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A cadmium-binding protein in rat liver identified as ornithine carbamoyltransferase

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A cadmium-binding protein of M_r about 40000 (40K Cd-BP_a) was detected in rat liver by Western blotting [Aoki, Kunimoto, Shibata & Suzuki (1986) Anal. Biochem. **157**, 117–122]. It was characterized and identified as ornithine carbamoyltransferase (OCTase, EC 2.1.3.3) on the basis of the coincidence of their physicochemical and enzymological features. The amino acid sequence of the *N*-terminal and those of three tryptic digests in 40K Cd-BP_a were identical with those of OCTase. The M_r values of the denatured and native forms of 40K Cd-BP_a (39000 and 110000 respectively) were the same as those of OCTase. 40K Cd-BP_a showed, as OCTase activity, a specific activity of 230 μmol/min per mg of protein and K_m of 0.6 mM for ornithine, this value also being essentially the same as that for OCTase. A rabbit antibody against OCTase reacted with 40K Cd-BP_a. The native form of 40K Cd-BP_a bound to 0.8 molar equiv. of cadmium, with a dissociation constant of 7.6×10^{-6} M.

INTRODUCTION

Cadmium is known to accumulate specifically in the liver and kidney, and to affect cellular activity in numerous ways. Cytotoxicity is among the effects exerted by cadmium. However, the mechanisms for the effects of this metal have yet to be clarified in detail. Cadmium ions taken up by cells induce the formation of metallothionein and bind to it. Those bound to any other type of protein may possibly be responsible for the toxic effect [1,2]. The characterization of proteins to which cadmium is bound (cadmium-binding protein, Cd-BP) needs to be carried out to clarify the mechanism of its toxicity, but these proteins have yet to be identified.

We have developed a method for detecting proteins with high affinity toward cadmium by using Western blotting. The proteins were separated by SDS/polyacrylamide-gel electrophoresis and then transferred to a membrane. The cadmium-binding activity of each protein was determined on the basis of the extent to which radioactive cadmium bound to the membrane [3]. By this method, three Cd-BPs, M_r about 40000, 29000 and 24000 (40K, 29K and 24K Cd-BPs), were detected in rat liver [3]. They were present in the parenchymal cells, and 40K Cd-BP was noted to have the highest affinity for cadmium among these Cd-BPs [4].

To gain some understanding of the function of Cd-BPs, one of these proteins, 40K Cd-BP, was purified, and it was found to consist of two isoproteins, 40K Cd-BP_a and 40K Cd-BP_b. The amino acid sequence of the former was partially determined and found to be the same as that of ornithine carbamoyltransferase (OCTase, EC 2.1.3.3), the enzyme involved in the synthesis of urea. The physicochemical and enzymological properties of 40K Cd-BP_a were the same as those of OCTase, and consequently it was identified as such.

MATERIALS AND METHODS

Materials

The following reagents were used: $^{109}CdCl_2$ (1000 mCi/mg) from New England Nuclear (Boston, MA, U.S.A.); CM-cellulose (CM52) from Whatman (Maidstone, Kent, U.K.); tosylphenylalanylchloromethane ('TPCK')-treated trypsin from Worthington (Cochranville, PA, U.S.A.); bovine serum albumin from Sigma (St. Louis, MO, U.S.A.). Purified rat OCTase and rabbit anti-(bovine OCTase) IgG were kindly provided by Dr. C. J. Lusty (The Public Health Research Institute, New York, NY, U.S.A.) and Dr. M. Tatibana (Chiba University, Chiba, Japan) respectively.

Detection of Cd-BP

Cd-BPs were detected by Western blotting as reported previously, with some modifications [3]. Protein mixtures were separated on SDS/polyacrylamide slab gel [12.5% (w/v) acrylamide] by the method of Laemmli [5] after SDS treatment in the absence of 2-mercaptoethanol. The proteins thus separated were transferred to a nitrocellulose membrane (Bio-Rad, Richmond, CA, U.S.A.) at a constant voltage of 60 V for 2 h, or to a Durapore membrane (Millipore, Bedford, MA, U.S.A.) at a constant voltage of 30 V for 30 min, followed by 150 V for 2 h in a transfer chamber (distance between electrodes 5 and 3.5 cm respectively). The proteins adsorbed on to the membrane were incubated in 10 mM-Tris/HCl buffer, pH 7.4 (25 °C), containing 1 μCi of $^{109}CdCl_2$/ml, 0.1 mM-zinc acetate and 0.1 M-KCl for 10 min, and then washed for 2 × 1 min with 10 mM-Tris/HCl buffer, pH 8.0 (25 °C), containing 0.1 M-KCl. Either dried membrane was subjected to autoradiography. The proteins were stained with Amido Black.

Abbreviations used: Cd-BP, cadmium-binding protein; 40K Cd-BP, cadmium-binding protein of M_r about 40000; OCTase, ornithine carbamoyltransferase (EC 2.1.3.3).

