Characterization of Dimethylargininase from Bovine Brain: Evidence for a Zinc Binding Site†

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ABSTRACT: Dimethylargininase (EC 3.5.3.18) is involved in the regulation of the levels of the natural occurring free arginine derivatives L-N^{ω},N^{ω}-dimethylarginine and L-N^{ω}-methylarginine, which are reversible inhibitors of nitric oxide synthase. A dimethylargininase has been isolated from bovine brain tissue and was characterized by using immunological, kinetic, and spectroscopic techniques. Western blot analysis using polyclonal antibodies revealed that the enzyme is widely distributed in bovine with the highest relative concentrations found in brain and kidney tissue. A similar tissue distribution has also been reported for the other so far isolated dimethylargininase from rat kidney [Ogawa, T., Kimoto, M., and Sasaoka, K. (1989) *J. Biol. Chem. 264*, 10205−10209]. The bovine enzyme is a monomeric, globular protein (molecular mass ≈ 31.2 kDa) containing one tightly bound Zn^{2+} ion, which can be removed by dialysis against 1,10-phenanthroline. The determination of kinetic constants for both the native (holo-protein) and the zinc-depleted (apo-protein) enzyme at 37 °C established that the dimethylargininase is not a zinc hydrolase. The specific activity was 0.66 unit/mg for the holo-protein and 0.19 unit/mg for the apo-protein. The secondary structure determination of the native enzyme by circular dichroism revealed 41% α-helix and 32% β-sheet and β-turn structure. In the apo-enzyme, a small, but significant decrease in the α-helical content (5%) was observed, consistent with a marked decrease in enzymatic activity to 30%. Upon preincubation of both enzyme forms at 50 °C, only the holo-enzyme showed a residual enzymatic activity. In thermostability studies, a 7 °C lower apparent T_m value was observed for the apo-enzyme compared to the 66 °C for the holo-enzyme, suggesting that the zinc ion has a structure-stabilizing role. Besides the tightly bound zinc, additional Zn^{2+} ions inhibit the enzyme competitively with a K_i value of 2.0 μM. A possible interrelationship between dimethylargininase and nitric oxide synthase is discussed.

The three methylated derivatives of L-arginine, L-N^{ω}-methylarginine (monomethylated L-arginine, MMA),[1] L-N^{ω},N^{ω}-dimethylarginine (asymmetric dimethylated L-arginine, ADMA), and L-N^{ω},$N^{\omega\prime}$-dimethylarginine (symmetric dimethylated L-arginine, SDMA), have been known for a long time to constitute an integral part of the primary structure of a number of proteins after their posttranslational modification (*1*, *2*). These derivatives have also been found as free amino acids in various mammalian tissues (*3*, *4*) and body fluids (*1*, *5*, *6*). Increased levels of free MMA and ADMA in plasma and urine have been reported in several clinical disorders such as muscular dystrophy (*7*), renal failure (*6*), hypercholesteremia (*8*, *9*), and schizophrenia (*10*). Although much data have been collected on the concentrations of the free methylated L-arginine derivatives, not much is known about their catabolism in vivo.

Nitric oxide synthase (NOS) produces the radical nitric oxide (NO) from L-arginine (*11*), which is responsible for many essential biological functions (*11*−*13*). It has been shown, moreover, that the methylated derivatives of L-arginine, MMA and ADMA, but not SDMA, are reversible inhibitors of NOS (*6*, *12*, *14*, *15*). Thus, it has been suggested that MMA and ADMA may act as endogenous regulators of NOS in mammals (*16*−*18*). Since the demonstration that MMA inhibits the NO formation in endothelium cells (*19*), a pharmacological potential of both MMA and ADMA as inhibitors of NOS was recognized. Thus, in in vivo studies, the blood pressure of dogs with a septic shock caused by endotoxin treatment could be restored by intravenous MMA application (*20*). In rats, an intravenous administration of ADMA increased the mean arterial blood pressure, but the blood pressure decreased when intracerebroventriculary was applied (*21*).

Several years ago a L-N^{ω},N^{ω}-dimethylarginine dimethylaminohydrolase (also named dimethylargininase, EC 3.5.3.18) was purified from rat kidney (*22*, *23*). This monomeric enzyme (molecular mass ≈ 31 kDa) is involved in the regulation of the free MMA and ADMA levels in organisms. The dimethylargininase hydrolyzes MMA and ADMA to form L-citrulline and dimethylamine, and L-citrulline and monomethylamine, respectively, while other arginine analogues cannot serve as substrates (*22*, *23*). Based on

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[1] Abbreviations: MMA, L-N^{ω}-methylarginine (monomethylated L-arginine); ADMA, L-N^{ω},N^{ω}-dimethylarginine (assymmetric dimethylated L-arginine); SDMA, L-N^{ω},$N^{\omega\prime}$-dimethylarginine (symmetric dimethylated L-arginine); DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); TCEP, tris(2-carboxyethyl)phosphine; ESI-MS, electrospray mass spectrometry; NOS, nitric oxide synthase.

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immunoblotting using monoclonal antibodies, the enzyme is also present in several other rat tissues including brain (*24*), and a cross-reactivity with some human tissues has also been reported (*25*). Besides the kinetic constants, so far very little is known about the enzyme itself.

Previously we have reported the isolation and characterization of a novel zinc-containing protein from bovine brain (*26*). At that time, the sequence comparison of four large peptides with protein and gene databases did not reveal any significant similarities to known proteins. Recently, the cDNA sequence of the dimethylargininase from rat kidney was published (*27*). This protein sequence exhibits a very high similarity to our protein, allowing the conclusion that the novel bovine brain protein is also a dimethylargininase.

