Isolation and characterization of a novel monomeric zinc- and heme-containing protein from bovine brain

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Received 30 July 1996; revised version received 2 September 1996

Abstract An acidic zinc- and heme-containing protein was isolated from the soluble fraction of bovine brain and has been purified to homogeneity. The zinc-heme protein is a monomeric globular protein with a molecular mass of 31 200 Da as determined by electrospray mass spectrometry. The protein was isolated with 0.90 ± 0.05 zinc per protein and with substoichiometric amounts of heme. Amino acid sequences of four peptides (ca. 20% of the protein) were determined and the comparison of these sequences with those of protein and gene sequence databases revealed no significant correlation with any known protein. Thus, it is concluded that it is a novel protein of currently unknown biological function.

Key words: Zinc protein; Heme; Brain tissue; Electrospray mass spectrometry

1. Introduction

Among the transition metals in the brain, zinc is the second most abundant trace metal after iron. Besides its role as a structural and/or catalytic component in proteins [1], it is involved in replication, transcription and translation of the genetic material [2,3]. Zinc is specifically secreted by certain neurons of the central nervous system and likely serves as an endogenous signaling substance [4]. In the course of our studies on the brain-specific zinc- and copper-containing member of the metallothionein gene family, metallothionein-3 or neuronal growth inhibitory factor [5,6], we were able to purify another low molecular weight zinc-containing protein from the soluble fraction of bovine brain. Further characterization showed that this monomeric protein contains zinc and was purified with substoichiometric amounts of heme, a combination which is known in multiprotein complexes like cytochrome *c* oxidase [7], but has never been reported for a monomeric protein. Here we report the purification and first characterization of the novel zinc- and heme-containing protein isolated from bovine brain.

2. Materials and methods

Bovine brains were obtained immediately after slaughter and stored at −20°C before use. All purification steps were performed at 4°C unless otherwise stated. In order to remove metal impurities buffers were passed over a Chelex 100 (Biorad) column.

2.1. Chromatographic separations

Conventional column chromatography was performed using a LKB chromatography unit. Fast protein liquid chromatography (FPLC) was performed with a Pharmacia 500/LKB system equipped with a programmable gradient controller. For ion exchange chromatography DEAE-Sephacel resin from Sigma in conventional columns and a Millipore DEAE MemSep 1000 chromatography cartridge attached to the FPLC system were used. Gel permeation chromatography was performed Sephadex G-75 (Pharmacia) columns. Protein pools from each chromatographic step were concentrated and desalted in Amicon ultrafiltration cells (Molecular/Por membranes from Spectrum, molecular weight cut-off 20 kDa). Analytical gel permeation was performed on a Superdex 75 (1.6×60 cm) column (Pharmacia) attached to the FPLC system. The column was calibrated using the following proteins obtained from Sigma: cytochrome *c* (12.4 kDa), myoglobin (17.8 kDa), α-chymotrypsinogen A (25.0 kDa) and bovine serum albumin (66.0 kDa). The Superdex 75 column was eluted with 20 mM Tris-HCl, 0.1 M NaCl, pH 7.6, at a flow rate of 1 ml/min at 20°C. The purity of the isolated protein was examined by both native and SDS polyacrylamide gel electrophoresis with subsequent Coomassie blue staining.

2.2. Metal analysis

For the determination of metal content small aliquots of the samples were diluted with 0.1 M HCl and analyzed using flame atomic absorption spectrometry (Instrumentation Laboratory, IL Video 12).

2.3. Amino acid analysis

The amino acid composition of the protein (samples of 50–200 pmol) was analyzed using an Applied Biosystems Inc. 420A/H amino acid analyzer. The cysteine content was determined as cysteic acid after performic acid oxidation of the sample. The tryptophan content was determined both by quantitative amino acid analysis in the presence of dodecanethiol and by magnetic circular dichroism (MCD) spectroscopy using the typical MCD band of the tryptophan residue at 290 nm [8]. The latter analysis was performed on the essentially heme-free protein.

