Katoh, S. (1979) *Encycl. Plant Physiol., New Ser. 5*, 247–252.
Katoh, S., Shiratori, I., & Takamiya, A. (1962) *J. Biochem. (Tokyo) 51*, 32–40.
Ke, B. (1972) *Biochim. Biophys. Acta 267*, 595–599.
Lappin, A. G., Segal, M. G., Weatherburn, D. C., & Sykes, A. G. (1979) *J. Am. Chem. Soc. 101*, 2297–2301.
Lien, S., & San Pietro, A. (1979) *Arch. Biochem. Biophys. 194*, 128–137.
Mathis, P. (1983) in *The Biology of Photoreception* (Cosens, D. J., & Vince-Price, D., Eds.) Vol. 36, pp 223–248, Cambridge University Press, Cambridge, U.K.
Mathis, P., & Haveman, J. (1977) *Biochim. Biophys. Acta 461*, 167–181.
Mathis, P., & Sētif, P. (1981) *Isr. J. Chem. 21*, 316–320.
Mauk, A. G., Scott, R. A., & Gray, H. B. (1980) *J. Am. Chem. Soc. 102*, 4360–4363.
Mullet, J. E., Burke, J. J., & Arntzen, C. J. (1980) *Plant Physiol. 65*, 814–822.
Nakatani, H. Y., & Barber, J. (1977) *Biochim. Biophys. Acta 461*, 510–512.
Nelder, J. A., & Mead, R. (1965) *Comput. J. 7*, 308–313.
Olsen, L. F., & Cox, R. P. (1982) *Biochim. Biophys. Acta 679*, 436–443.
Olsen, L. F., & Pedersen, J. Z. (1983) *Photobiochem. Photobiophys. 5*, 1–10.
Olsen, L. F., Cox, R. P., & Barber, J. (1980) *FEBS Lett. 122*, 13–16.
Overfield, R. E., & Wraight, C. A. (1980a) *Biochemistry 19*, 3322–3327.
Overfield, R. E., & Wraight, C. A. (1980b) *Biochemistry 19*, 3328–3334.
Overfield, R. E., Wraight, C. A., & DeVault, D. (1979) *FEBS Lett. 105*, 137–142.
Plesnicar, M., & Bendall, D. S. (1970) *Biochim. Biophys. Acta 216*, 192–199.
Rosen, D., Okamura, M. Y., & Feher, G. (1980) *Biochemistry 19*, 5687–5692.
Rottenberg, H., Grunwald, T., & Avron, M. (1972) *Eur. J. Biochem. 25*, 54–63.
Selak, M. A., & Whitmarsh, J. (1984) *Photochem. Photobiol. 39*, 485–489.
Sētif, P., & Mathis, P. (1980) *Arch. Biochem. Biophys. 204*, 477–485.
Takabe, T., Ishikawa, H., Niwa, S., & Itoh, S. (1983) *J. Biochem. (Tokyo) 94*, 1901–1911.
Takabe, T., Ishikawa, H., Niwa, S., & Tanaka, Y. (1984) *J. Biochem. (Tokyo) 96*, 385–393.
Tamura, N., Itoh, S., Yamamoto, Y., & Nishimura, M. (1981) *Plant Cell Physiol. 22*, 603–612.
Telfer, A., Hodges, M., & Barber, J. (1983) *Biochim. Biophys. Acta 724*, 167–175.
Van Best, J. A., & Mathis, P. (1978) *Biochim. Biophys. Acta 503*, 178–188.
Wood, P. M., & Bendall, D. S. (1975) *Biochim. Biophys. Acta 387*, 115–128.
Yocum, C. F. (1982) in *Methods in Chloroplast Molecular Biology* (Edelman, M., et al., Eds.) pp 973–981, Elsevier Biomedical Press, Amsterdam.
Yoneda, G. S., & Holwerda, R. A. (1978) *Bioinorg. Chem. 8*, 139–159.

Pseudocatalase from *Lactobacillus plantarum*: Evidence for a Homopentameric Structure Containing Two Atoms of Manganese per Subunit†

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ABSTRACT: An improved procedure for the isolation of the pseudocatalase of *Lactobacillus plantarum* has been devised, and the quaternary structure and manganese content of this enzyme have been reexamined. Sedimentation equilibrium of the native enzyme at several salt concentrations gave a molecular weight of 172 000. The subunit weight, obtained by sedimentation equilibrium in 6.4 M guanidinium chloride, with or without prior reduction and carboxymethylation, was 34 kilodaltons. The amino acid composition indicated 150 Arg + Lys, and after exhaustive tryptic digestion, 32 peptides were resolved. These data suggest that the pseudocatalase is a homopentamer. Cross-linking with dimethyl suberimidate, followed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, yielded five major bands, another indication of pentameric structure. The manganese content was found to be 1.8–2.4 per subunit. $s_{20,w}$ was found to be 9.6 S and f/f_0 = 1.2, suggesting a globular structure of Stokes radius 44 Å.

An azide-insensitive or "pseudo" catalase was first noted in certain species of bacteria which cannot synthesize heme (Delwiche, 1961; Johnson & Delwiche, 1964; Jones et al., 1965). We subsequently isolated this enzyme from *Lactobacillus plantarum* and found it to be an oligomeric manganienzyme (Kono & Fridovich, 1983a). Although the visible spectrum of the mangani-catalase resembled that of the mangani-superoxide dismutase, there was no overlap in the substrate specificities of these enzymes. The physiological function of this catalase was explored (Kono & Fridovich, 1983b), and studies of its structure and metal content suggested a hexamer containing one atom of manganese per

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subunit (Kono & Fridovich, 1983c).

The considerable utility of this enzyme for the elimination of H_2O_2 from reaction mixtures containing CN^-, N_3^-, or S^{2-}, its stability to freezing and thawing, and the likelihood that Mn(III) and Mn(V) are involved in the catalytic cycle stimulated further study. We now report an improved procedure for isolation of this enzyme and data indicating that it is a homopentamer containing two atoms of manganese per subunit.

Experimental Procedures

Materials. Dimethyl suberimidate (DMS)[1] and bis(sulfosuccinimidyl) suberate were from Pierce, guanidinium chloride (ultrapure) and SDS (electrophoresis grade) from Schwarz/Mann, molecular weight standards from Sigma, and chromatofocusing resin (PBE-94) and polybuffer (PB47) from Pharmacia. Pyridine was distilled over ninhydrin prior to use. All other materials were the best grades commercially available.