In this study, a detailed characterization of dimethylargininase from bovine brain is presented. This includes the secondary structure determination by circular dichroism, and the analysis of the cysteine/cystine content of the dimethylargininase from bovine brain. In addition, evidence for one tightly bound zinc ion in the native enzyme is presented. Furthermore, both the kinetic constants and the tissue distribution of the bovine enzyme, obtained from immunochemical studies, are compared with those reported for the rat enzyme.

MATERIALS AND METHODS

L-Citrulline, L-N^{ω}-methylarginine (MMA), and L-N^{ω},N^{ω}-dimethylarginine (ADMA) were from Sigma. L-N^{ω},$N^{\omega\prime}$-Dimethylarginine (SDMA) was purchased from Calbiochem. Thiosemicarbazide, 2,3-butanedione oxime, and diphenylthiocarbazone were from Fluka. Tris(2-carboxyethyl)phosphine (TCEP) was a product of Pierce.

Enzyme Preparation. The protein was isolated as described by Fundel et al. (*26*) with the following changes. After the gradient anion exchange chromatography on DEAE-Sephacel, the protein in 20 mM Tris/HCl, 0.11 M NaCl, pH 7.5, was concentrated in an ultrafiltration cell (Amicon Inc., USA; membrane MWCO 20 kDa; Spectrum, USA), and the salt concentration was increased to 0.25 M NaCl. Subsequently, the concentrated protein (ca. 3 mg) was put onto a zinc-loaded metal chelate affinity chromatography column (Chelating Sepharose Fast Flow, Pharmacia; 0.5 × 6 cm). The resin was prepared by washing with 20 mM EDTA (20 mL), with a solution of 20 mM $ZnCl_2$ in 25 mM acetic acid, pH 4.0 (15 mL), and with the final buffer (20 mM Hepes/NaOH, 0.5 M NaCl, pH 7.8, 50 mL). Under these conditions, the dimethylargininase does not bind to the column and was collected in the run-through fractions, whereas other proteins, especially bovine hemoglobin, possessing a low-affinity metal binding site on the protein surface, bind to the column. Ten milliliters of 20 mM Hepes/NaOH, 0.5 M NaCl, pH 7.8, was used in the sample loading and elution. The protein solution from the metal chelate affinity chromatography was concentrated by ultrafiltration (Centricon, MWCO 10 kDa, Amicon Inc.) and applied to a gel filtration column (3 × 10 cm; Superdex 75, Pharmacia). The single zinc-containing peak in the 30−35 kDa range was collected. It should be noted that no other metal ion but zinc has been detected in the protein prior to zinc chelating chromatography (*26*).

The purity of the protein was routinely checked by SDS−PAGE according to the method of Laemmli (*28*) followed by staining with Coomassie Brillant Blue R-250. The zinc concentration was determined by atomic absorption spectroscopy (IL Video 12) and that of the protein by absorption spectroscopy using the extinction coefficient $\epsilon_{280} = 14\,420$ M^{-1} cm^{-1}. The ϵ_{280} value was calculated using the average molar absorptivities of 5540 M^{-1} cm^{-1} for Trp and 1480 M^{-1} cm^{-1} for Tyr (*29*), and the determined amino acid composition of the protein (1 Trp, 6 Tyr) (*26*). In several preparations, the zinc-to-protein ratio was consistently in the range of 1.0 ± 0.1. The amino acid sequence of the blocked *N*-terminal peptide was obtained by electrospray mass spectrometry (ESI-MS) on a Sciex API III^+ triple-quadrupole electrospray instrument (Sciex, Ontario, Canada). The tryptic peptide was isolated by reverse-phase HPLC as previously described (*26*).

Preparation of Zinc-Depleted Enzyme (Apo-Enzyme). In the preparation of the zinc-depleted enzyme, precautions were taken to avoid any metal contamination as much as possible. Thus, all solutions were kept in plastic bottles, and all glass vessels were washed with 10 M nitric acid and rinsed extensively with metal-free water. Prior to use, the dialysis tubing (MWCO 12−15 kDa) was treated as described by Auld (*30*). The zinc-free protein was prepared by dialysis of the native zinc enzyme (ca. 40−80 μM) at 4 °C against a 50-fold molar excess of 1,10-phenanthroline (2 mM) in a metal-free buffer (5 mM MES/NaOH, 100 mM NaCl, pH 6.5) containing 1 mM 2-mercaptoethanol. The 1,10-phenanthroline was added as a 0.2 M solution in DMSO. The dialysis buffer was changed 4 times (every 2 h), and the dialysis was completed overnight. For the CD studies, the apo-enzyme was dialyzed further against a 50 mM metal-free phosphate buffer, pH 6.2 at 4 °C (changed 2 times), to remove 1,10-phenanthroline and 2-mercaptoethanol. The obtained zinc-depleted protein contained only a small amount of residual zinc (0.10 ± 0.03 molar equiv) and will be further denoted as the apo-enzyme.

Preparation of Metal-Free Buffers. Where necessary, solutions were rendered metal-free as follows. Diphenylthiocarbazone was recrystallized from a $CHCl_3$/hexane mixture and as a solution in $CHCl_3$ extracted with 25 mM Na_4EDTA (*31*). The buffers were extracted with 0.85 mM diphenylthiocarbazone in $CHCl_3$ as described (*32*). All buffers treated this way were subsequently extracted twice with $CHCl_3$. Traces of organic solvent in the water phase were removed by bubbling with nitrogen. The zinc content of the buffers was below the detection limit of the atomic absorption machine (ca. 0.12 μM).