2.4. Peptide analysis

Cyanogen bromide cleavage was performed according to Bonkovsky et al. [9] with minor modifications. The peptides obtained upon CNBr cleavage were applied to reversed-phase HPLC (Aquapore RP-300 C_8 column, Brownlee Labs). Conditions were: buffer A 0.1% (v/v) TFA; buffer B 0.075% (v/v) TFA, 80% (v/v) acetonitrile. A linear gradient of 0–60% B (1.2 ml/min, 90 min) was used for the separation of peptides. For the tryptic digestion, the protein (0.5 mg protein in 200 µl 0.1 M NH_4HCO_3, 0.1 mM $CaCl_2$, pH 8.3) was heat-denatured at 95°C for 10 min. Subsequently, 5 µg of trypsin (sequencing grade, Boehringer, Mannheim) was added and the sample was incubated for 1 h at 37°C. Another 5 µg of trypsin were added and the reaction was stopped after 30 min by acidification. Separation of peptides was performed on a C-18 reverse phase HPLC (Aquapore ODS 300 Brownlee Labs.) with a linear gradient of 0–40% acetonitrile. Buffers A and B were the same as described above for cyanogen bromide peptides. Peptides were sequenced by automated Edman degradation using an Applied Biosystems Inc. 470A/900A protein/peptide sequencer equipped with an 120-A on-line amino acid analyzer.

The determined peptide sequences have been used in database

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Abbreviations: TFA, trifluoroacetic acid; MCD, magnetic circular dichroism; ESI MS, electrospray ionization mass spectrometry

PII S0014-5793(96)00997-0

searches for identity with known protein or gene sequences. The following databases have been searched: protein databases SwissProt (version 33.0, updated weekly), PIR (NBRF protein database version 48.0), NRL_3D (Brookhaven Data, version 20.0) and the gene databases EMBL (version 46.0, updated weekly) and GenBank (version 94.0). FASTA and TFASTA algorithms [10] (GCG Genetics Computer Group sequence analysis software package version 8.1) were used with a gap creation penalty of 2.0 and a gap extension penalty of 2.0.

2.5. Peroxidase assay

Peroxidase activity was measured according to Ngo and Lenhoff [11]. For comparison horseradish peroxidase (HRP) and myoglobin (Mb) (both from Sigma) were tested as well. The assay was performed in 0.1 M potassium phosphate at pH 6.5 and pH 7.4. Due to the low heme content of the zinc-heme protein the comparison is based on the heme concentration assuming $\varepsilon_{soret} = 130\ mM^{-1}\ cm^{-1}$ and not on the protein concentration.

2.6. Polyacrylamide gel electrophoresis (PAGE)

SDS-PAGE was performed according to Laemmli [12] using precast single percentage gels (12%) obtained from BioRad. The proteins were stained with Coomassie blue G-250. Native PAGE was performed using the same gels but excluding SDS and reducing agents from the buffers and avoiding the heating step. Molecular weight markers used for SDS-PAGE were: ribonuclease A (13.7 kDa), myoglobin (17.8 kDa), carbonic anhydrase (29.0 kDa), glyceraldehyde-3-phosphate dehydrogenase (36.0 kDa) and bovine serum albumin (66.0 kDa) (all from Sigma). Heme staining of heme proteins in native gels was performed using 3,3',5,5'-tetramethyl-benzidine/H_2O_2 according to the method of Thomas et al. [13]. Subsequently, the gels were destained and stained for proteins with Coomassie blue. For the heme staining of the zinc-heme protein (Fig. 4) larger amounts of protein were loaded on the gel due to the low heme content.

2.7. Magnetic circular dichroism (MCD) spectroscopy

The MCD measurements for quantification of tryptophan were performed at room temperature with a Jasco J-500C spectropolarimeter interfaced to an IBM-PS/2 computer and equipped with an electromagnet delivering a constant magnetic field of 1.5 T.

2.8. Electrospray mass spectrometry (ESI MS)

Molecular masses of the zinc-heme protein were determined by ESI MS using a Sciex API III$^+$ triple-quadrupole electrospray instrument (Sciex, Ontario, Canada). The purified protein was exchanged into 10 mM ammonium acetate buffer (pH 6.4), yielding a protein concentration of about 15–20 pmol/μl. For the measurements of the denatured protein at pH 3.5, the sample was mixed with 50% water/50% acetonitrile/0.1% acetic acid (v/v) in a 1:1 ratio and 5 μl were flow-injected into a stream of 50% water/50% acetonitrile/0.1% acetic acid. Spectra were acquired in the positive-ion mode using an ion spray voltage of approximately 5000 V and an orifice voltage of 70 V. For ESI MS analysis at pH 6.4, the aqueous protein solution was mixed with acetonitrile (1:1) and injected into a carrier solution consisting of 90% water/10% acetonitrile. The orifice voltage was slightly reduced to 60 V to obtain gentle ionization conditions; all other acquisition parameters were identical. The reconstituted molecular mass profiles were obtained by using the Sciex deconvolution algorithm.

