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BIOCHEMICAL AND STABILITY PROPERTIES OF RECOMBINANT HUMAN MnSOD

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The light absorption spectral properties of recombinant human MnSOD, which contains an N-terminal additional methionyl residue, were investigated as a function of pH in the range 4.5–10.5. Whereas the extinction coefficient, ε_M, at the UV maximum (282 nm) was essentially independent of pH, the ε_M values of the visible spectrum maximum (482 nm) displayed a bell-shaped dependence with a plateau between pH 6.5 and 8. Those spectral changes were reversible and the enzymatic activity was not affected by exposure to buffered solutions at 25° C in the pH range 5–10.5. The stability of MnSOD was determined between 25 and 60° C at two different pH: 6.5 and 8.2. The enzyme was found to be considerably more stable at pH 6.5 than at pH 8.2, both toward aggregation and degradation. The gel permeation properties of MnSOD were investigated: the enzyme is a tetramer, with a subunit of 22.2 kD; however, it elutes from a Superose 12 column (Pharmacia) with an apparent molecular weight of ~60 kD. Under dissociative conditions (such as guanidine-HCl), molecular weights corresponding to the dimer and monomer could also be demonstrated. It thus appears that the tetramer adopts a non-globular shape, which causes the deviation from the Stokes radius corresponding to its molecular weight.

KEY WORDS: Biochemistry of human liver manganese-SOD, stability of recombinant MnSOD, light absorption characteristics of MnSOD, pH-dependence of spectral properties of MnSOD, subunit composition of MnSOD under dissociative conditions, aggregation of MnSOD upon "aging", abnormal Stokes radius of human MnSOD.

ABBREVIATIONS: kD - Kilodaltons; GuCl - Guanidine hydrochloride; MnSOD - Manganese SOD; SDS-PAGE - Sodium dodecyl sulfate polyacrylamide gel electrophoresis; UV - Ultraviolet.

INTRODUCTION

Human liver MnSOD is a tetrameric enzyme, whose subunit is made up of 198 amino acids.[1] Its amino acid sequence has been determined,[2] it has been cloned[3] and the cDNA-derived sequence was found to correspond, with minor changes, to the protein sequence. It has also been recently produced and purified in gram quantities in *E. coli*.[4] The availability of large amounts of purified and fully active MnSOD, which differs from the authentic enzyme only by the presence of an extra methionyl residue at the N-terminus of the sequence,[4] renders feasible studies on its biological effect in animal models,[5] as a means to evaluate its therapeutic potential. In order to understand the behavior of MnSOD upon storage and *in vivo*, biochemical studies on the properties of the protein are imperative. Since the Mn (III) ion constitutes a natural chromophore, use has been made of the light absorption characteristics of MnSOD in order

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to assess its behavior under various conditions, such as pH and temperature, as an indication of its stability. In addition, the study has also addressed the question of the protein structure under dissociative conditions, in order to shed light on the stability of both the metal-protein and subunit-subunit interactions.

MATERIALS AND METHODS

Chemicals

Recombinant human methionyl-MnSOD was expressed in *E. coli* and purified as previously described.[4] Its purity (> 98%) was assessed by SDS-PAGE. GuCl was from Sigma. All other chemicals used were analytical grade.

Light Absorption Spectra

The MnSOD solutions were thoroughly dialyzed at room temperature against 20 mM of the following buffers: pH 4.5–6: K-acetate; pH 6.5–7.5: K-phosphate; pH 8–9 Tris-HCl; pH 9.5–10.5: Na-carbonate/bicarbonate. Spectra were obtained at room temperature at concentrations of 5–25 mg/ml in the visible region and 0.2–1 mg/ml in the UV region on a Philips UV/Vis scanning spectrophotometer Model PU8720 (bandwidth 2 nm) equipped with a printer/plotter. The spectra were measured in Pye Unicam UV Silica cells of 10 mm path-length.

Protein Concentration

Protein concentration was determined by the modified Bradford method.[6]

Enzymatic Activity in Solution and in Activity Gels

MnSOD activity was determined on appropriate dilutions of the various test solutions by monitoring the reduction of cytochrome *c* by superoxide radicals generated by the xanthine/xanthine oxidase couple.[3] Activity was also visualized on 15% polyacrylamide gels under non-denaturing conditions.[8]

Molecular Weight Determination by Gel Permeation Chromatography

Apparent molecular weight determinations were performed on a Superose 12 column (HR10/30, Pharmacia Fine Chemicals) attached to either a FPLC apparatus, equipped with a liquid chromatography controller LCC-500 and recorder (Pharmacia Fine Chemicals) or to a HPLC system (Waters Associates), consisting of 2 pumps (Model 501), an injector (Model U6K) and an automated gradient controller (Model 580) equipped with a variable wavelength detector – spectro-Monitor 3000 (LDC/Milton Roy) – and a Chromato-Integrator (Merck-Hitachi. Model 2000). The column was calibrated by the following molecular weight standards, whose retention times were determined at the various GuCl concentrations: bovine serum albumin (67 kD), ovalbumin (43 kD), chymotrypsinogen (25 kD) and ribonuclease (13.7 kD). The standards were dissolved in the appropriate buffer (1 mg/ml) and allowed to equilibrate at room temperature for at least 1 hour prior to injection onto the column.

Flow rates were 0.4 ml/min in the absence of GuCl, 0.2 ml/min at 1 and 2 M GuCl and 0.1 ml/min at 4 and 6 M GuCl.