Immunological Characterization. Antibodies against the purified dimethylargininase from bovine brain were obtained by immunization of rabbits (Eurogentec, Belgium). For the reaction with the blotted protein, the obtained antiserum was used in a 10 000-fold dilution without further purification. For the analysis of enzyme distribution, a small amount (ca. 5−10 g) of the following tissues from a single cattle was used: total brain, brain cortex, blood, heart, lung, muscle, liver, kidney, spleen, great gut, and small gut. The tissues were homogenized in 9 mL of 20 mM Tris/HCl, 0.1 M NaCl, pH 7.5, per gram wet weight and centrifuged for 1 h at 15000*g*. The total protein concentration in the supernatant was determined using the BCA protein assay (Sigma). SDS−PAGE with subsequent Coomassie Brilliant Blue R-250 staining was performed according to Laemmli (*28*)

on 10−20% gradient gels. For Western blots, samples were separated on SDS−PAGE and subsequently electroblotted onto nitrocellulose paper (0.2 μm, Schleicher & Schüll, Germany). The protein transfer was checked by Ponceau S staining of the blot. The immunoassay was performed with a BioRad assay kit, which is based on the method of Towbin et al. (*33*). The blocking solution contained 2% (w/v) BSA and the antibody containing buffer 1% (w/v) BSA. Goat anti-rabbit IgG conjugated with alkaline phosphatase was used in a 10 000-fold dilution as secondary antibodies. The color developing reagent contained 5-bromo-4-chloro-3-indoyl phosphate (BCIP) and nitroblue tetrazolium (NBT).

Enzymatic Assay. The product of the enzymatic reaction, L-citrulline, was determined according to the method of Boyde and Rahmatullah (*34*). The standard conditions for the assay were as follows: 76 μL of 0.5−1.5 μM enzyme in 100 mM phosphate buffer, pH 6.2, was mixed with 4 μL of 100 mM substrate solution and incubated at 37 °C between 10 and 60 min. The reaction was stopped by adding 4 μL of 6 M TCA. The precipitated protein was removed by centrifugation, and 80 μL of the clear supernatant was mixed with 800 μL of the color developing reagent. This reagent was always freshly prepared by adding 1% (v/v) of 0.11 M thiosemicarbazide to 0.50 mM 2,3-butanedione oxime followed by the addition of a double volume of 1.5 mM $FeCl_3$ in 2.9 M phosphoric acid/4.5 M sulfuric acid. The sample−reagent mixture was heated for 5 min at 95 °C. Subsequently, upon cooling to room temperature, the absorption at 530 nm was measured. A standard curve with citrulline between 0 and 400 μM was used because of the linear dependence of extinction to concentration in this range. One unit of the enzyme is defined as the amount of enzyme that produces 1 μmol of L-citrulline per minute under the given conditions. Addition of metal ions, 1,10-phenanthroline or EDTA, was without any effect on the color development in the enzymatic assay.

Reaction of Native and Denatured Enzyme with Thiol Reagents. 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB) was used to examine the reactivity of the sulfhydryl groups in the native and denatured enzyme (*36*). The reaction with DTNB was performed at pH 7.3 in 0.5 M potassium phosphate buffer containing 0.5 mM EDTA. The concentrated protein and DTNB solutions were added to the phosphate buffer at a final concentration of 2−3 μM protein and 0.5 mM DTNB. The absorption at 412 nm (ϵ_{412} = 13 600 M^{-1} cm^{-1}) was detected continuously over a time period of 30 min and corrected against a blank to which no protein was added. For the reaction under denaturating conditions, the buffer contained 3 M guanidine hydrochloride. The enzyme from two independent preparations was used in these studies, and each measurement was performed at least in duplicate.

Thiol Modification with Iodoacetic Acid and upon Reduction with TCEP. The number of free sulfhydryl groups was also determined by modification with iodoacetic acid; 500 μL of a 6 μM solution of the protein in an argon-saturated denaturating buffer (6 M guanidine hydrochloride, 0.5 M Tris/HCl, 5 mM EDTA, pH 8.6) was incubated in the dark for 30 min with 2 mM iodoacetic acid at room temperature. In parallel, a second protein sample in the same buffer was first incubated with 2 mM TCEP for 30 min, and, subsequently, 10 mM iodoacetic acid was added (*37, 38*). The mixture was incubated for 30 min in the dark. The reactions were stopped by the sample acidification with 500 μL of 2% (v/v) TFA. The modified protein was desalted over a G-25 fast desalting column (Pharmacia) equilibrated with 0.1% (v/v) TFA. The protein samples were concentrated using a Speedvac concentrator to about 10 pmol/mL and analyzed by ESI-MS.

Table 1: Comparison of the Partial Sequence of Dimethylargininase from Bovine Brain[a] with That of the Rat Kidney Enzyme[b]

N-terminus	
bovine	Acetyl-A **S** L **G** H P **A** **T** F G R
rat	Acetyl-A **G** L **S** H P **S** **V** F G R
peptide I	
bovine	Q H Q L Y V G V L G S K
rat (AA 46-57)	Q H Q L Y V G V L G S K
peptide II	
bovine	m K E A L E K L Q L N I V E M
rat (AA 105-119)	M K E A L E K L Q L N I V E M
peptide III	
bovine	m A G P N L I A I G S S E S A Q K **A** L x I
rat (AA 180-200)	M A G P N L I A I G S S E S A Q K **A** L K I

[a] *N*-terminal sequence, this work; other peptide sequences are from (*26*). [b] Sequence data from (*27*). Differences are indicated in boldface letters; x = unidentified amino acid.