Assays. Catalase activity was determined spectrophotometrically (Beers & Sizer, 1952) in 16.7 mM potassium phosphate, 20 mM H_2O_2, and 0.1 mM EDTA at pH 7.0 and at 25 °C. The assay volume was 3.0 mL, and 1 unit is that amount which causes the decomposition of 1 μmol of H_2O_2/min. $E^{H_2O_2}_{240nm} = 43.6\ M^{-1}\ cm^{-1}$ (Hildebrant & Roots, 1975). Electrophoretograms were stained for catalytic activity by the method of Gregory & Fridovich (1972). Protein was measured colorimetrically (Lowry et al., 1951) with bovine serum albumin as the standard or, when dealing with the pure Mn-catalase, by using $E^{1\%}_{280nm} = 10.7\ cm^{-1}$ (Kono & Fridovich, 1983a).

Electrophoresis was performed on polyacrylamide slab gels both in the presence (Weber & Osborn, 1969) and in the absence (Davis, 1964) of SDS, at 25 mA/slab. Protein bands were visualized by staining with Coomassie Blue R-250 and destaining in methanol/acetic acid/H_2O [3:1:9 (v/v)].

Ultracentrifugation was performed in a Beckman Model E analytical ultracentrifuge equipped with ultraviolet optics and a photoelectric scanner. The data obtained at sedimentation equilibrium were analyzed as described by Yphantis (1964). Guanidinium chloride at 6.4 M was used to separate subunits. Some samples of enzyme were reduced and alkylated prior to this analysis (Feeney, 1971). Partial specific volume ($\bar{v}$) was calculated from the amino acid composition (Cohn & Edsall, 1943), and in the presence of guanidinium chloride, this was corrected as described by Lee & Timasheff (1979). The Stokes radius and frictional coefficient of the native protein were calculated from the sedimentation velocity data by using the method described by Van Holde (1975).

Cross-linking was used to study subunit composition as described by Davies & Stark (1970). Dimethyl suberimidate (DMS) or bis(sulfosuccinimidyl) suberate (BSS) was used at 2.5 or 10.0 mg/mL, respectively, in 0.2 M triethanolamine hydrochloride at pH 8.5 and 25 °C. The enzyme was at 1.0 mg/mL, and 30-μL aliquots of the reaction mixture were removed at intervals and were quenched with 10 μL of 2.0 M NH_4Cl. SDS and β-mercaptoethanol were then added to 1%; the samples were heated to 100 °C for 2 min and were then dialyzed 15 h against 10 mM sodium phosphate, 1% SDS, and 1% β-mercaptoethanol prior to electrophoresis.

Amino Acid Analysis. Homogeneous enzyme (~1.0 mg) was dialyzed against 1000 volumes of 50 mM Tris-HCl, 2.5 M guanidinium chloride, 20 mM 8-hydroxyquinoline, and 100 mM EDTA at pH 3.5 for 48 h, followed by dialysis against several changes of water containing 10 g/L Chelex-100. The apoenzyme was then lyophilized and redissolved in 6.0 N HCl and 0.1% phenol. Aliquots were heated to 110 °C for 24, 48, or 96 h (Spackman et al., 1960), and the hydrolyzates were analyzed on a Beckman System 6300 automatic amino acid analyzer. Cysteine and its disulfide were measured as cysteic acid after performic acid oxidation (Moore, 1963) or as carboxymethylcysteine following alkylation with iodoacetic acid (Means & Feeney, 1971). Tryptophan was determined colorimetrically (Edelhoch, 1967).

Trypsinization and Peptide Mapping. Following reduction and alkylation, 100 μg of enzyme was dialyzed against 0.2 M NH_4HCO_3 and then lyophilized. The protein was redissolved in 200 μL of 0.2 M NH_4HCO_3 (pH 8.0), and 5 μL (0.5 μg) of freshly prepared TPCK-treated trypsin was added and the digestion allowed to proceed for 3 h at 37 °C, at which time another 5 μL of trypsin was added. After 6 h of total incubation with constant stirring, the digest was lyophilized, redissolved in 10 μL of 2% NH_4OH, and spotted onto the origin of a 20 × 20 cm cellulose TLC plate (Eastman, 13255). Electrophoresis in one direction at 440 V for 2.5 h (4 °C) in pyridine/acetic acid/H_2O (10:3:300) at pH 6.0 was followed by drying the plate and ascending chromatography in the second direction in 1-butanol/pyridine/acetic acid/H_2O (50:33:1:40). The plate was then dried and sprayed with 0.2 mg/mL fluorescamine in acetone to make the peptide spots visible under UV light (Work & Burdon, 1981).

Metals. Neutron activation, at a flux of 1.5×10^{13} N cm^{-2} s^{-1} for 3 min, was done by the Nuclear Energy Services Division of the Nuclear Engineering Department of North Carolina State University in Raleigh, NC. The decay of induced radioisotopes was monitored in a series of 300-s counts on an Ortec 35% GeLi detector coupled to an ND6620 computerized γ detector system. Calibration was provided by irradiation of Mn standards in parallel with the unknowns. Enzyme was dialyzed against 50 mM potassium phosphate and 0.1 mM EDTA, pH 7.0, containing 10 g/L Chelex-100 prior to analysis. Dialyzate was used as the blank, and the protein was analyzed at several dilutions to expose protein matrix effects, which proved negligible.

Atomic absorption was done with a Perkin-Elmer Model 107 atomic absorption spectrophotometer equipped with an HGA-2000 graphite furnace. Samples (10 μL) were dried at 150 °C (40 s), ashed at 600 °C (26 s), and atomized at 2500 °C (6 s). Samples to be analyzed were dialyzed 18 h against changes of 10 mM potassium phosphate and 0.1 or 1.0 mM EDTA, at pH 7.0. Dialysis against 1.0 mM EDTA caused a 10–20% loss of Mn and an 8–15% loss of activity. The last dialyzate was used as a blank. All samples and Mn standards were made 1% in HNO_3 (AR grade). Data reported are the average of 10 measurements ± the standard deviation.

One series of measurements were also made with a Perkin-Elmer Model 3030 atomic absorption spectrophotometer equipped with an HGA-600 programmable graphite furnace and with AC-Zeeman effect background correction. This graphite furnace was equipped with a pyrolytically-coated graphite tube with a L'vov platform. A Perkin-Elmer AS-60 autosampler was used to introduce 20-μL samples. Both unknowns and standards were made 1% in HNO_3, and $Mg(NO_3)_2$ was added as a matrix modifier. Samples were dried at

[1] Abbreviations: kDa, kilodalton(s); DMS, dimethyl suberimidate; SDS, sodium dodecyl sulfate; BSS, bis(sulfosuccinimidyl) suberate; EDTA, ethylenediaminetetraacetic acid; TLC, thin-layer chromatography; DEAE, diethylaminoethyl; PAGE, polyacrylamide gel electrophoresis; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone.