SDS-PAGE

Protein integrity was established by SDS-PAGE on 15% gels, stained with Coomassie Brilliant Blue.[9]

Preparation of MnSOD Samples at Various GuCl Concentrations

Two 1 ml aliquots of a MnSOD solution (20 mg/ml) were exhaustively dialyzed against either buffer without GuCl – 20 mM Tris HCl, pH 7.8 containing 150 mM NaCl – or the same buffer with 4 M GuCl. The 6 M GuCl sample was prepared by adding buffer containing 7.5 M GuCl to the 4 M GuCl sample of MnSOD. The 1 & 2 M GuCl samples were prepared by addition of 6 M GuCl-containing buffer to the MnSOD sample in buffer without GuCl.

Stability Studies

Two solutions of MnSOD which had been kept frozen at – 20° at 10 mg/ml in 10 mM of either Na-acetate, pH 6.45 or Na-bicarbonate, pH 8.2, were thawed and 1 ml aliquots were incubated (in screw-cap tubes) in paraffin oil bathes at 37 and 60° C. At various times (up to 7 days), aliquots were removed from the bath, cooled on ice, assayed for activity – both in solution and on activity gels – and applied to SDS-PAGE.

RESULTS

pH-Profile of the Light Absorption Spectral Properties of MnSOD

The spectra of MnSOD in the visible region at several pH values (5, 7 and 10) are shown in Figure 1. As can be seen from the spectra themselves and from Figure 2, the ratio between the absorbancies at the minimum and the maximum decreases with increasing pH from pH 5 to pH 10. The change in ratio values is due to a combination of two effects: pH-dependent increase in the absorbance at the minimum and a bell-shaped curve for the pH-dependence of the absorbance at the maximum at 482 nm (Figure 3). The data used in this Figure have been normalized with respect to protein concentration, as determined by the modified Bradford method,[1] and the ordinate represents therefore the molar extinction coefficients, ε_M, at the various pH values. These seem to be invariant in the pH range 6–8, and from the ascending and descending limbs of the curve pK_a values of ~ 5.9 and ~ 8.9 can be calculated. Similarly, the dependence of the ratio of the absorbancies at 340 and 482 nm (Figure 4), which is invariant in the pH range 5–6, displays a titration curve with a $pK_a \sim 9.35$. The pH-dependence of the ratio of absorbancies at the minimum and the maximum of the UV spectrum (Figure 5) is also invariant in the range 5–7.5 and the apparent pK_a value derived from the descending limb of the curve is ~ 9.0. The pH-dependence of the molar extinction coefficient, ε_M, at the maximum of the ultraviolet spectrum,

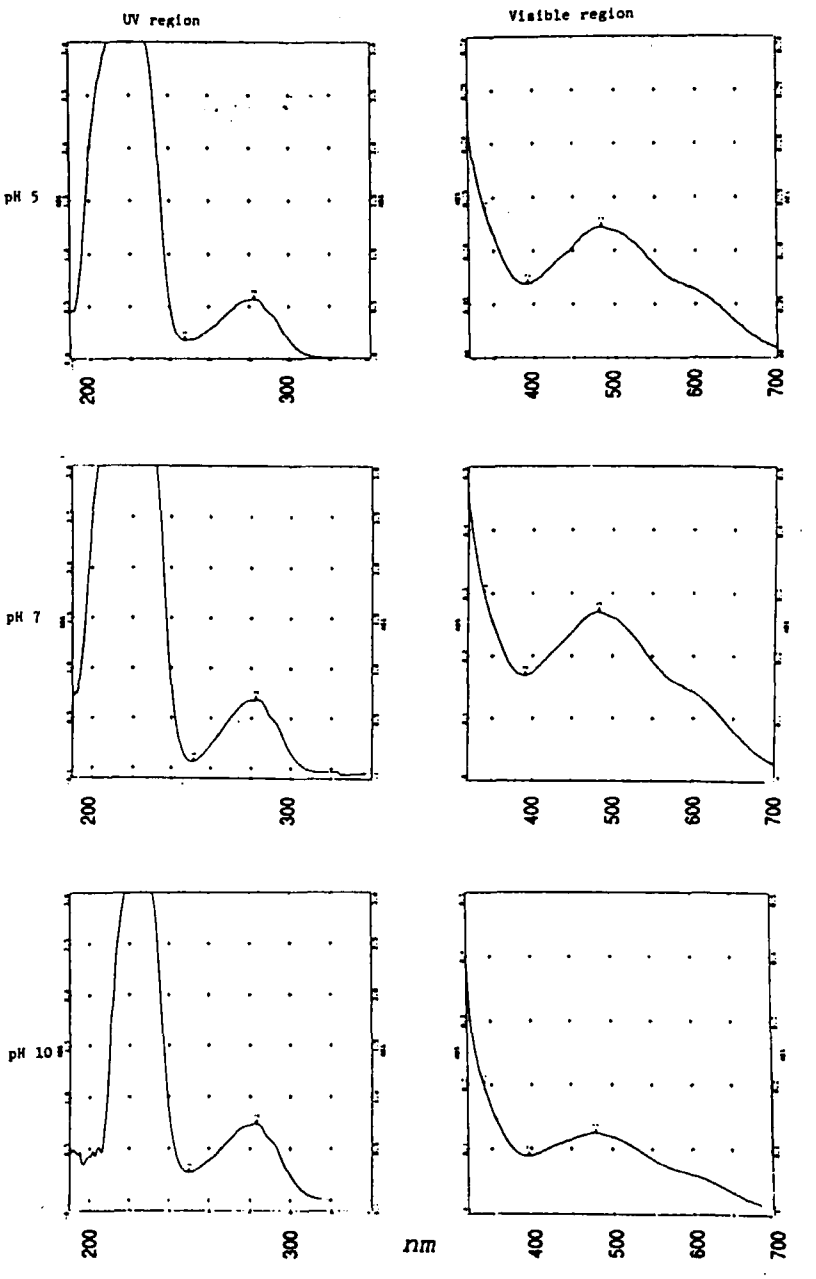

FIGURE 1 Light absorption spectra of MnSOD at 3 different pH values. The left and right panels contain spectra in the UV and the visible regions at pH 5, 7 and 10, respectively. For the preparation of the samples, see Methods. In the UV region, spectra were measured between 190 and 350 nm and in the visible region between 350 and 700 nm.