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Gel-permeation separation of proteins by h.p.l.c. and detection of cadmium bound to proteins

Sample proteins were subjected to h.p.l.c. (Beckman series 340 instrument) using a gel-permeation column (Asahipak GS-520; 500 mm × 7.6 mm diameter; Asahi Chemical Industries, Tokyo, Japan) as reported previously [6]. The column was eluted with 10 mM-Tris/HCl buffer (pH 7.4, 25 °C) containing 0.9% NaCl and 0.05% NaN_3. The cadmium bound to the proteins was detected by the direct introduction of the eluate into an atomic-absorption spectrophotometer (model 170-50A; Hitachi, Tokyo, Japan).

Purification procedure

All operations were carried out at 0–5 °C unless otherwise stated.

Step 1: preparation of crude extract of rat liver. A 40 g portion of untreated rat (Wistar, male) liver was minced and homogenized in 2 vol. of 50 mM-Tris/HCl buffer, pH 7.4 (25 °C), containing 0.25 M-glucose, with a glass–Teflon homogenizer. The homogenate was centrifuged at 170000 *g* for 60 min, and the resulting supernatant was designated as the crude extract.

Step 2: $(NH_4)_2SO_4$ precipitation. Solid $(NH_4)_2SO_4$ was added to the crude extract with constant stirring to give 40% saturation, followed by additional stirring of the solution for 30 min. The precipitate was removed by centrifugation at 10000 *g* for 20 min. The supernatant was mixed with solid $(NH_4)_2SO_4$ to give 70% saturation, and then centrifuged at 10000 *g* for 20 min after stirring for 30 min. The precipitate was dissolved in a small volume of 10 mM-Tris/HCl buffer, pH 7.4 (25 °C), and dialysed against 100 vol. of the same buffer with two changes of solution overnight.

Step 3: heat treatment. The dialysis residue obtained in Step 2 was diluted to 120 ml with 10 mM-Tris/HCl buffer, pH 7.4 (25 °C), and mixed with 1.43 vol. of 50% (w/v) glucose (final concn. 25%) and 0.43 vol. of 0.5 M-Tris/HCl buffer, pH 6.9 (25 °C) (final concn. 75 mM). The solution was warmed quickly and maintained at 55 °C for 5 min, quickly chilled in an ice bath and centrifuged at 10000 *g* for 10 min.

Step 4: CM-cellulose chromatography. The supernatant obtained in Step 3 was dialysed against 10 vol. of 10 mM-Bistris/HCl buffer, pH 6.0, containing 10% (v/v) ethylene glycol, with two changes of solution overnight. The dialysis residue was applied on to a CM-cellulose column (30 cm × 2 cm diameter) equilibrated with 10 mM-Bistris/HCl buffer, pH 6.0, containing 10% ethylene glycol, at a flow rate of 15 ml/h. After the unadsorbed proteins were washed out, those adsorbed were eluted with 400 ml of a linear gradient of 0–0.5 M-NaCl in the same buffer at the same flow rate. 40K Cd-BP_a was eluted at 0.19 M-NaCl, and the fraction containing it ('CM-cellulose fraction'; about 30 ml) was stored at −70 °C until use.

Step 5: h.p.l.c. with TSK Phenyl-5PW column. For this, 0.5 vol. of the CM-cellulose fraction obtained in Step 4 was dialysed against 50 vol. of 10 mM-Tris/HCl buffer, pH 7.4 (25 °C), containing 1 M-$(NH_4)_2SO_4$ and 10% ethylene glycol. The dialysis residue was subjected to h.p.l.c. (Beckman) using a TSK gel Phenyl-5PW column (Toyo Soda Manufacturing Co., Tokyo, Japan) (15 cm × 2.15 cm diameter) equilibrated with 10 mM-Tris/HCl buffer, pH 7.4 (25 °C), containing 1 M-$(NH_4)_2SO_4$ and 10% ethylene glycol, at a flow rate of 4 ml/min. The column was washed for 12 min with the same buffer and then eluted with a linear concentration gradient of $(NH_4)_2SO_4$ in the same buffer from 1 to 0 M for a period of 40 min. 40K Cd-BP_a was eluted at 20 min after the start of the gradient, and the fraction containing it (Phenyl-5PW fraction) was stored at −20 °C after dialysis against 50 vol. of 10 mM-Tris/HCl buffer, pH 7.4 (25 °C), containing 40% (v/v) glycerol and 10% ethylene glycol.