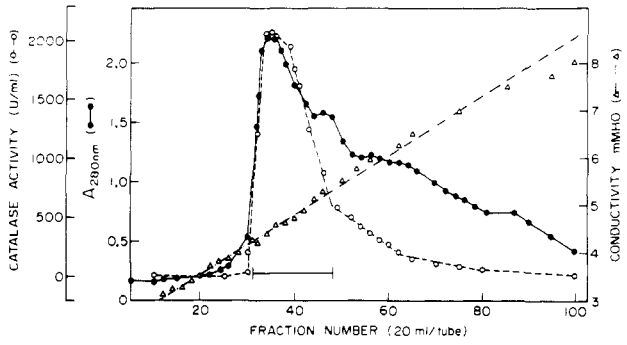

FIGURE 1: Chromatography on DE-52 cellulose. The sample (3100 mg of protein in 290 mL) was applied to a 5.5 × 38 cm DE-52 column equilibrated with 50 mM potassium phosphate and 0.1 mM EDTA at pH 7.0. The column was washed with equilibration buffer, and then a linear gradient from 0.05 to 0.25 M potassium phosphate and 0.1 mM EDTA (pH 7.0) was used to develop the column. (Total gradient volume 4.0 L.) Twenty-milliliter fractions were collected, and the most active fractions (—) were pooled. (●) A_{280nm}; (○) catalase activity (units per milliliter). (Flow rate = 15 cm h^{-1}.)

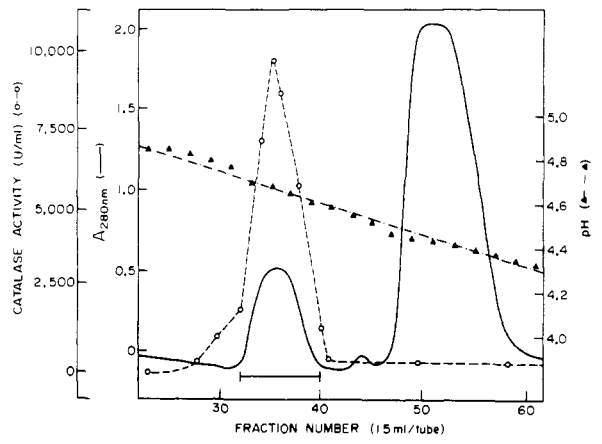

FIGURE 2: Chromatofocusing of Mn-catalase. The sample [750 mg in 30 mL of 0.025 M piperazine hydrochloride (pH 5.5)] was applied to a 1.5 × 89 cm column of PBE 94 equilibrated with 0.025 M piperazine hydrochloride (pH 5.5). The column was washed with equilibration buffer, and a linear pH gradient from 5.5 to 4.0 was generated by elution with polybuffer 74 (diluted 1:10, adjusted to pH 4.0 with HCl). Fifteen-milliliter fractions were collected, and the most active fractions were pooled. (●) A_{280nm}; (○) catalase activity (units per milliliter).

150 °C (60 s), ashed at 1400 °C (60 s), and atomized at 2200 °C (5 s). Prior to use, all enzyme samples were dialyzed against 50 mM potassium phosphate and 0.1 mM EDTA at pH 7.0 in the presence of 10 g/L Chelex-100. The data reported are the average of five measurements ± the standard deviation.

Isolation of Mn-Catalase. *Lactobacillus plantarum*, ATCC 14431, was grown aerobically in 200-L batches of commercial APT medium for 18 h at 37 °C and were then collected with a Cepa super centrifuge and were stored at −70 °C until used. Cell paste (1.0 kg) was thawed 15 h at 4 °C in 3 L of 50 mM potassium phosphate and 0.1 mM EDTA at pH 7.0. The cell suspension was passed 6 times through a Gaulin 15 M8TA laboratory homogenizer at 450 kg/cm^2. The resultant homogenate was centrifuged at 14000*g* for 45 min. Streptomycin sulfate was added (1.0 mg/mg of protein) to the turbid yellow supernatant, and after being stirred at 4 °C for 45 min, the precipitate was removed at 14000*g* for 45 min. One liter (bed volume) of DEAE-cellulose (DE-52), which had been equilibrated in the homogenization buffer, was added with stirring. After 20 min, a small aliquot of the slurry was clarified by centrifugation and was assayed for residual catalase. An additional 0.5 L of DE-52 was added, and the assay for unbound catalase was done again. This was repeated until at least 95% of the original catalase had been adsorbed to the DE-52. Typically, this required 2.5 L of DE-52.

The DE-52 was collected on a Büchner funnel and was washed with several cake volumes of 50 mM potassium phosphate and 0.1 mM EDTA, pH 7.0. The washed DE-52 was extracted by stirring with 2-L portions of 0.2 M potassium phosphate and 0.1 mM EDTA for 30 min at 25 °C. The extract was collected by vacuum filtration, and the DE-52 was extracted again. This was repeated 4 times, yielding 8 L of extract, which was then concentrated to 0.7 L over an Amicon XM-100A ultrafilter. $(NH_4)_2SO_4$ was added to 50% of saturation, and the precipitate which salted out after 2 h of stirring at 4 °C was eliminated by centrifugation. The supernatant was brought to 80% saturation with $(NH_4)_2SO_4$, and after 2 h, the precipitate was collected by centrifugation, suspended in a minimal volume of 50 mM potassium phosphate and 0.1 mM EDTA, pH 7.0, and dialyzed for 12 h against several changes of this buffer.

The dialyzed enzyme was clarified by centrifugation and was applied to a 5.5 × 38 cm column of DE-52 equilibrated with the dialysis buffer. The column was eluted with 4.0 L of potassium phosphate, pH 7.0, applied in a linear gradient from 0.05 to 0.25 M. The most active fractions, as shown in Figure 1, were pooled and were concentrated to 30 mL over an Amicon PM-30 ultrafilter. The enzyme was then dialyzed for 12 h against changes of 25 mM piperazinium chloride (pH 5.5) and was clarified by centrifugation before being applied to a 1.5 × 89 cm column of PBE-94 previously equilibrated with the dialysis buffer. This chromatofocusing column was washed with 200 mL of the piperazine buffer and was then eluted with polybuffer PB74, which had been diluted 10-fold and adjusted to pH 4.0 with HCl. Fractions with the greatest catalase activity were pooled as shown in Figure 2 and were brought to 80% saturation with $(NH_4)_2SO_4$.

The light pink precipitate was collected by centrifugation, dissolved in a minimal volume of the neutral phosphate–EDTA buffer, and dialyzed against changes in this buffer for 12 h. The final yield of pseudocatalase was 59 mg. Homogeneity was evident in that this preparation exhibited a single band of protein, which coincided with the band of activity, when electrophoresed in the native state on 7.5% or 10.5% polyacrylamide gels. A single protein band was also seen on 6% gels in the presence of SDS. Finally, gel exclusion chromatography on Sephacryl S-200 revealed a single symmetrical elution profile. This new isolation procedure is preferable to that originally described (Kono & Fridovich, 1983c) in that the overall recovery is 3 times greater and the final specific activity achieved is 20% higher. The results of the new procedure are summarized in Table I. The enzyme isolated by the new procedure was identical with that described earlier with respect to electrophoretic mobility, $E^{1\%}_{280nm}$, and optical spectra.