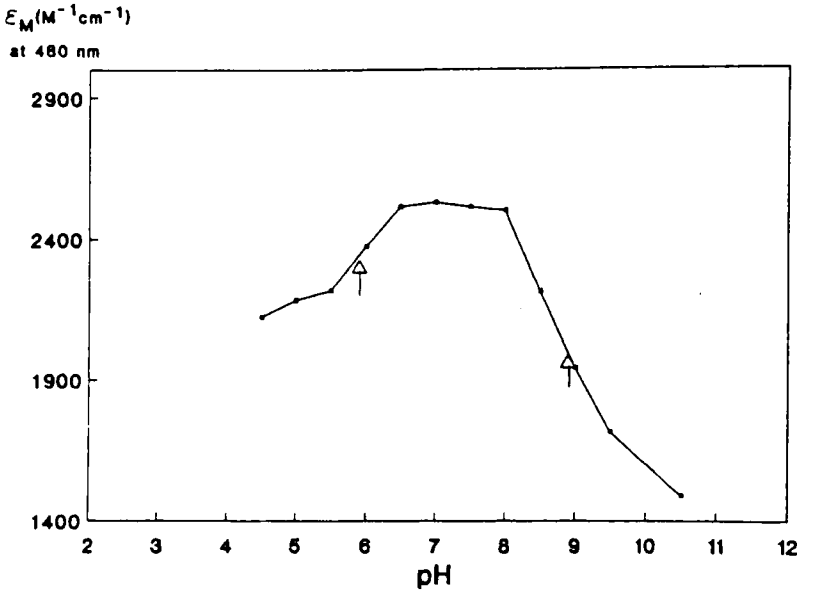

FIGURE 2 pH-Dependence of the extinction coefficients – ε_M – at the maximum wavelength (~482 nm) of the visible region of the absorption spectrum. The data were obtained by dividing the absorbancies – measured as in Figure 1 – by the protein concentration. The 2 arrows indicate the positions of the pK_a values derived from the apparent titration curves generated by the data.

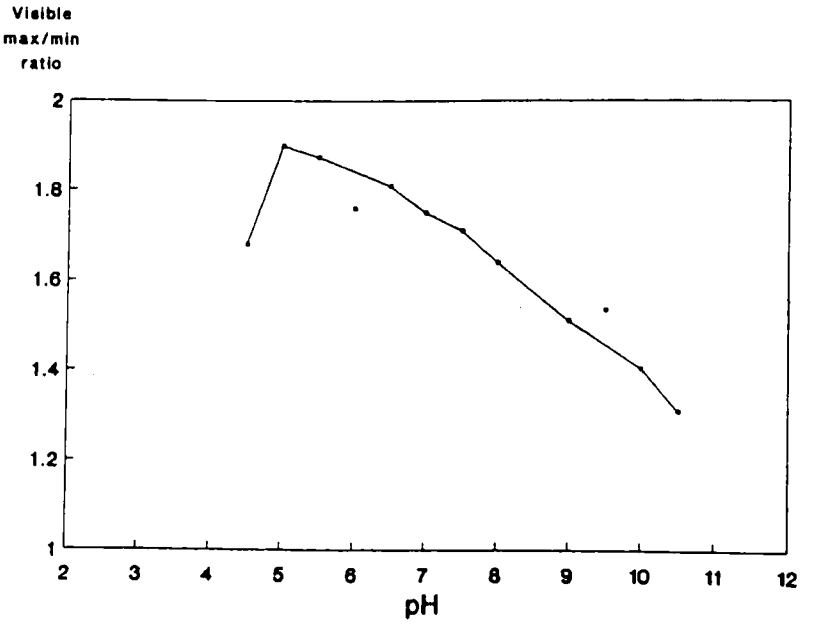

FIGURE 3 pH-Dependence of the ratio of the absorbancies at the maximum – ~480–482 nm – (max) and minimum – ~390 nm – (min) wavelengths of the visible spectra. Each point on this plot is derived from a spectrum (such as in Figure 1) of a MnSOD solution in one of the appropriate buffers (see Methods).