3. Results and discussion

By following the low molecular weight zinc-containing proteins in the soluble fractions of bovine brain, we were able to isolate a novel protein. In the following a purification scheme for this protein and its characterization are presented.

3.1. Protein isolation

The protein was purified to homogeneity using a combination of several anion exchange and gel permeation chromatographic steps (Fig. 1).

Step 1 – Homogenization and bulk anion exchange chromatography: 2000 g of bovine brain tissue was homogenized with 3 v/w of 20 mM Tris-HCl, pH 7.6, and stirred for 30 min,

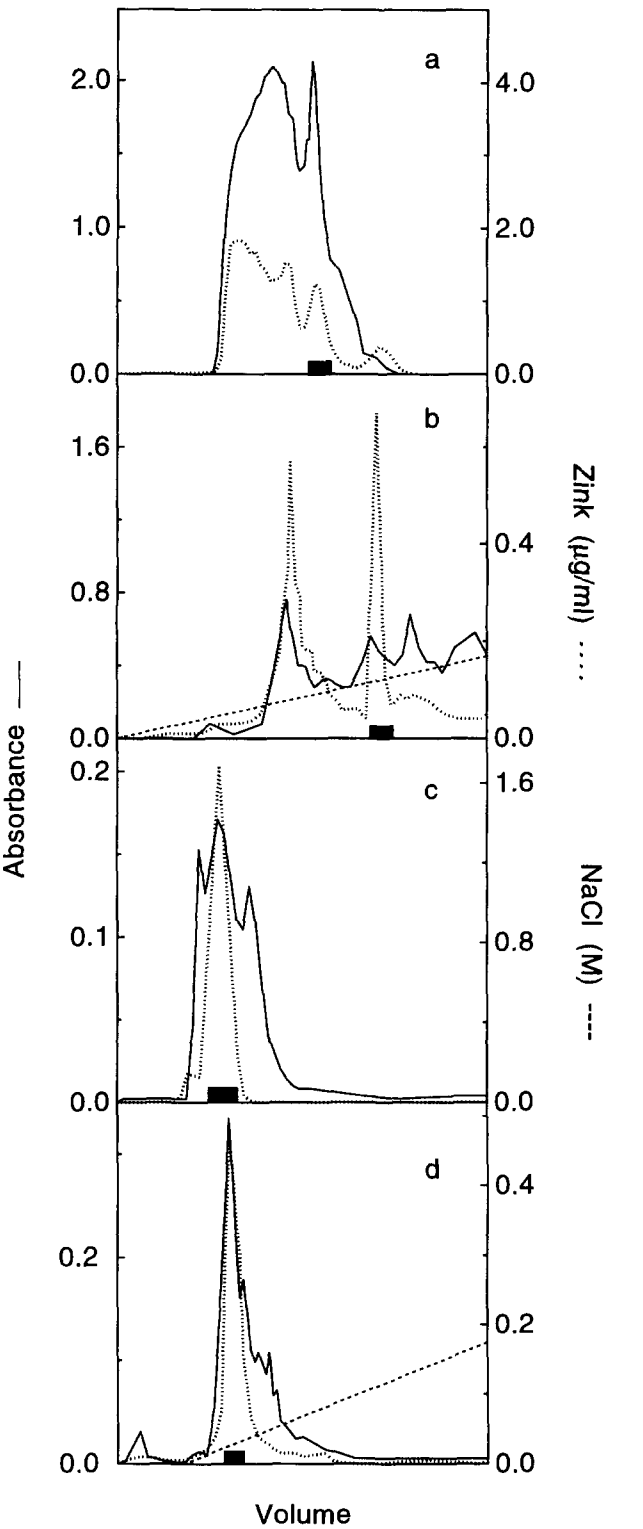

Fig. 1. The purification steps for the zinc-heme protein from the soluble fraction of bovine brain. In all chromatographic steps 20 mM Tris-HCl, pH 7.6, was used. (a) Gel filtration (Sephadex G-75) in 0.1 M NaCl; (b) ion exchange (DEAE-Sephacel) with a linear gradient of 0–0.2 M NaCl; (c) gel filtration (Sephadex G-75) in 0.1 M NaCl; (d) anion exchange (DEAE MemSep 1000 cartridge) with a linear gradient of 0–0.2 M NaCl. ■, pooled fractions.