RESULTS

Manganese Content. Table II summarizes the results obtained by atomic absorption spectrophotometry, with and without Zeeman effect background correction, while Table III presents the results of neutron activation analysis. We are led to the conclusion that the pseudocatalase contains between 9 and 12 atoms of tightly bound Mn per molecule.

Molecular Weights of Enzyme and Subunits. We had previously used sedimentation equilibrium and an assumed $\bar{v}$ = 0.73 to arrive at a molecular weight of 172 000 ± 2000 for the native enzyme (Kono & Fridovich, 1983c). This was

Table I: Purification of Mn-Catalase from *L. plantarum*

purification step	volume (mL)	protein (mg)	act. (units)	sp act. (units/mg of protein)	recovery (%)	purification factor
crude extract	2920	1.8×10^4	7.7×10^5	43	100	1.0
streptomycin sulfate	3090	1.5×10^4	7.7×10^5	51	100	1.2
batchwise DE-52	750	4.5×10^3	6.8×10^5	151	88	3.5
50–80% ammonium sulfate	290	3.1×10^3	6.4×10^5	210	83	4.9
gradient DE-52	210	7.5×10^2	5.2×10^5	693	67	16.1
chromatofocusing	10	59[a]	4.6×10^5	7.8×10^3	59	181.0

[a] Protein was determined by dry weight.

Table II: Mn Content of Mn-Catalase Determined by Graphite Atomic Absorption[a]

sample	catalase act. (units/mL)	sp act.[b]	[protein][c] (M)	[Mn] (M)	Mn/protein[e]
dialysis vs. 0.10 mM EDTA	255	6990	$(2.12 \pm 0.02) \times 10^{-7}$	$(2.25 \pm 0.04) \times 10^{-6}$[d]	10.6 ± 0.20
	224	6770	$(1.92 \pm 0.03) \times 10^{-7}$	$(1.72 \pm 0.04) \times 10^{-6}$[d]	8.95 ± 0.20
dialysis vs. 1.0 mM EDTA	255	7340	$(2.02 \pm 0.04) \times 10^{-7}$	$(2.01 \pm 0.03) \times 10^{-6}$[d]	9.95 ± 0.15
	235	7006	$(1.95 \pm 0.02) \times 10^{-7}$	$(1.71 \pm 0.03) \times 10^{-6}$[d]	8.77 ± 0.15
dialysis vs. 0.1 mM EDTA + Chelex-100	46	7200	$(9.12 \pm 0.03) \times 10^{-8}$	$(1.09 \pm 0.05) \times 10^{-6}$[f]	11.9 ± 0.6

[a] Samples were obtained from different preparations and prior to analysis were dialyzed against 0.01 M potassium phosphate (pH 7.0) buffer with the indicated additional chelators. [b] Expressed as units per milligram of protein. [c] Assuming M_r = 172000. [d] Determined by graphite atomic absorption. [e] Expressed as moles per 172 kDa. [f] Determined by graphite atomic absorption equipped with Zeeman background correction.

Table III: Mn Content of Purified Mn-Catalase Determined by Neutron Activation Analysis[a]

sample[e]	sp act.[b]	Mn/protein[c,d]
A	7.2×10^3	12.4 ± 2.1
B	6.9×10^3	9.4 ± 0.2

[a] 100-μL samples were irradiated for 3 min at 1.5×10^{13} N/cm^2. The decay was monitored in 300-s counts on an Ortec 35% GeLi detector. Quantitative results were obtained by comparison of the decay time of the unknown to that of a Mn standard irradiated with the samples. Data reported are the average ± standard deviation. [b] Expressed as units per milligram of protein. [c] Expressed as moles per 172 kDa. [d] Data were corrected for dialysate blank which was found to contain $<2 \times 10^{-9}$ M Mn. [e] Samples were obtained from different preparations and contained 50 mM potassium phosphate (pH 7.0) buffer supplemented with 0.10 mM EDTA.

reexamined by using $\bar{v}$ = 0.722 calculated from the amino acid composition and using several solvents to establish that the enzyme was not deviating from ideality due to electrostatic interactions. The results are summarized in Table IV and in Figure 3. The molecular weight in the presence of 1.0 M KCl was larger than that seen in its absence. We suspected that this might be due to Cl^- binding and gained confidence in this interpretation by noting that 0.5 M K_2SO_4, unlike 1.0 M KCl, did not cause a positive deviation of the molecular weight. When we assumed that each cationic site on the enzyme at pH 7.0 would bind one Cl^- ($\bar{v}_{Cl^-}$ = 0.425 mL/g) and corrected the molecular weight on this basis, we obtained a value within the expected range (Table IV). We conclude that the molecular weight of the native enzyme is 169000 ± 4200.

The subunit molecular weight was measured by sedimentation equilibrium in 6.4 M guanidinium chloride. This was done in three ways in order to increase our confidence in the results. Thus, in the first case, the native enzyme was exhaustively dialyzed against 6.4 M guanidinium chloride prior to ultracentrifugation. In the second case, enzyme was reduced and carboxymethylated prior to dialysis against 6.4 M guanidinium chloride. In the third case, the apoenzyme was prepared, reduced, carboxymethylated, and then dialyzed against the guanidinium chloride. The results of bringing these samples to sedimentation equilibrium are shown in Figure 4. The linearity of the Dintzis plots indicates that all subunits are of identical size. The partial specific volume was calculated from the amino acid composition and was corrected for hydration and for guanidinium chloride binding (Hade & Tanford, 1967; Thomas & Edelstein, 1971; Lee & Timasheff, 1979). $\bar{v}$ prior to correction was 0.722 mL/g and was 0.718 mL/g after correction. Using the latter value and the slope of the Dintzis plots, we found the subunit weight to be 33.57 ± 0.5 kDa. These results are summarized in Table V. When the molecular weight of the native enzyme (169000 ± 4200) and that of the subunit (33500 ± 500) are considered in conjunction, we are led to a homopentameric structure for this enzyme.

Hydrodynamic Properties. The rate of sedimentation of the native enzyme was measured at 26000 and at 30000 rpm, respectively (Figure 5). From these data and from $\bar{v}$ = 0.722 mL/g and M_r = 172000, the hydrodynamic data in Table VI were calculated. Since the Stokes radius of the Mn-catalase is calculated to be 44 Å, while for an unhydrated sphere of equivalent weight a radius of 36.6 Å would be expected, we conclude that the native enzyme is nearly globular in shape.