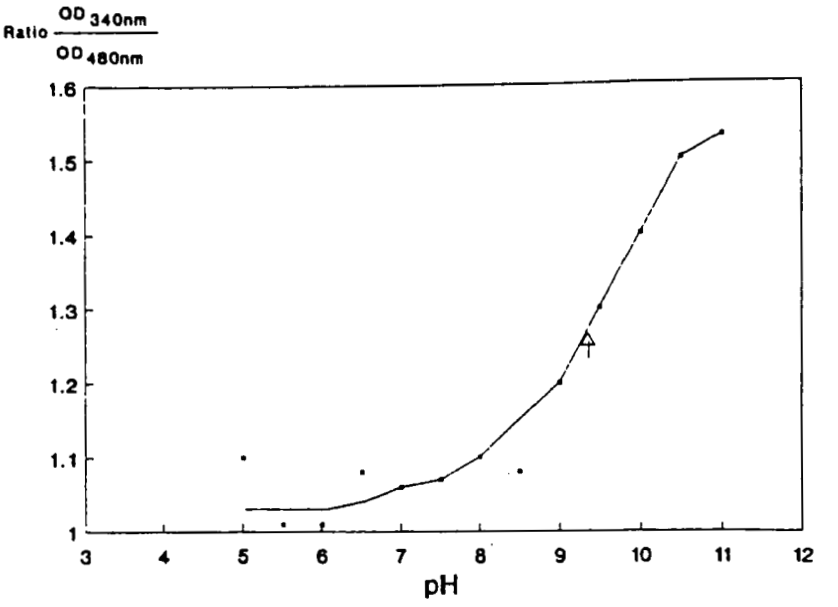

FIGURE 4 pH-Dependence of the ratio of the absorbancies at 340 and at 480 nm – the maximum of the visible spectrum. The wavelength at 340 nm was chosen as representing a transition between the visible and ultraviolet regions of the spectrum. Each point on this plot is derived from a spectrum (such as in Figure 1) of a MnSOD solution in one of the appropriate buffers (see Methods). The arrow indicates the position of the pK_a value derived from the apparent titration curve generated by the data.

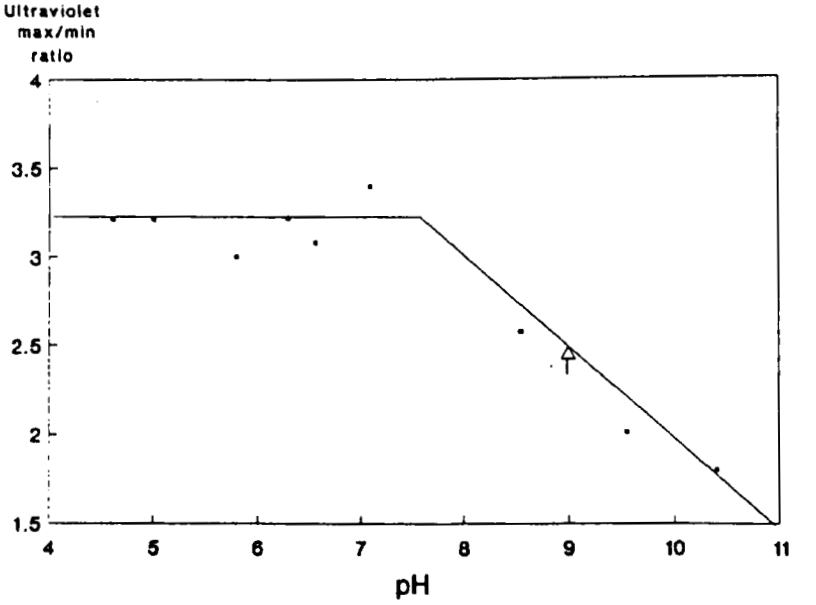

FIGURE 5 pH-Dependence of the ratio of the absorbancies at the maximum – 282 nm – (max) and minimum – ~251 nm – (min) wavelengths of the UV spectra. Each point on this plot is derived from a spectrum (such as in Figure 1) of a MnSOD solution in one of the appropriate buffers (see Methods). The arrow indicates the position of the pK_a value derived from the apparent titration curve generated by the data.

i.e., at 282 nm, is shown in Figure 6. There seems to be little change with pH, except perhaps for a small increase in the values in the range 6–8. Table I is a summary of the absorption coefficients in both the visible and UV regions of the spectrum in the invariant pH range 6.5–8, as well as at the extrema of pH: 5 and 10.5.

pH-Dependence of the Stability of MnSOD

No change in enzymatic activity measured at pH 7.8 (see Methods) was observed for MnSOD, which had been exposed for 24 hours at 25° C to various buffered solutions (see above) in the pH range 5–10.5 (at pH 4.5 – under these conditions – a decrease of 12% in the activity was observed). At 37° C there was also little effect of the pH on the enzymatic activity of MnSOD after 24 hours (Table II). More drastic conditions – incubation at 60° C for up to a week – were applied at 2 pH values: 6.45

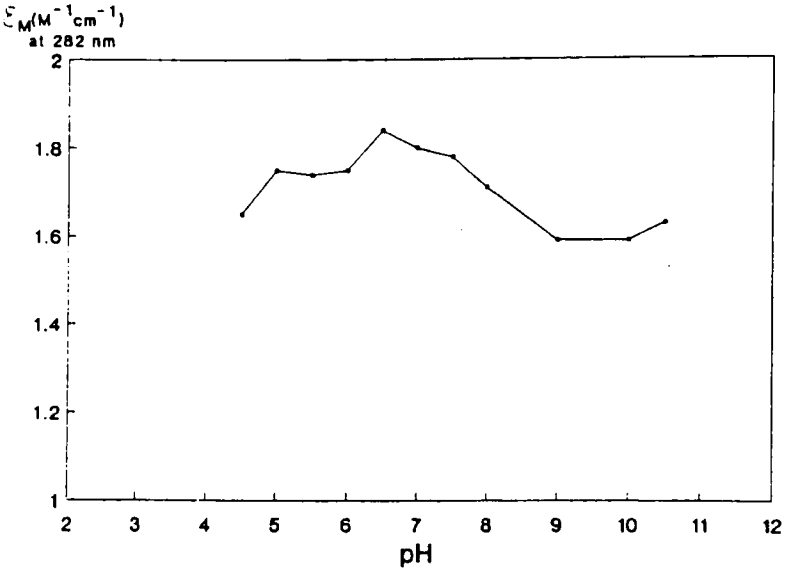

FIGURE 6 pH dependence of the extinction coefficients – ε_M – at the maximum wavelength of the UV region of the absorption spectrum. The data were obtained by dividing the absorbancies – measured as in Figure 1 – by the protein concentration.