Spectroscopic Measurements. Circular dichroism (CD) measurements were performed on a Jasco (Model J-715) spectropolarimeter using a 0.1 cm cylindrical quartz cuvette. The CD data are expressed as mean residual molecular ellipticity, [θ] (deg $dmol^{-1}$ cm^2), using a total of 286 residues (*26*). For the secondary structure determination by CD spectroscopy, the Protein Secondary Structure Estimation Program (Jasco, Japan) was used. The program is based on the method described by Yang (*39*), and the fractions of α-helix, β-sheet, β-turn, and unordered structures are estimated by a least-squares method. The thermal denaturation studies were performed in a water-jacketed, cylindric quartz cuvette (pathlength 0.1 cm), and the temperature was regulated with a circulating water bath operated under computer control. Thermal denaturation was followed by monitoring the ellipticity at 222 nm. The temperature was increased with a heat rate of 1 °C/min from 15 to 85 °C. The protein solutions (ca. 4 μM) were prepared in a 25 mM metal-free phosphate buffer, pH 6.2. The first derivative of the melting curves was calculated for a more accurate determination of T_m. The measurements were performed in duplicate.

RESULTS

N-Terminal Sequencing and Sequence Comparison with the Rat Kidney Enzyme. The *N*-terminus of the purified protein was shown to be resistant to a direct Edman degradation (*26*). After tryptic digestion and peptide separation by reverse-phase HPLC, one *N*-terminally blocked peptide could be identified. This peptide was sequenced using tandem mass spectrometry on an ESI-MS instrument (Table 1). Between Ile and Leu, which have the same mass, could be differentiated using a quantitative amino acid analysis of the peptide. The analysis of mass spectra is consistent with an acetylated Ala residue found also in the rat kidney enzyme (*27*). In Table 1, the *N*-terminal peptide sequence and three other previously reported peptide sequences (*26*) of the bovine brain protein are compared with the corresponding peptides of the rat kidney sequence (*27*).

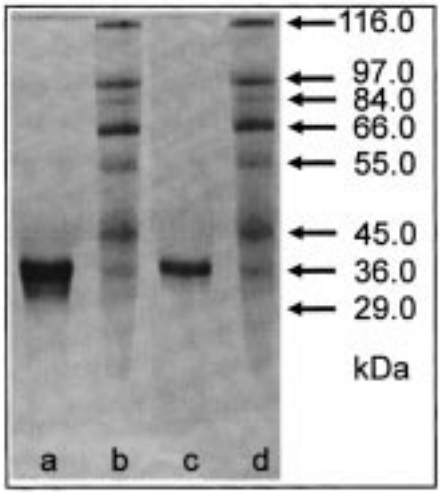

FIGURE 1: SDS−PAGE (12%, Coomassie stained) of bovine dimethylargininase, lanes a, 12 μg and c, 5 μg, and molecular mass markers, lanes b and d.

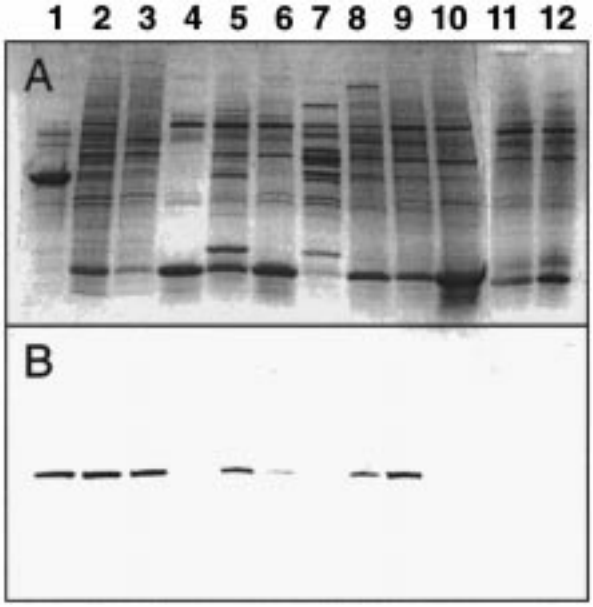

FIGURE 2: (A) SDS−PAGE (10−20%, Coomassie stained) and (B) immunoblots of dimethylargininase in bovine tissue extracts: 1, partially purified enzyme; 2, total brain; 3, brain cortex; 4, blood; 5, heart; 6, lung; 7, muscle; 8, liver; 9, kidney; 10, spleen; 11, great gut; 12, small gut. For Coomassie staining of the polyacrylamide gels (A), 8 μg of total protein was loaded at each lane except lane 1 which contained 3 μg. For Western blots (B) 60 μg of proteins was loaded except for lanes 1 (80 ng), 2 (10 μg), and 3 (20 μg).

The overall sequence identity is very high with a few substitutions in the *N*-terminal peptide. Both apo-proteins have a closely similar molecular mass, with molecular mass = 31 207 Da for bovine and molecular mass = 31 414 Da for the rat dimethylargininase, and very similar amino acid compositions (23, 26).

