of leucine in the parotid hypocalcemic protein was high and 48 mol/mol of protein.[6)] In our experiment, the α-helix of the protein in 6.6 M urea was about 9% and the amino acid residues included in the α-helix of 9% were approximately 40, and were larger than 8—18 residues of the nucleus required for refolding, as stated by Wetlaufer. The hypocalcemic protein denatured with urea must have been renatured with these 40 amino acid residues as the nucleus during dialysis. We could not measure the CD spectrum of the protein in 6 M Gdn-HCl due to the effect of guanidine, but the content of α-helix must be lower than that in 4 M Gdn-HCl, in which α-helix might be about 4% (16 amino acid residues). The nucleus required for the refolding might be destroyed in 6 M Gdn-HCl and the sample must have refolded irregularly after dialysis. In the present work, we studied the secondary structure, such as α-helix, β-structure, and random form, by the CD measurements but the three-dimensional structure of the protein treated with Gdn-HCl might also differ from that of the native protein, and the three-dimensional structure is absolutely necessary for producing the activity.

Reduction with Mercaptoethanol

The content of SH residue in the present hypocalcemic protein was determined to be 0.2 mol/mol of protein using Ellman's reagent. After reduction with mercaptoethanol in the presence of urea at pH 2 and 5.8, the content of SH in the protein was determined to be 2.2 mol/mol of protein, and thereby the content of sulfide residue was estimated as 1.1 mol/mol of protein, which agreed well with the content of cystine, 1.4 mol/mol of protein, from the results of amino acid analysis. The disulfide residue might be situated at the relatively outer part of the protein because the amount of disulfide in the protein reduced in the absence of urea were fairly large, 0.7 mol/mol of protein.

CD spectrum of the reduced sample is shown in Fig. 4, and the α-helix content was distinctly lower than that of the native protein. The hypocalcemic activity of the treated sample is shown in Table I. Activity of the reduced hypocalcemic protein decreased but was clearly detected. The reduction and gel filtration of the sample used for the CD measurements and bioassay were carried out in saline at pH 5.8. Under this condition, we failed to find any indication for oxidation of SH residue in the reduced hypocalcemic protein by the quantitative analyses of sulfhydryl residue. The reason for the reduced sample to have the activity, though weak, is not due to renaturation by air oxidation but may rather be attributable to some change in the conformation. The disulfide bond in the protein must be not the active site but may play an important role in maintaining the highly ordered conformation to produce the hypocalcemic activity. These results of bioassay were consistent with those obtained by Mizutani[5c)] who reported that the partially purified sample reduced with cysteine-HCl retained the activity.

CD Spectra of the Protein in Solution at Various pH

The dependence of $[\theta]_{222}$ on pH of the solution is shown in Fig. 5. The decrease in the values at pH 4—5, shown by the broken line, must be due to isoelectric precipitation of the

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17) B. Honig, A. Ray, and C. Levinthal, *Proc. Natl. Acad. Sci. U.S.A.*, **73**, 1853 (1976).

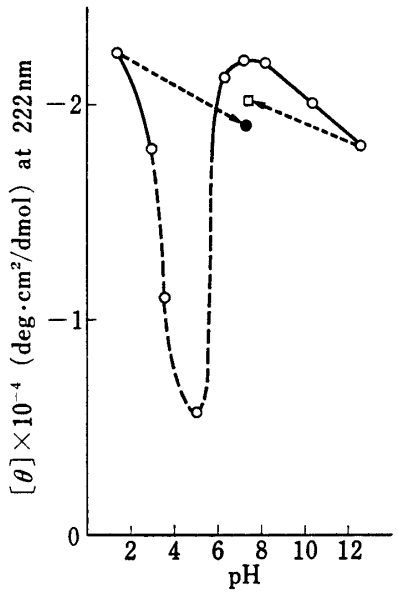

Fig. 5. Effect of pH on Mean Residue Ellipticity at 222 nm

□, the sample adjusted at pH 7.38 after the treatment at pH 12.6; ●, the sample adjusted at pH 7.38 after the treatment at pH 1.4.

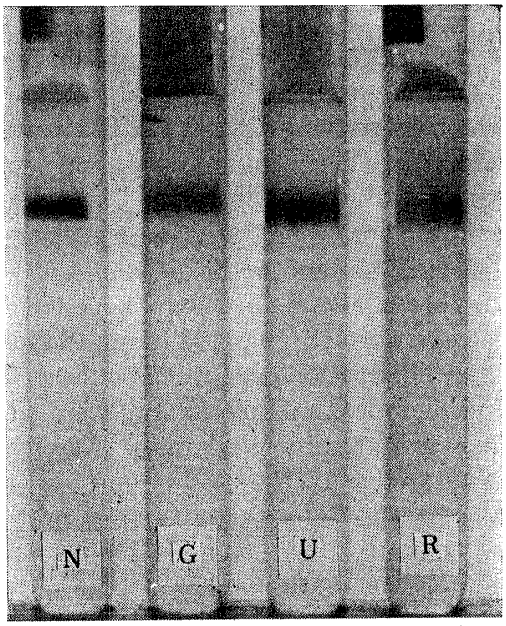

Fig. 6. Comparison of Disc Electropherograms

N, the native hypocalcemic protein; G, the sample dialyzed after the denaturation with 6M Gdn-HCl; U, the sample dialyzed after the denaturation with 6.6M urea: R, the reduced protein.

protein. The hypocalcemic protein showed a high content of α-helix in both acidic (pH 1.4) and alkaline (pH 12.6) solutions. When the sample was neutralized after incubation at pH 1.4 or 12.6, the value of $[\theta]_{222}$ of the acid-treated sample was lower than that of the native and alkali-treated samples. Ito and Mizutani[5b] reported that the partially purified sample treated at pH 12.6 was active but the sample treated at pH 1.4 was inactive. Therefore, conformation of the sample treated at pH 1.4 may differ from that of the native protein.

Analytical Disc Electrophoresis

Disc electrophoretic patterns of the samples dialyzed after incubation in 6.6 M urea or in 6 M Gdn-HCl, of the reduced sample, and of the native substance (Lot No. 1) are shown in Fig. 6. Since mobility of the main band of these samples was similar, it was considered that scission of the peptide bond of these proteins did not occur, even though the hypocalcemic activity of the sample dialyzed after incubation with Gdn-HCl and the reduced sample was low. Consequently, the protein might be inactivated through variation of the native conformation.

We compared the effect of denaturants on the structure of the protein, using the mean residue ellipticity at 222 nm (Figs. 1b, 3, and 5), because the absorbance of guanidino group prevented measurements below 210 nm. The estimation of α-helix content, based on empirical equations for ellipticity at 222 nm,[7b] might not fit the protein having a high β-structure content. However, the α-helix content of the native hypocalcemic protein was relatively high (54%) from the estimation using the value at 208 nm. Accordingly, the content of β-structure in the native protein was low (26%) and it could not influence estimation of the α-helix content of various samples using the values at 222 nm.

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