TABLE I
Spectral characteristics of MnSOD as a function of pH

pH	Visible region			Ultraviolet region		
	λ_{max} (nm)	$10^{-3}.\varepsilon_M$ ($M^{-1}.cm^{-1}$)	Ratio (max/min)	λ_{max} (nm)	$10^{-5}.\varepsilon_M$ ($M^{-1}.cm^{-1}$)	Ratio (max/min)
5	484	2.185	1.90	282	1.75	3.21
6.5–8	483	2.520	1.64*	282	1.80	2.90*
10.5	479	1.490	1.31	282	1.63	1.80

*At pH 8.

TABLE II
Stability of MnSOD at 37°C

pH (Buffer)	Residual activity* (%)
4.5 (acetate)	80
6.0 (MES)	94
8.0 (HEPES)	90
10.0 (carbonate)	100

*The original activity (at a 300-fold dilution) was 30 U/ml.

and 8.2. The results shown in Figure 7 are based on the activity remaining in solution. Most of the losses in activity were due to precipitation of denatured protein, since no significant changes were observed in the specific activity of the supernatant. However, several degradation bands were observed in SDS-PAGE gels (not shown) and minor changes in the pattern of isoforms also appeared in activity gels, in particular at pH 8.2 after incubation of 4 or more days at 60°C – same as for "aged" MnSOD (Figure 8).

Dissociative Behavior of Tetrametric in MnSOD under Denaturing Conditions

Under native conditions the apparent molecular weight of human liver MnSOD is ~89,000 daltons, as determined by sedimentation equilibrium,[1] i.e., it is a tetrameric

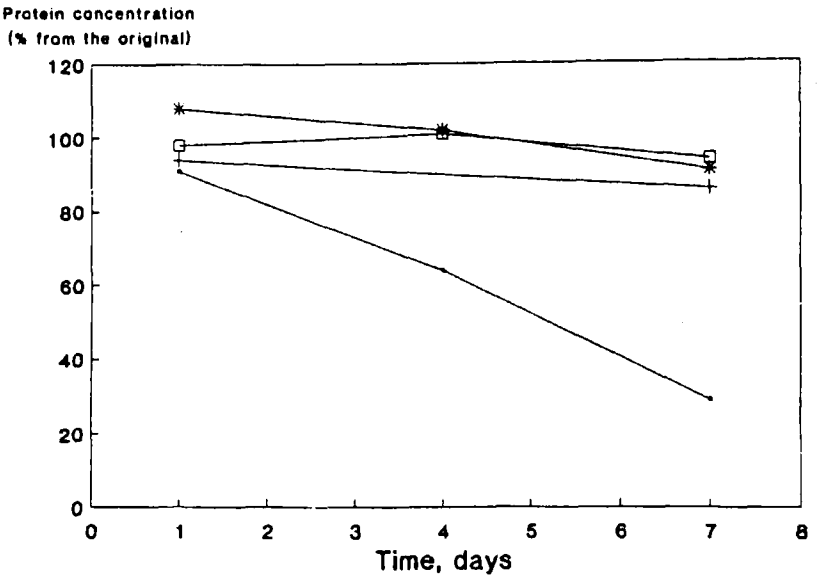

FIGURE 7 Stability of MnSOD at 37 and 60°C at 2 different pH values. The data shown refer to the protein concentration remaining in the supernatant after incubation for the indicated time at the 2 temperatures. The specific activity of the soluble protein was unchanged.