The bovine protein was previously described as a zinc protein, which contains substoichiometric amounts of heme resulting in an absorption in the Soret region (*26*). However, small amounts of heme previously observed were, at least partly, due to contamination by bovine hemoglobin. Using metal chelate affinity chromatography, applied in addition to the previously described purification procedure (*26*), the traces of hemoglobin can be quantitatively separated from the dimethylargininase, resulting in a protein with almost no absorption in the Soret region. A SDS−PAGE of the purified protein at two different concentrations is shown in Figure 1. The purified protein exhibits a single band at ca. 34 kDa (Figure 1, lanes a and c). The quantitative amino acid analysis of this protein revealed a closely similar amino acid composition to that reported in our previous studies (*26*).

Immunochemical Characterization. Polyclonal antibodies were raised against the purified protein in rabbits. The sensitivity and possible cross-reactivity of the obtained antiserum were tested by Western blot analysis using the purified protein and crude brain homogenates in various concentrations. Using a 10 000-fold dilution of the antiserum, approximately 0.04 μg of the purified dimethylargininase could still be detected as a single immunoreactive band at 34 kDa. A single immunoreactive band at 34 kDa was also found with crude brain extracts (50−100 μg). An apparent molecular mass of 34 kDa, obtained for this protein by SDS−PAGE, corresponds to a molecular mass of 31.2 kDa determined by electrospray mass spectrometry (*26*). Thus, the obtained antiserum shows a relatively high sensitivity toward the dimethylargininase and no cross-reactivity with other bovine brain proteins.

Tissue Distribution of the Enzyme. Although the protein had been isolated from bovine brain, we wished to examine its presence in other bovine tissues. Figure 2 shows the results of a Western blot analysis of dimethylargininase performed on various bovine tissues. In all cases, either one immunoreactive band or none was detected. The highest relative amount of the enzyme was found in total brain, brain cortex, and kidney. A significant amount was also present in heart, liver, and lung, whereas no band was observed with blood, muscle, spleen, and intestine extracts. These studies indicate that the protein is widely distributed in bovine.

Cysteine/Cystine Content of Dimethylargininase. The sulfhydryl groups in the protein were analyzed by different thiol reagents. DTNB was used to examine the reactivity of the sulfhydryl groups in the native and denatured enzyme. In both cases, about 5 reactive sulfhydryl groups (4.7 ± 0.3) were modified at comparable reaction times under the conditions employed.

The number of free sulfhydryl groups was also determined by reaction with iodoacetic acid, a carboxymethylating agent for thiolate groups. To examine the presence of disulfide bonds in the protein, the reaction was performed under denaturating conditions (6 M guanidine hydrochloride) in the absence and the presence of a reducing agent. For this purpose, a portion of the protein sample was first incubated with TCEP and subsequently with iodoacetic acid. TCEP is a water-soluble phosphine, which has proved to be an excellent reducing agent for disulfide bonds (*37*, *38*). The protein samples were analyzed by ESI-MS. Both the reduced and nonreduced carboxymethylated protein revealed a major signal at 31 497 Da. The mass difference of 290 Da between the latter value and that of the noncarboxymethylated apo-protein, obtained under identical experimental conditions (31 207 Da), is consistent with five carboxymethylated Cys residues. These results indicate that there are no disulfide bridges in the protein.

Determination of Secondary Structure Components. The circular dichroism spectrum of native dimethylargininase between 250 and 190 nm recorded in 25 mM metal-free

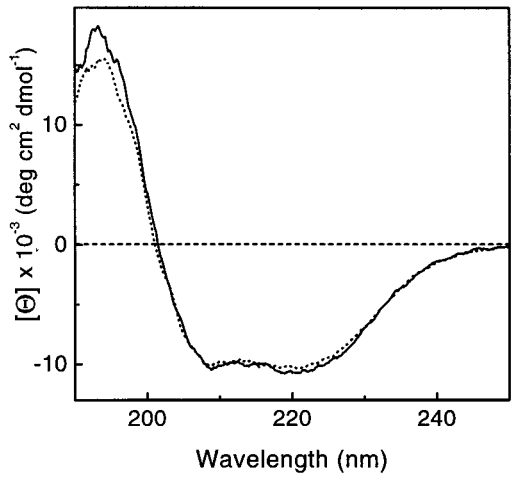

FIGURE 3: Circular dichroism spectra of native dimethylargininase (solid line) and of the apo-protein (dotted line) in 25 mM metal-free phosphate buffer, pH 6.2.

phosphate buffer (pH 6.2) is shown in Figure 3 (solid line). The spectral features in the far-UV region mainly originate from amide transitions of the adjacent peptide chromophores. The spectral deconvolution of the protein CD profile as a linear sum of the predetermined basis spectra allows the determination of secondary structure components of the protein. The CD features of the enzyme with negative maxima at 208 and 222 nm are characteristic for a protein with a relatively high α-helical content. The analysis of this CD profile using the method of Yang (*39*) resulted in the following secondary structure content: 41% α-helix, 17% *β*-sheet, 15% *β*-turn, and 27% random coil. The zinc-depleted apo-protein, also shown in Figure 3 (dotted line), exhibits a very similar CD spectrum. A closer inspection shows minor CD changes characterized by a decrease of the CD bands at 193 and 222 nm. The analysis of the apo-protein spectrum reveals a reduction of the α-helical content and an increase in that of *β*-sheet, i.e., 36% α-helix, 19% *β*-sheet, 16% *β*-turn, and 29% random coil. These structural changes are relative small given the errors associated with secondary structure predictions by CD spectroscopy in general. However, since the same trend was observed in several measurements using protein from independent preparations, we conclude that these differences are real and that small structural differences between these two forms exist.