then centrifuged for 1 h at 20000×*g*. A suspension of DEAE-Sephacel (ca. 1 kg) in 20 mM Tris-HCl pH 7.6 was added to

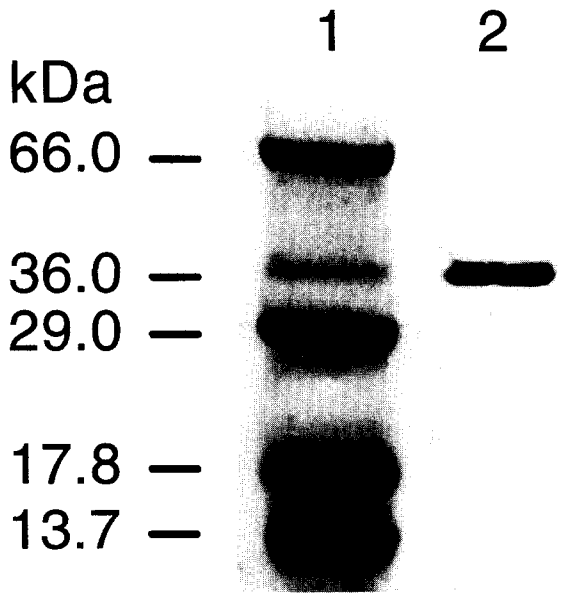

Fig. 2. SDS-PAGE of the zinc-heme protein. Lane 1, molecular weight markers; lane 2, 7 μl of zinc-heme protein after purification step 5.

the supernatant and stirred for another 30 min. Subsequently, the slurry was centrifuged in a Sorvall H6000-A centrifuge for 15 min at 2400×*g*. The supernatant was discarded. The resin with the adsorbed proteins was resuspended in three volumes of the same buffer and recentrifuged. The latter washing step was repeated three times. Subsequently, the slurry was poured into a column (50×4 cm) and eluted with 20 mM Tris-HCl, 0.3 M NaCl, pH 7.6. The zinc-containing fractions were pooled (data not shown).

Step 2 – Gel permeation chromatography: The pool from step 1 was concentrated by ultrafiltration and applied to a Sephadex G-75 column equilibrated with 20 mM Tris-HCl, 0.1 M NaCl, pH 7.6. The elution of the proteins was followed both spectroscopically (at 254 nm) and by analysis of the zinc content. The zinc-containing chromatographic peak (between 20–40 kDa) was pooled (Fig. 1a). In this chromatographic step most of the abundant hemoglobin could be removed.

Step 3 – Gradient anion exchange chromatography: The pool from step 2 was concentrated and desalted by washing with 20 mM Tris-HCl, pH 7.6, in an ultrafiltration cell and loaded onto a DEAE-Sephacel column equilibrated with 20 mM Tris-HCl, pH 7.6. About 1/2 column volume of the equilibration buffer was eluted before the salt gradient was started. Proteins were eluted with a linear gradient of 0–0.2 M NaCl (20 mM Tris-HCl, pH 7.6). The zinc-containing peak eluted at about 0.11 M NaCl was retained (Fig. 1b).

Step 4 – Gel permeation chromatography: The concentrated pool from step 3 was applied to a Sephadex G-75 column equilibrated with 20 mM Tris-HCl, 0.1 M NaCl, pH 7.6 (Fig. 1c). The major zinc-containing peak was processed further.

Step 5 – Gradient anion exchange chromatography: The concentrated fractions from step 4 were desalted by dialysis (24 h) against 5 mM Tris-HCl, pH 7.6. Aliquots of the sample were applied to a DEAE MemSep 1000 cartridge attached to a Pharmacia FPLC system and equilibrated with 10 mM Tris-HCl, pH 7.6, at room temperature. A linear gradient between 0–0.2 M NaCl was used. The zinc-heme protein eluted at about 0.05 M NaCl (Fig. 1d). On average about 15 mg of the purified protein was obtained from 2000 g of brain tissue. The purity of the isolated zinc-heme protein was examined using native and SDS-PAGE. In both gels a single band was detected (Figs. 2 and 4).