pH6.45: 37°C ⊟ 60°C ⊢
pH 8.2: 37°C ⊣ 60°C ■

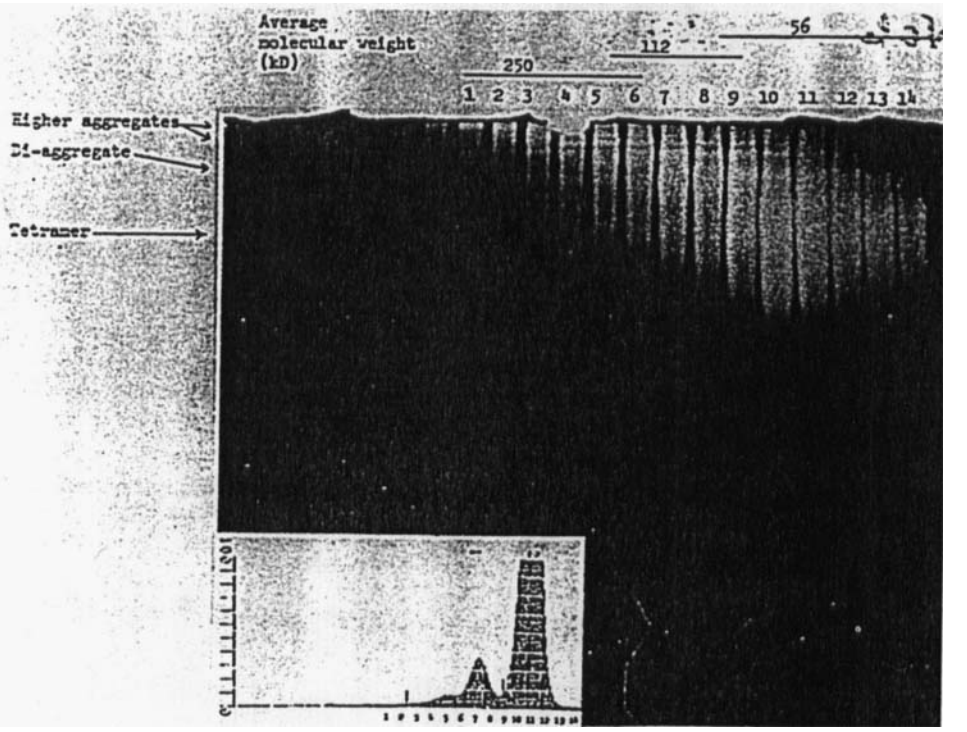

FIGURE 8 Pattern of MnSOD isoforms obtained on a non-denaturing activity gel. Hundred μl of a sample (20 mg/ml) of a several months-"aged" MnSOD was run on a Superose 12 column in 20 mM Tris-HCl, pH 7.8, containing 150 mM NaCl (for running conditions, see Methods). One minute fractions were collected as shown in the inset. Aliquots of each fraction (20 μl) were applied to a 10% acrylamide native gel, which was stained for activity (see Methods). The three molecular weight regions indicated ($\sim$240, 112 and 56 kD) correspond, respectively, to high molecular weight, di-aggregate (peak 1), and tetramer (peak 2) fractions separated on the column (see Inset). The isoforms observed on the activity gel are thus a reflection of the various molecular weight fractions separated on the column.

protein. However, under these conditions, the apparent molecular weight by gel permeation chromatography on a Superose 12 column (Pharmacia Fine Chemicals) is $\sim$ 57,000 daltons (Figure 9). A small peak ($<$ 3% of the protein) is also observed; it has an apparent molecular weight of $\sim$ 110kD and is a 'diaggregate' of the native tetrameric form. Upon aging for several months at room temperature, or under more drastic conditions, i.e., incubation at elevated temperatures as above, the amount of this di-aggregate increases and, concomitantly, a "ladder" of this and higher molecular weight isoforms can be detected in activity gels (Figure 8). Various dissociative conditions were applied in order to detect the dimeric and monomeric isoforms of MnSOD. Thus, at 4–6 M GuCl, MnSOD is mainly in the monomeric form, whereas at intermediate GuCl concentrations (1–2 M) it is in the dimeric form (Figure 10). Under these denaturative conditions there seems to be no appreciable loss of Mn, as judged from the absorption spectra in the visible region. However, the shape of the spectra, as well as the extinction coefficients, are significantly affected (Figure 11 and Table III).

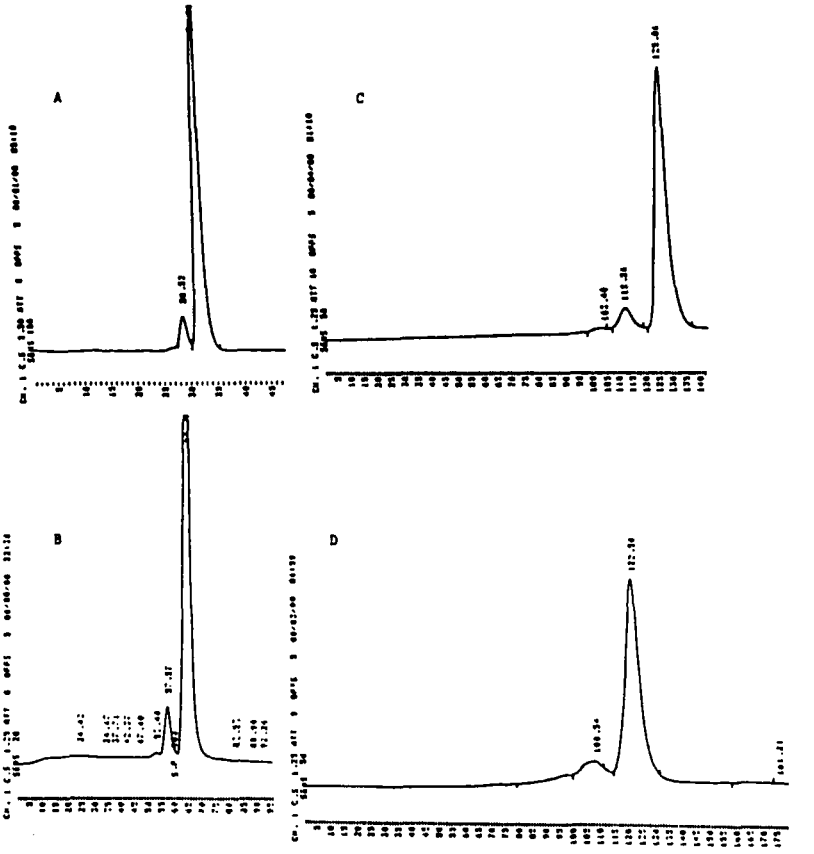

FIGURE 9 Gel permeation chromatography of MnSOD at various GuCl concentrations. Samples were either dialyzed or brought to the appropriate GuCl concentration with 7.5 M GuCl in buffer (see Methods). Before being injected onto the Superose 12 column all samples were preincubated for 1 hour at room temperature. Prior to running the standard molecular weight markers and the samples the column was thoroughly equilibrated at the appropriate GuCl concentration. The apparent molecular weights calculated for MnSOD peaks from calibration curves at the various GuCl concentrations are as follows: A. Buffer alone, 105 and 57 kD; B. 2 M GuCl, 53 and 32 kD; C. 4 M GuCl, 39 and 24 kD; D. 6 M GuCl, 45 and 23 kD.