Enzymatic Activity. The dependence of enzymatic activity on enzyme concentration and time, respectively, was found to be linear in the concentration range used. Since the dependence of the initial rates of the enzymatic reaction as a function of substrate concentrations was hyperbolic, for the determination of kinetic constants the model of Michaelis and Menten was employed (not shown). This kinetic model has also been used in the previous studies of the rat enzyme by Ogawa et al. (*23*). The kinetic and inhibitory constants for the bovine dimethylargininase were obtained using the method of Hanes and Wolf (*35*).

In our previous paper, we showed that the bovine brain protein contains one tightly bound zinc ion, whereas no metal ion was reported for the dimethylargininase from rat kidney (*22*, *23*). To establish whether the zinc ion plays a catalytic or structural role, the enzymatic activity of the holo- and apo-protein from bovine brain was compared. In Table 2 the kinetic constants for both substrates ADMA and MMA

Table 2: Comparison of the Kinetic Constants of the Dimethylargininase from Bovine Brain (Holo- and Apo-Forms) with Those of the Rat Kidney Enzyme

	bovine brain		
	holo	apo	rat kidney[a]
substrate ADMA			
K_m (mM)	1.6 ± 0.3	0.81 ± 0.12	0.18 ± 0.01
V_{max} (units/mg of protein)	0.66 ± 0.10	0.19 ± 0.02	0.28 ± 0.02
k_{cat} (s^{-1})	0.34 ± 0.06	0.101 ± 0.013	0.154
k_{cat}/K_m (M^{-1} s^{-1})	210 ± 50	120 ± 20	855
substrate MMA			
K_m (mM)	1.31 ± 0.12		0.36 ± 0.01
V_{max} (units/mg of protein)	0.51 ± 0.07		0.17 ± 0.02
k_{cat} (s^{-1})	0.27 ± 0.03		0.094
k_{cat}/K_m (M^{-1} s^{-1})	200 ± 30		260

[a] Kinetic values of the rat kidney enzyme are from (*23*). Measurements were performed under the standard assay conditions described under Materials and Methods.

are summarized and compared with those reported for the rat kidney enzyme. Both the holo- and apo-enzyme from bovine brain were found to be active, suggesting that the zinc ion does not have a catalytic function in the enzyme. In the case of the bovine holo-enzyme, the kinetic constants for ADMA and MMA are closely similar (Table 2). An effect of zinc is evident from the changes in K_m and V_{max} values obtained with ADMA for the apo-enzyme and holo-enzyme. Thus, K_m and V_{max} values of the apo-enzyme are lower compared to the holo-enzyme. However, although the apo-enzyme exhibits a 3 times lower specific activity, a slightly lower K_m value of this form results in comparable values of the second-order rate constant k_{cat}/K_m for both forms of the enzyme. Attempts to fully restore the activity of the apo-form by addition of various concentrations of zinc in the presence and absence of 2-mercaptoethanol were so far unsuccessful, suggesting that some irreversible changes occur upon zinc removal. The activity of both the holo- and apo-protein was also measured in the presence of a large excess of the chelating agents 1,10-phenanthroline and EDTA, respectively. When 1 μM protein was incubated with 1 mM of chelating substances in 100 mM metal-free phosphate buffer, pH 6.2 at 4 °C for 12 h, no significant alterations in the corresponding catalytic activities have been observed.

The kinetic constants and substrate specificity of the bovine brain enzyme are overall comparable to those reported for the rat kidney enzyme (Table 2). Thus, while ADMA and MMA serve as good substrates for the bovine protein, no measurable activity was found with SDMA (<1%) precluding the determination of the kinetic constants. The pH profile of the dimethylargininase from bovine brain, using ADMA as the substrate, showed a bell-shaped curve with a maximum at pH 6.2. This value compares well with that of 6.5 reported for the rat enzyme (*23*). Furthermore, an inhibition of the bovine enzyme by similar agents reported for the rat enzyme was also observed (see below).

Inhibition by Zinc. It has been shown that dimethylargininase from rat kidney is inhibited by SH-blocking reagents, e.g., *p*-chloromercuribenzoate, and divalent metal ions such as Cu^{2+}, Cd^{2+}, and Zn^{2+} at 0.1 mM concentrations (*23*). As one tightly bound zinc is present in the structure of bovine brain dimethylargininase, the effect of several metal ions on the activity of this enzyme was examined. The relative activities under standard assay conditions using ADMA as

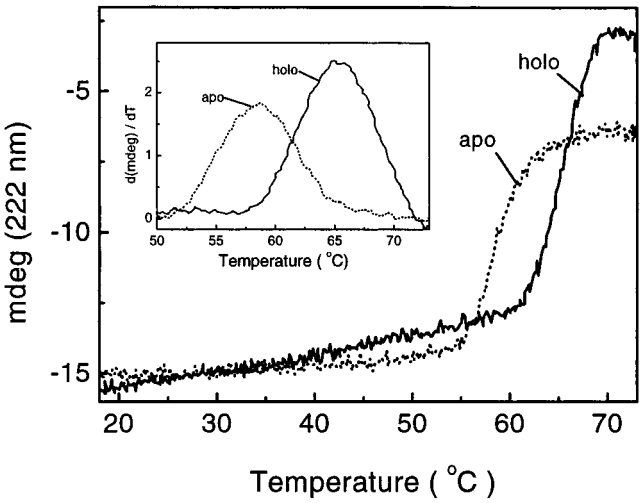

FIGURE 4: Temperature dependence of the circular dichroism band at 222 nm for the holo- (solid line) and apo-dimethylargininase (dotted line). 4 μM enzyme was in 25 mM metal-free phosphate buffer, pH 6.2. The inset shows the first derivatives of the melting curves of both forms of the protein.

the substrate in the presence of 10 mM Mg^{2+} or 2 mM Mn^{2+} were 93 and 95%, respectively. In contrast, the presence of only 20 μM Zn^{2+} in the assay reduced the relative activity significantly to 40%. Further measurements showed that the inhibition by zinc is competitive with a K_i value of 2.0 $\pm$ 0.8 μM.