3.2. Protein characterization

In all preparations the purified protein showed a Soret absorption maximum, characteristic for heme proteins, occurring at 407–412 nm. Based on the quantitative amino acid analysis and assuming a molar extinction coefficient of $\varepsilon_{soret} = 130\ mM^{-1}\ cm^{-1}$, the amount of heme in the different preparations was estimated to be in the range of 0.05–0.12 equivalents per protein monomer. The results suggest the presence of this cofactor in the protein (see below).

Since the zinc-heme protein was isolated from the soluble fraction of brain homogenate and in the absence of detergents it is unlikely to be a membrane associated protein. The electrophoretic behavior of the zinc-heme protein on native polyacrylamide gels together with its relatively strong binding to anion exchange resin indicate that the net charge of the protein is negative. From SDS-PAGE an apparent molecular mass of 34 kDa was calculated (Fig. 2). As a similar apparent molecular mass of 33 kDa for the native protein was also obtained in analytical gel filtration studies (data not shown), it is concluded that the zinc-heme protein is monomeric and of a globular shape. The purified protein was analyzed for zinc and copper by flame-atomic absorption spectrometry. Based on the protein concentration determined by quantitative amino acid analysis 0.90 ± 0.05 mol zinc per mol protein was found indicating the presence of a zinc binding site. No copper was detected.

3.2.1. Electrospray ionization mass spectrometry. ESI MS is an established technique to obtain the exact molecular weight of smaller proteins and peptides [14,15] and has also been shown to be effective in characterizing noncovalent interactions [16] including metal-protein interactions [17,18].

Fig. 3 shows the ESI MS spectra of the purified zinc-heme protein at pH 3.5 and pH 6.4. Analysis of the protein under denaturing conditions at pH 3.5 (top panel) yielded a mass spectrum with only one predominant protein form of an average molecular mass of 31 200 Da. The spectrum acquired at pH 6.4 (bottom panel) contains two distinct charge state contributions. The minor distribution at relative low mass-to-charge values is similar to that acquired at pH 3.5 and corresponds to the unfolded, denatured state of the protein. The more intense distribution at higher m/z values represent the folded state of the protein and has split peaks. Mass conversion of the latter charge-state distribution gave a minor signal at 31 207 Da and a major signal at 31 270 Da, corresponding to the protein with and without one zinc ion bound, respectively, as the mass differences reveals (found mass difference 63 Da, expected mass difference 63.38 Da). These results clearly establish the presence of one single zinc binding site in the native protein, and there is no indication for any other bound metal ion(s).

3.2.2. Amino acid composition and partial sequencing. The amino acid composition of the purified protein (Table 1) shows a relatively high content of hydrophobic amino acids with proline, alanine, valine, methionine, isoleucine, leucine and phenylalanine representing about 35% of the total amino