Cross-Linking. Native enzyme was exposed to DMS or BSS at pH 8.5, and cross-linked products were analyzed by SDS–PAGE (Means & Feeney, 1971; Davies & Stark, 1970). Figure 6 presents typical results. Prior to exposure to the cross-linking agent, only the monomer band was seen. After 30 min of reaction, five bands became evident. A progressively longer reaction times, additional minor bands appeared. The mobilities of the major cross-linked bands differed from those

Table IV: Sedimentation Equilibrium of Mn-Catalase in Various Solvent Systems[a]

solvent	density (g/mL)	rpm	$M(1 - \bar{v}'\rho)$	$M_{protein}$[b,c]
0.10 M potassium phosphate (pH 7.0)	1.008	9000	$(4.6 \pm 0.12) \times 10^4$	171806 ± 4400
1.0 M KCl and 0.01 M potassium phosphate (pH 7.0)	1.043	9000	$(4.46 \pm 0.14) \times 10^4$	180567 ± 5668 165246 ± 5187[d]
0.50 M K_2SO_4 and 0.01 M potassium phosphate (pH 7.0)	1.061	12000	$(3.98 \pm 0.07) \times 10^4$	170000 ± 3000

[a] Protein concentration was ~0.19 mg/mL. [b] The partial specific volume ($\bar{v}$) was calculated from the amino acid composition to be 0.722 mL/g. M represents molecular weight in Tables IV and V. [c] 100% of the optical density was recovered. [d] Corrected for Cl^- binding.

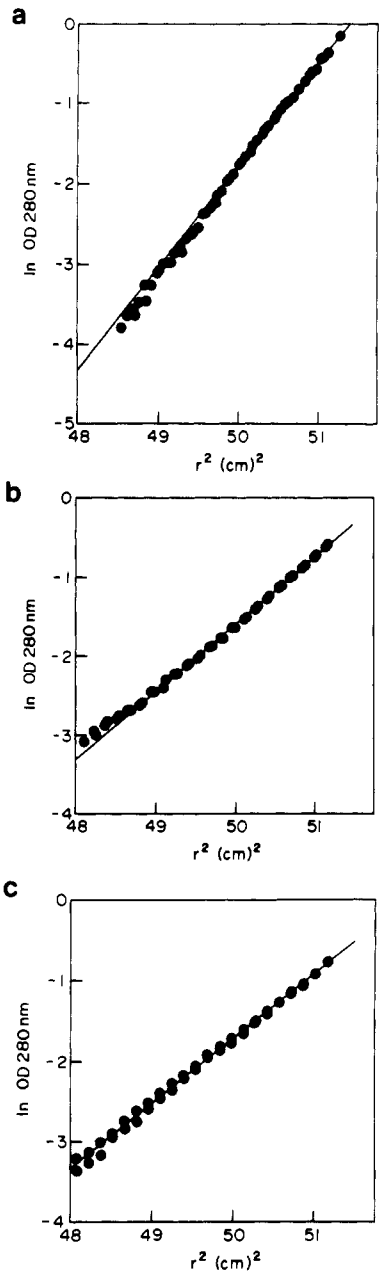

FIGURE 3: (a) Sedimentation equilibrium of native Mn-catalase in 0.01 M potassium phosphate containing 0.50 M K_2SO_4. The protein was brought to equilibrium at 12000 rpm, and $\bar{v}$ = 0.722 mL/g was used to calculate the molecular weight. (b) Sedimentation equilibrium of native Mn-catalase in 0.10 M potassium phosphate (pH 7.0). The protein was brought to equilibrium at 9000 rpm, and $\bar{v}$ = 0.722 mL/g was used to calculate the molecular weight. (c) Sedimentation equilibrium of native Mn-catalase in 0.01 M potassium phosphate containing 1.0 M KCl. The protein was brought to equilibrium at 9000 rpm, and $\bar{v}$ = 0.722 mL/g was used to calculate the molecular weight.

Table V: Sedimentation Equilibrium of Mn-Catalase in 6.4 M Guanidine Hydrochloride[a]

treatment	density (g/mL)	$M_p(\bar{v}$ = 0.722)[b,d]	$M_p(\bar{v}$ = 0.718)[e]
none	1.149	35135 ± 176	34211 ± 171
reduced, IAA treated[c]	1.148	34230 ± 585	33333 ± 569
apo, reduced, IAA treated[c]	1.150	34060 ± 1000	33161 ± 918

[a] Protein at ~0.19 mg/mL was brought to equilibrium at 30000 rpm. [b] 100% of the optical density was recovered. [c] IAA = iodoacetic acid. [d] Partial specific volume ($\bar{v}$) calculated from amino acid composition. [e] Partial specific volume ($\bar{v}$) corrected for hydration and guanidine hydrochloride binding.

Table VI: Sedimentation Velocity Measurements of Mn-Catalase[a]

parameter[b]	26000 rpm	30000 rpm
temp (°C)	22.2	21.7
density (g/mL)	1.0096	1.010
viscosity (η) (cP)	0.9791	0.9909
s ($\times 10^{13}$ S)	9.36	9.43
$s_{20,w}$ ($\times 10^{13}$ S)	9.54	9.61
r_s[c] (Å)	44.5	43.6
f[d] ($\times 10^8$)	8.21	8.14
f/f_0	1.22	1.19

[a] Protein was present at 0.35 mg/mL in 0.10 M potassium phosphate buffer, pH 7.0. [b] Based on a partial specific volume ($\bar{v}$) of 0.722 mL/g and a molecular weight of 172000. [c] Stokes radius. [d] Frictional coefficient.

Table VII: Amino Acid Composition for Mn-Catalase

amino acid	residues/mol of protein[a]	amino acid	residues/mol of protein[a]
aspartic acid	145	isoleucine[c]	40
threonine	70	leucine[c]	140
serine[b]	100	tyrosine	50
glutamic acid	210	phenylalanine	50
proline	80	histidine	55
glycine	140	lysine	80
alanine	120	arginine	70
valine[c]	65	tryptophan[d]	18
methionine	80	cysteine[e]	0

[a] Data have been normalized to 172 kDa. [b] Determined by extrapolation to zero time. [c] Determined from 48-h hydrolysis. [d] Determined spectrophotometrically. [e] See text.

expected. Thus, Figure 7 presents the expected mobilities of the cross-linked species as a function of molecular weight (O) and the mobilities actually observed (●). The presumed dimeric and trimeric species exhibited slower mobilities than expected. Similar results were obtained by using several additions of DMS or by using BSS (data not shown). Increasing degrees of cross-linking should result in progressive diminution in the yield of monomer, with a corresponding increase in the cross-linked species. Figure 8 presents densitometric scans of stained gels, which conform to this expectation. Repetition of these cross-linking studies over a 5-fold range of enzyme concentration gave similar results (data not shown).