DISCUSSION

The pH-dependence of the visible absorption spectrum of MnSOD implies a strong influence of ionizing groups present in the neighborhood of the chromophore, i.e., the Mn(III) ion. As seen in Figure 3, two pK_a values can be derived from the two limbs of the pH-dependence profile. The lower $pK_a = 5.9$ can be ascribed to the ionization of a histidine residue, probably one of the three histidines vicinal in the primary structure to the Mn ion ligand (His 26 – His 28 in the sequence of *T. thermophilus*, whose structure has been determined at a 2.4 Å resolution).[10,11] The identity of the second ionizing group is less obvious, since it seems also to play a role in the UV region, at least with respect to the maximum/minimum ratio of absorbancies (Figure 5). It could be a tyrosine residue, such as Tyr 34 (Tyr 36 in the thermophilic enzyme),

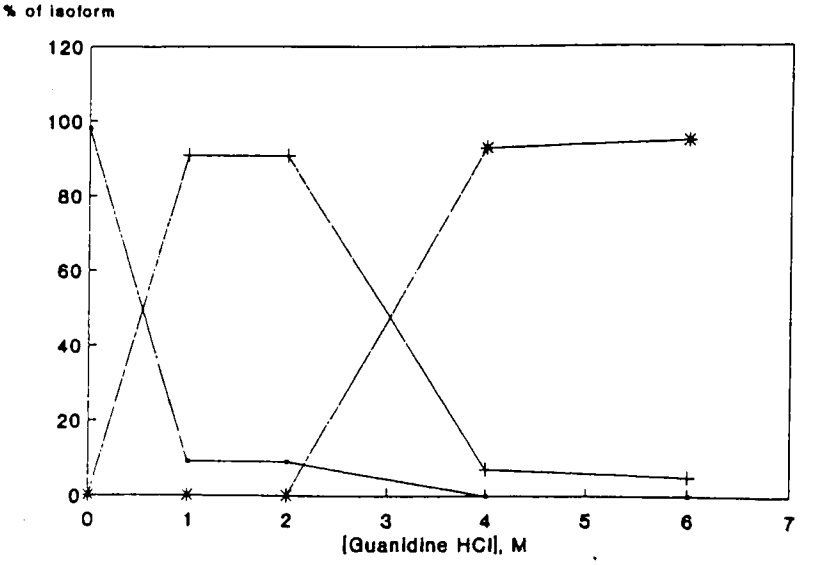

FIGURE 10 Effect of GuCl on the dissociation of MnSOD: percent of monomer, dimer and tetramer as a function of GuCl concentration. From elution profiles (on Superose 12) at the various GuCl concentrations (see Figure 9) the percent of: -•- monomer (22–24 kD), -+- dimer (36–45 kD), except at 2 M GuCl: 32 kD) and -■- tetramer (53–57 kD, an anomalous value — see Discussion) could be derived.

since it has been suggested to be one of the aromatic residues that line the cavity in which the Mn ion is embedded.[11] Support for this interpretation is based on the circular dichroism data on MnSOD from several sources, that seem to imply that the aromatic amino acids contributing to the intensity are situated close to the Mn-containing active center.[12]

As for the characteristics of the visible spectral bands in the pH range 6.5–8, the human enzyme is very similar to the other MnSODs characterized so far.[12] Thus, at 482 nm we find a molar absorption coefficient of 630 $M^{-1}.cm^{-1}$ per atom of manganese, and if one takes into account that the occupancy of the Mn sites is ~0.8 – as determined by atomic absorption (unpublished data), this value could even be higher by 25%, i.e., up to ~800 $M^{-1}.cm^{-1}$. This is in excellent agreement with the values reported for the enzymes from *E. coli* or thermophilic bacteria.[12] Our value is

TABLE III
Spectral characteristics of MnSOD as a function of GuCl concentration

[GuCl] (*M*)	Visible region			Ultraviolet region		
	λ_{max} (nm)	$10^{-3}.\varepsilon_M$ ($M^{-1}.cm^{-1}$)	Ratio (max/min)	λ_{max} (nm)	$10^{-3}.\varepsilon_M$ ($M^{-1}.cm^{-1}$)	Ratio (max/min)
0	483	2.25	1.57	282	1.69	2.62
2	481	2.20	1.54	281	1.70	2.64
4	479	1.74	1.35	280	1.31	2.59
6	480	1.42	1.32	280	1.19	2.97