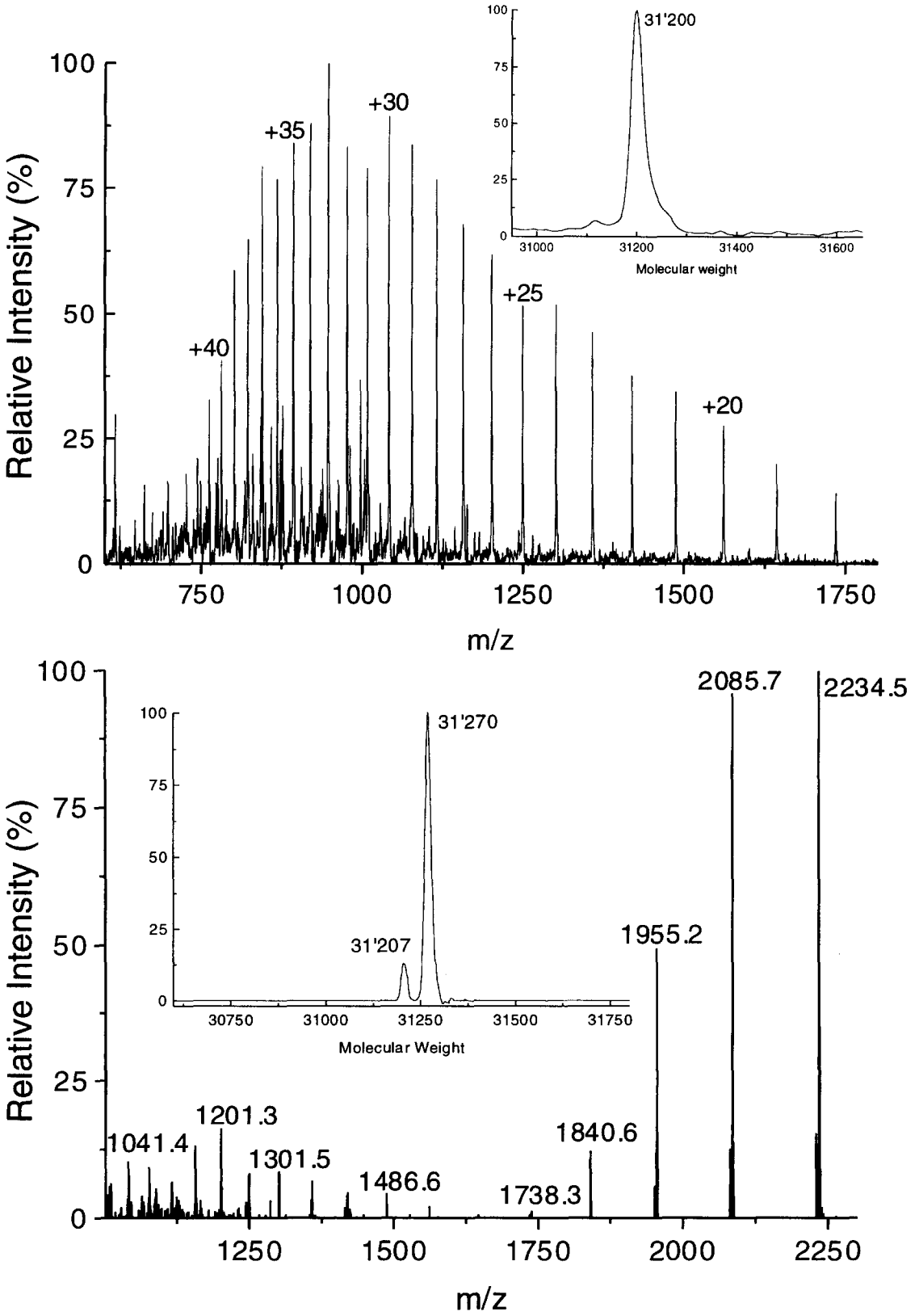

Fig. 3. Electrospray mass spectra of zinc-heme protein at pH 3.5 (top) and pH 6.4 (bottom).

acids. Attempts to perform amino acid sequencing by Edman degradation of the purified protein were unsuccessful indicating that the N-terminus is blocked. Partial sequence information was obtained following the cleavage of the protein with cyanogen bromide or trypsin. Four peptides were isolated by HPLC and sequenced (Table 2). A database search with the obtained peptide sequences using FASTA for the SwissProt and PIR protein databases and TFASTA for the EMBL and

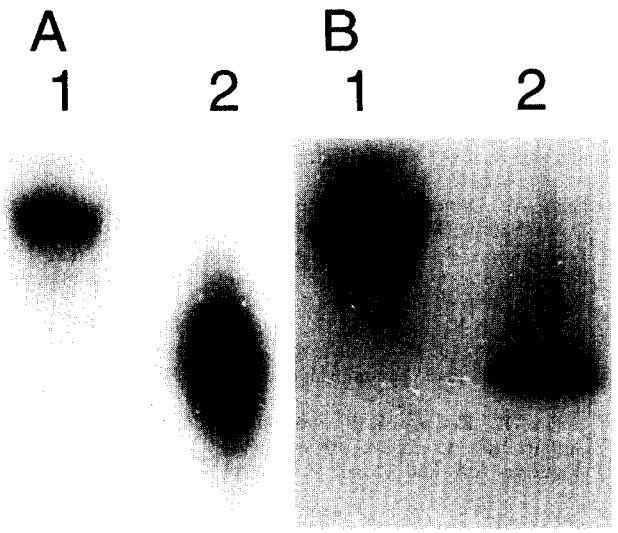

Fig. 4. Native PAGE of the zinc-heme protein. (A) Heme staining, lane 1, 5 μg bovine hemoglobin; lane 2, 60 μg zinc-heme protein; (B) Coomassie staining, lane 1, 5 μg bovine hemoglobin; lane 2, 7 μg zinc-heme protein. For details see Section 2.