Peptide Mapping. The amino acid composition was determined. The results from the 24-, 48-, and 96-h hydrolyses were averaged, the recovery was assumed to be 100%, and the data were normalized to a molecular weight of 172000. The results obtained on this basis are given in Table VII. These results are generally in good agreement with those reported earlier (Kono & Fridovich, 1983c), with the difference that we now find no evidence of cysteic acid after performic acid oxidation or of carboxymethylcysteine after reduction and alkylation, whereas we had previously reported two half-cystines per subunit (Kono & Fridovich, 1983c). Since we have now achieved a 20% higher specific activity than we had formerly, it appears possible that the previous result reflected a Cys-rich contaminant.

Since Arg + Lys = 150 (Table VII), we should anticipate 31 tryptic peptides from a homopentameric structure and 26 from a homohexamer. The peptide map actually obtained is shown in Figure 9. The origin showed only very slight fluorescence, suggesting that tryptic digestion had been complete, and the appearance of 32 resolvable spots supports a homopentameric structure.

Discussion

Previous work (Kono & Fridovich, 1983c) had indicated a native molecular weight of 172000 for the Mn-catalase and

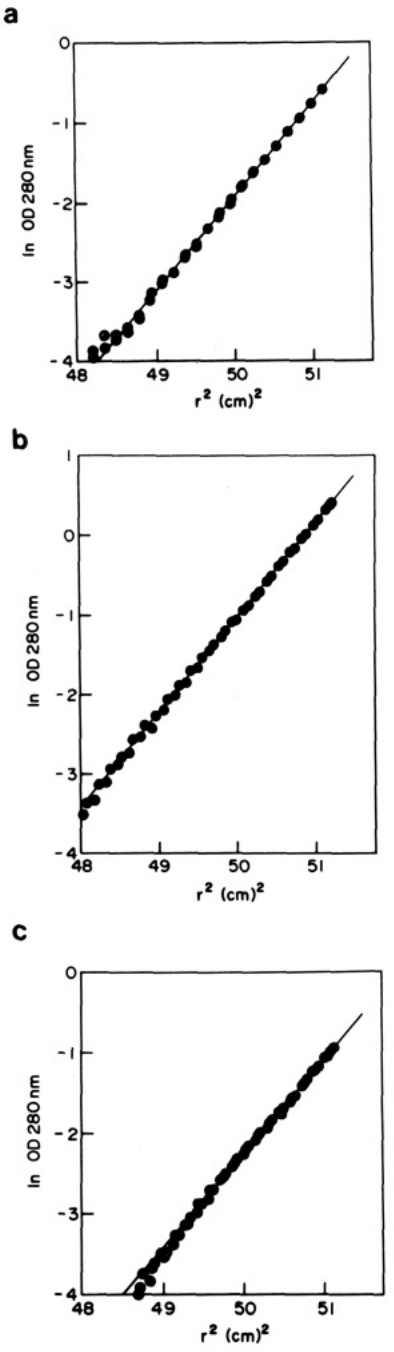

FIGURE 4: (a) Sedimentation equilibrium of reduced, iodoacetic acid treated Mn-catalase in 6.4 M guanidine hydrochloride. The protein was brought to equilibrium at 30000 rpm. (b) Sedimentation equilibrium of Mn-catalase in 6.4 M guanidine hydrochloride. The protein (~0.19 mg/mL) was brought to equilibrium at 30000 rpm. (c) Sedimentation equilibrium of apo, reduced, iodoacetic acid treated Mn-catalase in 6.4 M guanidine hydrochloride. The protein was brought to equilibrium at 30000 rpm.

a subunit weight of ~28 kDa by SDS–PAGE. We have now verified the native molecular weight in several solvent systems but find that the subunit molecular weight, when measured by equilibrium sedimentation in 6.4 M guanidinium chloride, is ~34 kDa. The latter result was obtained consistently whether the native protein was directly dissolved into the guanidinium chloride or was first converted to the apoenzyme, or was first reduced and carboxymethylated. This leads to the view that the Mn-catalase is composed of five, rather than six, subunits of equal size. Subunit weights based upon SDS–PAGE are subject to underestimation due to incomplete saturation of the protein with SDS. This is particularly likely

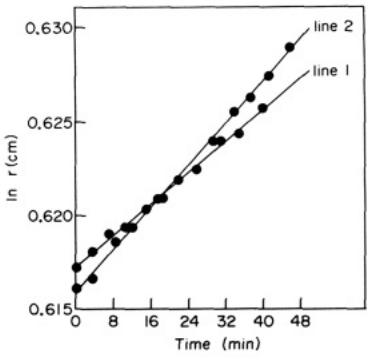

FIGURE 5: Sedimentation velocity of native Mn-catalase in 0.10 M potassium phosphate (pH 7.0) at 26000 rpm (line 1) and 30000 rpm (line 2). Protein was present at ~0.35 mg/mL. Temperature was 22.2 (line 1) and 21.7 °C (line 2).

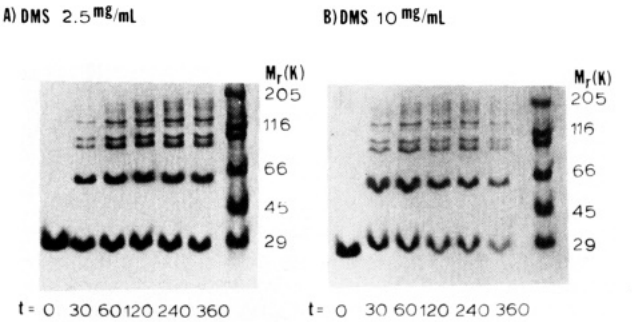

FIGURE 6: Chemical cross-linking of purified Mn-catalase with dimethyl suberimidate (DMS). Reactions were done in 0.20 M triethanolamine hydrochloride (pH 8.5). Protein was present at 1.0 mg/mL. Samples were dissociated in 1% SDS and 1% β-mercaptoethanol and electrophoresed in 6% slab SDS gels according to the method of Weber and Osborn. Time indicates minutes of incubation of protein with cross-linking reagent.