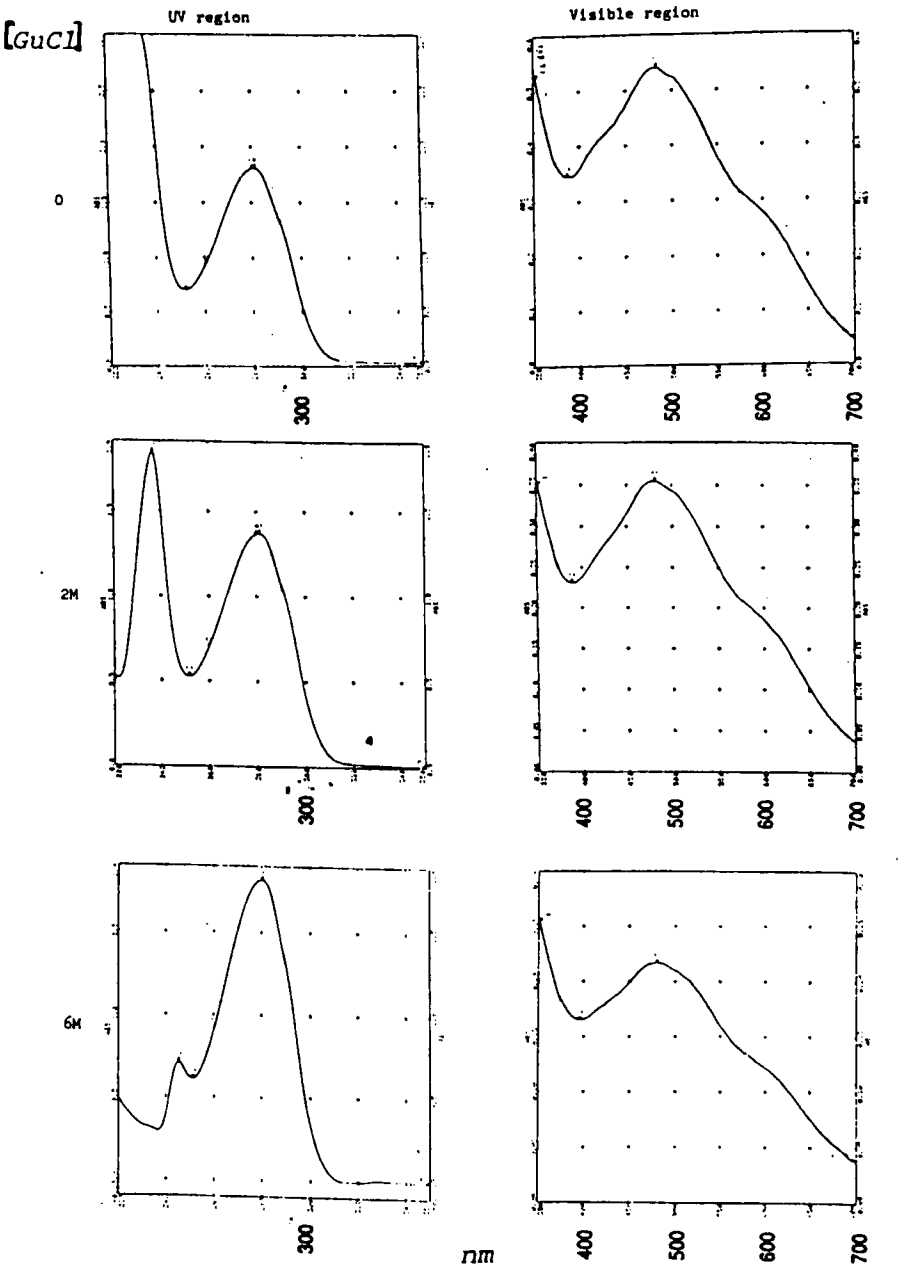

FIGURE 11 Light absorption spectra of MnSOD at 3 different GuCl concentrations. The left and right panels contain spectra in the UV and the visible regions at [GuCl] = 0, 2 & 6 M, respectively. For the preparation of the samples, see Methods and Legend of Figure 9. In the UV region, spectra were measured between 220 and 350 nm and in the visible region between 350 and 700 nm.

somewhat higher than the value reported for the human liver enzyme,[1] probably due to the difference in the methods used for protein determination.

MnSOD was found to be rather stable at 25 and 37° C – up to 24 hours (Table II) in the pH range 5–10.5. From pH 4.5 and below (not shown) there was a tendency for the Mn to leak out of the protein and for the resulting apoprotein to precipitate, as has been also previously reported by others for bacterial MnSODs.[13] The stability of MnSOD was studied under harsher temperature conditions, in the pH range (6.5–8) where it seemed most stable, based on the absorption data (see Figure 3). The results (Figure 7) indicate that the high temperature (60° C) affects the enzyme more at pH 8.2 than at pH 6.5 and that the denaturation is caused mainly by Mn loss (since there was no change in specific activity of the enzyme remaining in solution). Thus, the metal is most probably one of the best stabilizing factors in this protein. When it is lost, either as a result of low pH or high temperature, the apoprotein denatures and subsequently precipitates. Since GuCl is a strong denaturant, it was of interest to follow its effect on the stability of MnSOD. The results (Table III and Figure 11) indicate that although there is a distinct change in the shape of the spectrum, the metal ion remains bound to the protein. This is in agreement with the results of Bjerrum[12] for the *S. cerevisiae* MnSOD, who inferred from CD data that the addition of GuCl induced no change in activity in the neutral to weakly acidic pH range.

Because of the anomalous molecular weight obtained for human MnSOD by gel permeation chromatography under native conditions, dissociative conditions have been applied. Although at 4 M and 6 M GuCl the enzyme is fully dissociated — monomeric, at intermediate GuCl concentrations (1 and 2 M) one can clearly observe dimeric and even some tetrameric species (Figure 10). This implies that the native tetrameric protein is not compact and globular, such as would have been expected if it had a Stokes radius corresponding to its molecular weight.[14] This conclusion is in full agreement with the unusual structure found by Ludwig and coworkers[11] for the tetrameric MnSOD from *T. thermophilus*: a hollow molecule held together by 2 rather than 3 kinds of interfaces, implying a loose dimer of dimers. It is therefore expected that a similar structure will be found for the human enzyme, whose 3-dimensional structure determination is under way.[15,16]

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