GenBank nucleic acid databases revealed no significant match with any protein. The 40 best aligned database proteins of one peptide were not found among the 120 best aligned database proteins of the three other peptides. Moreover, an alignment of each peptide against the 120 best fitted database proteins of the three other peptides did not show any reasonable correspondence. Based on these criteria no significant similarity to any known protein exists, strongly suggesting the novelty of this protein.

3.2.3. Heme staining. To examine whether the heme constitutes an integral part of the protein the purified protein was stained on native polyacrylamide gels using 3,3′,5,5′-tetramethylbenzidine/H_2O_2. This method can detect low levels of the heme associated peroxidase activity and is used for the specific detection of heme proteins [13,19]. The native polyacrylamide gel (Fig. 4) was first stained for heme and, subsequently, with Coomassie blue for proteins. In both cases a single band at the same position was found. These results suggest that the protein is a new member of the family of heme proteins.

Table 1
Amino acid composition of the zinc-heme protein[a]

Amino acid	Residues (mol/mol protein)
Asx	27
Glx	33
Ser	19
His	7
Gly	20
Thr	14
Ala	28
Arg	13
Tyr	6
Val	24
Met	6
Phe	9
Ile	10
Leu	31
Lys	19
Pro	15
Trp	1
Cys	4
Total	286

[a]Determined based on a molecular mass of 31.2 kDa. Values for each amino acid represent the mean of 15 independent determinations performed on samples from several preparations, except Trp: $n = 3$, Cys: $n = 4$. The tryptophan content was also determined by MCD spectroscopy which revealed an identical value to the amino acid analysis. The cysteine was determined as cysteic acid.

Table 2
Amino acid sequences of four peptides of the zinc-heme protein

	1	10	20
I	(M)A G P N L I A I	G S S E S A Q K A L	X I
II	(M)K E A L E L L Q	L N I V E G	
III	Q X Q L Y V G V L	G S(K)	
IV	V Y E(A P)A(T)F G		

I and II were obtained after cyanogen bromide cleavage and III and IV after tryptic digestion. Methionine (M) has not been sequenced but is the cleaving site for CNBr. The parentheses indicate a tentative assignment, X stands for an unidentified amino acid.

The low heme content (0.05–0.12 equivalents) indicates heme loss during protein isolation. A similar heme instability during isolation has been reported for other heme proteins, e.g. brain nitric oxide synthase (68–83% heme content) [20] and initially also for soluble guanylate cyclase (0–20%) [21,22]. Although no apparent instability of the isolated zinc-heme protein was observed, attempts to isolate the protein with a higher heme content have so far been unsuccessful. Thus, variations in column materials, buffers, pH values and the presence of reducing agent (2-mercaptoethanol) lead to either a significantly lower yield of the protein with no increase in heme content or to a lower heme content. However, a zinc-to-protein ratio of about 1 was found consistently in the preparations.

As the heme staining method in gel electrophoresis relies on peroxidase activity, we tested the peroxidase activity of the zinc-heme protein at two pH values (6.5 and 7.4) and compared it to that of Mb and HRP. The measured peroxidase activity was referred to that of HRP, which was taken as 100%. The zinc-heme protein showed only very low peroxidase activity of 0.12% and 0.05% at pH 6.5 and 7.4, respectively, which compares well with that determined for Mb at both pH values, i.e. 0.26% and 0.07%. Thus, based on these results it is unlikely that the zinc-heme protein is a peroxidase.

The results of this work provide evidence for the existence of a novel monomeric protein which contains both zinc and heme. The presence of these two cofactors in a monomeric protein is unique and may thus represent a new protein structural motif. Interest in heme proteins found in the brain has increased considerably in recent years. Nitric oxide produced by the heme protein nitric oxide synthase has been found to be an important neurotransmitter [20,23–26] with soluble guanylate cyclase being the only receptor characterized thus far. Overall, although results concerning its tissue specific expression are lacking, the fact that this new protein has been isolated from mammalian brain tissue with a combination of cofactors that has never been reported before makes the zinc-heme protein a highly interesting subject for future studies.

Acknowledgements: The authors gratefully acknowledge Dr. P. Hunziker, Mrs. R. Sack and Mr. N.E. Birchler from the protein structure analysis core facility unit of the Institute of Biochemistry, University

of Zürich, for quantitative amino acid analysis, peptide sequencing and assistance with ESI MS. This work was supported by Swiss National Science Foundation Grants No. 31-40479-94 and 31-42181-94, and by the Horten Stiftung.

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