Temperature Stability. Substantial differences in the enzymatic activity were found when the holo- and apo-proteins were preincubated for 2 h at 50 °C. Under these conditions, the holo-enzyme retained ca. 56% of the original activity, whereas no residual activity of the apo-enzyme was found. This result suggests a structure-stabilizing effect of the zinc. The temperature stability of both the holo- and apo-enzyme was studied by using CD spectroscopy monitoring the ellipticity at 222 nm. The thermal denaturation curves of the holo- and apo-enzyme, obtained under experimental conditions comparable to those of the enzymatic assay (pH 6.2, phosphate buffer), are shown in Figure 4. For both forms, a relatively sharp structural transition is observed. The T_m values have been determined as the maximum of the first derivative of the thermal denaturation curves (inset of Figure 4). The apo-protein exhibits a significantly lower T_m value of 58.7 $\pm$ 1.0 °C compared to that of the holo-enzyme of 65.9 $\pm$ 1.0 °C. Under these conditions, the thermal denaturation of both protein forms was irreversible. Similar studies under anaerobic conditions and in the presence of 2-mercaptoethanol (0.1 mM) and/or zinc (25 μM) did not result in a reversible renaturation process. The irreversibility might be attributed to protein aggregation, since some protein precipitation at higher temperatures was observed. The irreversibility of the denaturation process precluded the determination of thermodynamic parameters.

DISCUSSION

In this paper, the first detailed characterization of dimethylargininase isolated from bovine brain is presented. In our previous studies, we have shown by ESI-MS and atomic absorption spectroscopy that the purified protein contains one tightly bound zinc per polypeptide chain (*26*). In general, zinc is known to constitute an integral part of a large number of enzymes involved in virtually all aspects of metabolism (*40*). Based on the X-ray analysis of a variety of zinc enzymes, three major zinc binding motifs exist, i.e., catalytic, cocatalytic, and structural (*41*). Here we demonstrate that the Zn^{2+} ion can be removed from the protein structure by dialysis against the strong chelating agent 1,10-phenanthroline. This made it possible to compare the holo- and apo-enzyme in order to investigate the possible function of the Zn^{2+} ion in the native enzyme.

From the comparison of a molecular mass of 31.2 kDa, obtained by ESI-MS, with an apparent molecular mass of 34 kDa, revealed by gel filtration and SDS−PAGE studies, the dimethylargininase is a monomeric protein of globular shape (*26*). The determination of secondary structure components of the holo-protein by CD spectroscopy revealed the presence of 41% α-helix and 32% β-sheet and β-turn structures and only a small amount of unordered structure. These features indicate a well-defined protein fold. The CD analysis of the apo-enzyme shows a small, but significant decrease in the α-helical content (ca. 5%), suggesting a minor structural alteration upon zinc removal. This is supported by differences in the substrate affinities (K_m values) and the turnover numbers with ADMA observed for the holo- and apo-enzyme (see Table 2). Before discussing these differences in more detail, a comparison of the kinetic data of the holo-enzyme from bovine brain with those reported for the rat enzyme should be made. Both enzymes show a specificity for the substrates ADMA and MMA, and do not convert SDMA. It should be noted, however, that the rat enzyme has been isolated in the presence of 2-mercaptoethanol. In our hands, the presence of DTT in the buffers during enzyme purification resulted in a substantial zinc loss. Consequently, as the zinc content of the rat enzyme was not determined, a detailed comparison of the kinetic constants cannot be made. Nevertheless, the kinetic constants for the bovine and rat enzyme are comparable (see Table 2). These similarities are to be expected in light of the high sequence identity between these proteins. At present, using a tryptic peptide map of the bovine brain enzyme in combination with ESI-MS and comparison with the sequence from the rat kidney enzyme, we have determined ca. 65% of the total protein sequence. The comparison shows a more than 95% sequence identity to that of the rat kidney enzyme (Gehrig and Vašák, unpublished data). It should be noted, however, that based on the sequence information 7 Cys residues are present in the enzyme from rat kidney (*27*). This is in contrast to the bovine enzyme in which by using thiol modification with DTNB or their carboxymethylation with iodoacetic acid a total of 5 Cys residues were found. Moreover, the obtained data suggest that no disulfide linkages exist.