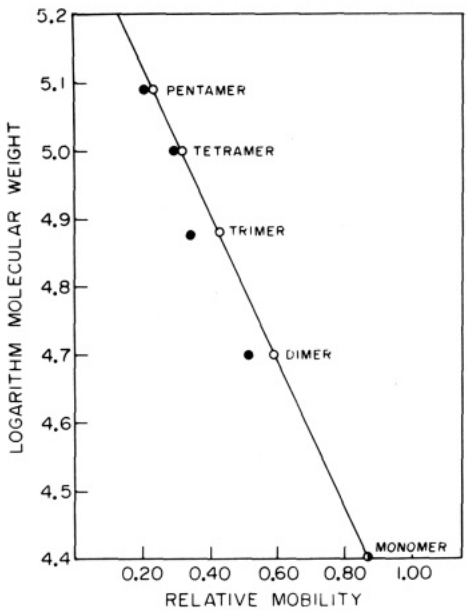

FIGURE 7: Plot of the logarithm of the molecular weight of DMS-cross-linked Mn-catalase species vs. the relative electrophoretic mobility in 6% SDS–PAGE gels. Reactions performed at pH 8.5 in 0.20 M triethanolamine hydrochloride. Protein concentration was 1.0 mg/mL, and DMS concentration was 2.5 mg/mL [(●) observed species; (○) predicted species].

to be a problem with acidic proteins (Lambin, 1978) such as the Mn-catalase.

Since we have resolved 32 tryptic peptides and would have predicted 31 for a homopentamer with 150 Arg + Lys, we propose that the Mn-catalase is a homopentamer. Since the

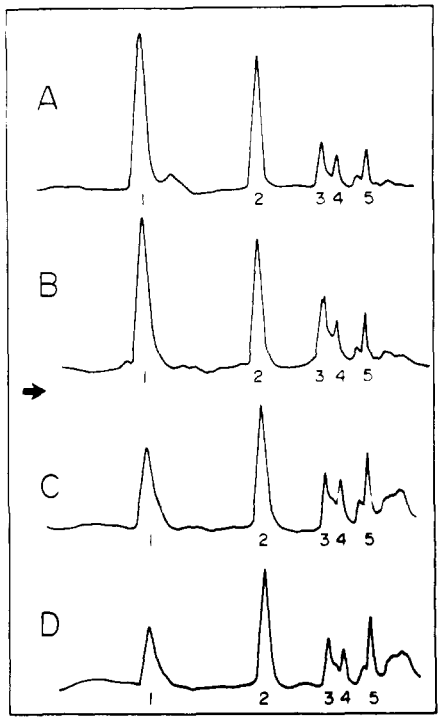

FIGURE 8: Densiometric scan of gels used for DMS cross-linking as described in Figure 6. Each lane was loaded with the same amount of protein, and the disappearance of the monomer peak as a function of time was noted. (A) 60-min incubation; (B) 120-min incubation; (C) 180-min incubation; (D) 360-min incubation; (1) monomer; (2) dimer; (3) trimer; (4) tetramer; (5) pentamer. At the arrow, DMS was added to restore the cross-linking reagent to 2.0 mg/mL.

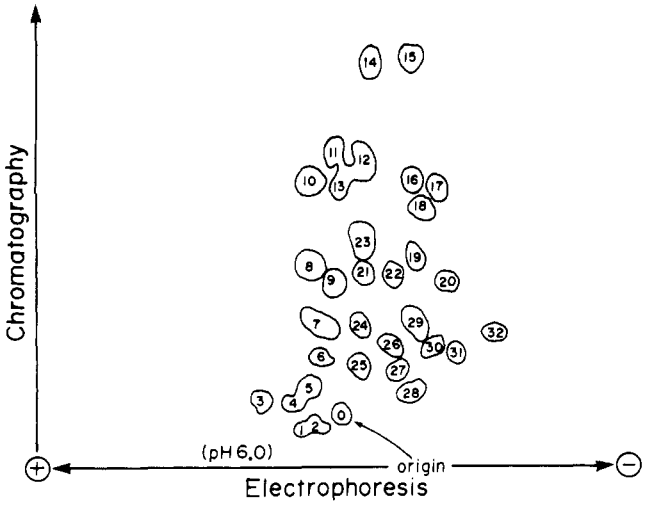

FIGURE 9: Fingerprint map of purified Mn-catalase following tryptic digestion. Tryptic digest (~100 μg) was subjected to electrophoresis at pH 6.0 for 2.5 h (400 V). Chromatography was performed by using 1-butanol/pyridine/acetic acid/water as the solvent. The peptides were derivatized with fluorescamine and visualized by long-wave ultraviolet light.

content of tightly bound manganese ranged from 9 to 12 atoms per molecule, it is clear that each subunit contains 2 atoms of Mn. This raises the possibility of one binuclear manganese cluster per subunit which, in turn, suggests fascinating mechanistic possibilities.

Cross-linking with bifunctional reagents is another method for investigating the subunit structure of oligomeric proteins (Davies & Stark, 1970) which has been applied successfully a number of times (Carpenter & Harrington, 1972; Hucho & Janda, 1974; Hucho et al., 1975; Hajdu et al., 1977; Siebert & Bowien, 1984). We noted five principal bands on SDS-PAGE of cross-linked Mn-catalase, in accord with a pentameric structure, and the putative pentamer did exhibit 5 times the molecular weight of the monomer. Yet, the putative dimeric and trimeric bands exhibited lower mobilities than expected. This deviation from ideality may be due to the circumstance that the cross-linked oligomers are apt to be branched structures.

Oligomeric proteins with odd numbers of subunits are not common (Dixon & Webb, 1975), and although a few heteropentameric proteins have been reported (Montie & Montie, 1971; Ugeda, 1969; Paetkau et al., 1968), this is the first homopentameric enzyme to be described. Further study of the Mn-catalase, particularly by X-ray crystallography, is clearly desirable.

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Registry No. Mn, 7439-96-5; pseudocatalase, 86352-20-7.

REFERENCES

Beers, R. F., Jr., & Sizer, I. W. (1952) *J. Biol. Chem. 195*, 133.

Carpenter, F. H., & Harrington, K. T. (1972) *J. Biol. Chem. 247*, 5580.

Cohn, E. J., & Edsall, J. T. (1943) *Proteins, Amino Acids, and Peptides*, Van Nostrand-Reinhold, Princeton, NJ.

Davies, G. E., & Stark, G. R. (1970) *Proc. Natl. Acad. Sci. U.S.A. 66*, 651.

Davis, B. J. (1964) *Ann. N.Y. Acad. Sci. 121*, 404.

Delwiche, E. A. (1961) *J. Bacteriol. 81*, 416.

Dixon, M., & Webb, E. (1979) *Enzymes*, 3rd ed., Academic Press, New York.

Edelhoch, H. (1967) *Biochemistry 6*, 1948.

Gregory, E. M., & Fridovich, I. (1972) *Anal. Biochem. 58*, 57.

Hade, E. A. K., & Tanford, C. (1967) *J. Am. Chem. Soc. 89*, 5034.

Hajdu, J., Wyss, S. R., & Hugo, A. (1977) *Eur. J. Biochem. 80*, 199.

Hildebrandt, A. G., & Roots, I. (1975) *Arch. Biochem. Biophys. 171*, 385.

Hucho, F., & Janda, M. (1974) *Biochem. Biophys. Res. Commun. 57*, 1080.

Hucho, F., Müllner, H., & Horst, S. (1975) *Eur. J. Biochem. 59*, 79.

Johnston, M. A., & Delwiche, E. A. (1964) *J. Bacteriol. 83*, 936.

Jones, D., Diebel, D. H., & Niven, C. F., Jr. (1965) *J. Bacteriol. 88*, 602.

Kono, Y., & Fridovich, I. (1983a) *J. Biol. Chem. 258*, 6015.

Kono, Y., & Fridovich, I. (1983b) *J. Bacteriol. 155*, 742.

Kono, Y., & Fridovich, I. (1983c) *J. Biol. Chem. 258*, 13646.

Lambin, P. (1978) *Anal. Biochem. 85*, 114.

Lee, J., & Timasheff, S. (1979) *Methods Enzymol. 61*, 49.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265.

Means, G. E., & Feeney, R. E. (1971) *Chemical Modification of Proteins*, Holden-Day, San Francisco, CA.

Montie, T. C., & Montie, D. B. (1971) *Biochemistry 10*, 2094.

Moore, S. (1963) *J. Biol. Chem. 238*, 235.

Paetkau, V. H., Younathan, E. S., & Lardy, H. A. (1968) *J. Mol. Biol. 33*, 721.

Siebert, K., & Bowien, B. (1984) *Biochim. Biophys. Acta 787*, 208.
Spackman, D. H., Stein, W. H., & Moore, S. (1960) *J. Biol. Chem. 235*, 648.
Ugeda, K. (1969) *Biochemistry 8*, 2366.
Van Holde, K. E. (1975) *Proteins (3rd Ed.) 1*, 226–287.
Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406.
Work, T. S., & Burdon, R. H. (1981) *Lab. Tech. Biochem. Mol. Biol. 9*, 149.
Yphantis, D. A. (1964) *Biochemistry 3*, 297–317.

Proton Nuclear Magnetic Resonance Study on the Dynamics of the Conformation of the Hinge Segment of Human G1 Immunoglobulin

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ABSTRACT: A proton nuclear magnetic resonance (NMR) study is reported for the dynamics of the conformation of the hinge segment of human G1 immunoglobulin. The hinge fragment (Thr^{223}-His-Thr-Cys-Pro-Pro-Cys-Pro-Ala-Pro-Glu-Leu^{234})$_2$ was obtained by tryptic digestion of F(ab')$_2$, a peptic fragment of IgG1. Comparisons of the NMR results obtained for the hinge fragment with those for the intact IgG1 and its fragments led us to conclude that (1) a significant change in conformation of the segment preceding the disulfide-linked Cys-Pro-Pro-Cys core is induced when the Fab portion is cleaved off and (2) the presence or absence of the Fc portion affects very little, if any, of the conformation of this part of the hinge. On the basis of the present NMR results along with those which we have obtained previously using the intact IgG1 and its fragments, it was concluded that the conformation of the segment preceding the Cys-Pro-Pro-Cys core of the intact IgG1 can be maintained only when it is flanked by the Fab portion and the Cys-Pro-Pro-Cys core. An X-ray crystallographic study [Marquart, M., Deisenhofer, J., Huber, R., & Palm, W. (1980) *J. Mol. Biol. 141*, 369–392] showed that segment Cys-220–Thr-225 forms a one-turn helix with little inherent stability. Upon loss of Fab or Fc, residual segments of the hinge would become too short to form the helix. In view of the NMR results obtained for Fab, Fc(t), and the hinge fragment, we suggest that the helical structure of the hinge as revealed by the X-ray crystallographic study is also retained in solution. We have previously shown on the basis of experiments using *spin diffusion* that the Lys-222–Thr-225 segment of the hinge is exposed to solvent and is primarily responsible for the internal flexibility of the IgG1 molecule. We suggest that the helical structure makes a major contribution to the expression of the flexibility of the IgG1 molecule. Spin coupling patterns for the α and β protons of Cys-226 and Cys-229 of hinge fragment I were analyzed under a variety of conditions of pH and temperature. It was concluded that the conformation about the C_α and C_β bond in Cys-226 and Cys-229 of IgG1 in solution changes very little, if any, throughout the conditions of pH and temperature examined and is quite similar to that in the crystal. Results of NMR measurements along with those obtained by using sodium dodecyl sulfate–polyacrylamide gel electrophoresis and gel filtration on an HPLC column show that (1) the segment, which follows the Cys-Pro-Pro-Cys core, is extended and (2) the presence of the disulfide-linked core is essential in maintaining the extended conformation. It was shown that the β-proton chemical shifts for Cys-229 change to a great extent on increasing the temperature, whereas very little shift was observed for Cys-226. Model building shows that there exists a significant degree of freedom of internal motion involving NH–C_α and C_β–S bonds of Cys-229 with the dihedral angle around the C_α and C_β bond held fixed. We suggest that the internal motion is responsible for the failure of observing the electron density beyond the Cys-Pro-Pro-Cys core in the X-ray crystallographic analyses of IgG1. We also suggest that the internal motion in conjunction with the extended conformation of the segment that follows the Cys-Pro-Pro-Cys core plays an important role in regulating the quaternary structure of the C_H2 domains for the optimum C1 binding.

Proteins of the human immunoglobulin G (IgG)[1] class can be differentiated into four subclasses, IgG1 through IgG4, each with a distinctive heavy chain, γ1, γ2, γ3, or γ4. The γ chains consist of four homology units, V_H, C_H1, C_H2, and C_H3. The hinge region, which is a peptide segment between the C_H1 and C_H2 domains, is highly susceptible to attack by proteolyses, and the cleavage can give rise to Fab, F(ab')$_2$, and Fc fragments[2] [see, e.g., Kabat (1976)]. The Fab region carries a recognition site for antigenic determinants, whereas the Fc region reacts with receptors of a variety of effector systems. On the basis of hydrodynamic and spectroscopic observations,

[1] Abbreviations: DSS, 4,4-dimethyl-4-silapentane-1-sulfonic acid sodium salt; Fab, an antigen-binding fragment; Fab(Lys-222), Fab region ending with Lys-222 as the C-terminal residue; Fc, a group of fragments that are composed of the C-terminal halves of the heavy chains; Fc(t), an Fc fragment obtained by tryptic digestion of human immunoglobulin G1; Fc(Leu-235), Fc region starting with Leu-235 as the N-terminal residue; HPLC, high-performance liquid chromatography; IgG, immunoglobulin G; NMR, nuclear magnetic resonance; PTH, phenylthiohydantoin; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; TFA, trifluoroacetic acid; TPCK–trypsin, trypsin pretreated with L-1-(tosylamido)-2-phenylethyl chloromethyl ketone.

[2] The nomenclature for immunoglobulin G and its fragments is as recommended in *Bull. W. H. O.* (1964).

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