It was not possible to remove the zinc from the protein by a simple incubation with chelators which indicates its relatively high binding affinity. However, the zinc could be removed by an extensive dialysis against 1,10-phenanthroline. A comparison of the kinetic constants between the holo- and apo-enzyme using ADMA as a substrate shows approximately double as high K_m and 3 times higher k_{cat} values for the holo-enzyme (Table 2). These changes in kinetic parameters give rise to the 2 times lower second-order rate constant k_{cat}/K_m for the apo-enzyme (Table 2). Thus, under the conditions of the assay (37 °C), the apo-enzyme remains active and, judging from the temperature

dependence studies (Figure 4), no protein denaturation occurs. Based on these results, a direct involvement of zinc in enzyme catalysis is highly unlikely. Hence, we conclude that the dimethylargininase is not a zinc hydrolase. More profound differences between the apo- and holo-forms were observed in thermostability studies followed by CD spectroscopy (Figure 4). The apparent T_m value of 58.7 °C for the apo-enzyme is significantly lower compared to 65.9 °C for the holo-enzyme. These differences in thermostability of both forms are consistent with a total loss of the activity of the apo-form upon preincubation at 50 °C compared to more than half residual activity of the holo-enzyme. These results would suggest that the tightly bound zinc in the dimethylargininase plays a structure-stabilizing role. However, the apo-enzyme preserves a substantial enzymatic activity and overall structure at 37 °C (see Figure 4). In addition, at physiological pH the holo-enzyme shows a very low activity due to its pH optimum of 6.2. Thus, activation of the enzyme by other so far unknown substances in vivo might be possible. The zinc binding site could be involved in this process. Further studies regarding the nature of the zinc binding site are currently in progress.

Besides the tightly bound zinc, there is evidence for at least one additional low-affinity metal binding site on the protein. Ogawa et al. (*23*) reported an inhibition of the enzyme by high concentrations (0.1 mM) of the divalent metal ions Cu^{2+}, Cd^{2+}, and Zn^{2+}. Moreover, the binding of these metal ions to cysteine thiolate(s) at the active site has been suggested based on enzyme inhibition by SH-modifying agents (*23*). As the dimethylargininase is a zinc protein, an effect of free zinc ions on the enzymatic activity was studied. We could show that the inhibition by Zn^{2+} is competitive with a K_i value of 2.0 μM. This suggests a direct interaction with residue(s) at the active site. Besides thiolates, a Zn^{2+} coordination with oxygen or nitrogen ligands is also often encountered in biological systems. Based on the pH optimum of 6.2, the involvement of acidic residues in enzyme catalysis, perhaps in stabilizing the guanidinium group of the substrate, is also likely.

Two metal-ion-dependent enzymes involved in the catalysis of a similar reaction as the dimethylargininase are known. One of them is the arginine deiminase (EC 3.5.3.6), which converts L-arginine to L-citrulline and NH_3. The enzyme is activated by Mg^{2+} and Mn^{2+} and is strongly inhibited by Zn^{2+} (*42*). The second group of enzymes are arginases (EC 3.5.3.1), which bind Mn^{2+} as cofactor and hydrolyze L-arginine to L-ornithine and urea. This reaction represents the first step in the urea cycle. The best characterized arginase is the liver arginase, a homotrimeric protein (35 kDa per subunit) with a binuclear spin-coupled Mn center (*43*). The arginase from *Saccharomyces cerevisiae* contains one tightly associated zinc, important for the structure stability, and a weakly bound Mn^{2+} ion that confers catalytic activity (*44*). However, in our activity measurements, no increase in catalytic activity was detected upon addition of relatively high concentrations of Mn^{2+} (2 mM) or Mg^{2+} (10 mM) to the native dimethylargininase under standard assay conditions. Thus, there is currently no indication that the catalytic reaction of dimethylargininase is metal ion dependent. In this connection, it may be noted that no protein sequences similar to that of the rat dimethylargininase have so far been identified in the protein and gene databases.

The distribution of the enzyme in bovine tissues was investigated using polyclonal antibodies and immunoblotting techniques. The protein was shown to be widely distributed in bovine tissues with the highest concentrations in the brain and kidney. Kimoto et al. (*24*, *25*) have analyzed the tissue distribution in rat and a few human tissues using monoclonal antibodies against the rat kidney enzyme. They found the enzyme in various rat tissues with relatively high amounts in kidney, brain, aorta, and pancreas (*24*). We could isolate relatively high amounts of dimethylargininase from bovine brain (8 μg/g tissue), a tissue where one of the highest NO production occurs. This and the presence of the free substrates ADMA and MMA, potent inhibitors of NOS (*6*, *12*, *14*, *15*), in brain (*3*) are consistent with the proposed function for this enzyme in the regulation of NOS. The inhibitory constants K_i for MMA are 0.94 μM for the endothelium NOS from bovine aorta (*14*) and 0.18 μM for the neuronal NOS from rat kidney (*15*). It remains to be shown how much the increased levels of ADMA and MMA affect the activity of NOS in the pathology of muscular dystrophy (*7*), renal failure (*6*), hypercholesteremia (*8*, *9*), and schizophrenia (*10*). In this connection, the observed competitive inhibition of dimethylargininase by zinc with a K_i value of 2.0 μM should also be discussed. A number of exo- and endometalloproteases are also inhibited by an excess of zinc, e.g., carboxypeptidase A, thermolysin, human neutrophil collagenase, and angiotensin converting enzyme. These observations and other studies raised the question about the influence of intracellular free zinc concentrations on the activity of metalloenzymes (*45*, and references cited therein). In normal states, free zinc concentrations in the human body range from nanomolar concentrations in the cytoplasm to millimolar amounts in some vesicles or in abnormal/toxicological states (*46*). Zinc is present in millimolar amounts in brain and is particularly concentrated in the cortex and regions of the hippocampus (*47*). It is well established that abnormalities in zinc metabolism occur in Alzheimer's disease and Parkinson's disease. In these instances, increased levels of zinc in the brain have been reported (*48*, *49*). Consequently, an inhibition of the dimethylargininase by zinc may have an effect on the function of NOS under these conditions. Hence, more biological and structural studies are needed to understand the physiological role of this enzyme.

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