Determination of amino acid sequences

For this purpose, the 40K Cd-BP_a in the Phenyl-5PW fraction was further purified. The Phenyl-5PW fraction (50–70 μg of protein) was applied on to a TSK gel TMS-250 column (75 mm × 4.6 mm diameter; Toyo Soda Manufacturing Co.). The column was eluted with the following solvent system at a flow rate of 0.5 ml/min: 0.05% trifluoroacetic acid in water (Soln. A) was mixed with 0.05% trifluoroacetic acid in acetonitrile (Soln. B) at a ratio of 19:1 and, at 5 min after injection of sample solution, the proportion of Soln. B was increased linearly, attaining 100% during 60 min. Protein elution was monitored by the A_{280} (Altex model 152 instrument). 40K Cd-BP_a was eluted at a retention time of 36 min. After freeze-drying, 40K Cd-BP_a was subjected to tryptic digestion. A 60 μg portion of the freeze-dried 40K Cd-BP_a was dissolved in 200 μl of 6 M-guanidine hydrochloride containing 2% (v/v) 2-mercaptoethanol and heated at 100 °C for 5 min. After dialysis against 1 litre of 20 mM-$(NH_4)_2CO_3$, the dialysis residue was mixed with 1 μl of a 1 mg/ml solution of tosylphenylalanylchloromethane-treated trypsin and 4 μl of 0.1 M-$CaCl_2$, and incubated at 37 °C for 18 h with two successive additions of 0.5 μl of the tosylphenylalanylchloromethane-treated trypsin solution. The digest was separated by h.p.l.c. (model 302 apparatus; Gilson, Villiers le Bel, France) on a reversed-phase column (SynChropak RP-P; 250 mm × 4.1 mm diameter; Synchrom, Linden, IN, U.S.A.). The column was eluted as follows, at a flow rate of 0.5 ml/min: Soln. A was mixed with Soln. B at a ratio of 19:1, and, at 5 min after injection of sample solution, the proportion of Soln. B was increased linearly, to 60% within 60 min. Elution of the peptide was monitored by the A_{215}, with a Gilson model 1001 u.v. detector. The amino acid sequence of the eluate, after freeze-drying, was determined.

Sequence analysis of the tryptic digests and *N*-terminal of 40K Cd-BP_a was conducted with a gas-phase sequencer (model 470 A; Applied Biosystems, Foster City, CA, U.S.A.) and on-line-connected h.p.l.c. (model 120A; Applied Biosystems). For determination of the *N*-terminal, 40 μg of 40K Cd-BP_a was sequenced directly.

Assay of OCTase

OCTase activity was measured as the rate of citrulline formation from ornithine and carbamoyl phosphate as described by Lusty *et al.* [7]. The reaction mixture (1 ml) contained 50 mM-diethanolamine/acetate, pH 8.1 (37 °C), 5 mM-L-ornithine hydrochloride, 5 mM-dilithium carbamoyl phosphate and the sample protein. After

incubation for 10 min, the reaction was terminated by addition of 0.5 ml of 5 M-$HClO_4$. The mixture was chilled on ice for 5 min and centrifuged at 1000 *g* for 10 min to remove the precipitates. A 200 μl portion of the resulting supernatant was then used to determine citrulline production, as described by Raijman [8]. One unit of OCTase activity is defined as the amount of enzyme that catalyses the formation of 1 μmol of citrulline in 1 min at 37 °C.

For determination of K_m, the reaction mixture (1 ml) contained 100 mM-Hepes/NaOH buffer, pH 7.7 (37 °C), 0.075–5 mM-L-ornithine, 5 mM-dilithium carbamoyl phosphate and 60 ng of purified 40K Cd-BP_a (Phenyl-5PW fraction) [7]. Citrulline concentration was determined from the A_{464} by using an absorption coefficient of 37 800 $M^{-1} \cdot cm^{-1}$ [7].

Electrophoresis

SDS/polyacrylamide-gel electrophoresis was performed as described by Laemmli [5], with a separating gel of 12.5% (w/v) polyacrylamide.

Non-denaturing gel electrophoresis was conducted as described by Davis [9]. The sample was applied to 5% (w/v) polyacrylamide gel (55 mm × 5 mm diameter) containing 0.38 M-Tris/HCl buffer, pH 8.8 (25 °C). For detection of OCTase activity, the gel was cut into slices (2 mm width), each of which was subsequently homogenized in a Dounce homogenizer with 1 ml of 10 mM-Tris/HCl buffer, pH 7.4 (25 °C), containing 100 μg of bovine serum albumin/ml and then left overnight. After removal of the gel pieces by centrifugation at 1000 *g* for 10 min, OCTase activity in the sample was measured. Proteins on the gel were stained with Coomassie Brilliant Blue R-250.

Determination of the M_r of native 40K Cd-BP by sucrose-density-gradient centrifugation

The sample solution (200 μl) was layered on to a 5 ml linear gradient of 5–20% (w/v) sucrose in 50 mM-Tris/HCl buffer, pH 7.4 (25 °C), and this was centrifuged for 13 h at 46000 rev./min (190000 *g* at $r_{av.}$ 8.4 cm) in a Beckman SW 50.1 rotor at 2 °C. Fractions were collected from the bottom of the tube, each of which was assayed for OCTase activity and subjected to SDS/polyacrylamide-gel electrophoresis.

Immune blotting to detect OCTase, using a Protein A–gold-colloid conjugate

The protein mixture was separated on an SDS/polyacrylamide slab gel after SDS treatment without 2-mercaptoethanol and transferred electrophoretically to a nitrocellulose membrane as described above. Immune-detection of OCTase on the membrane was carried out with a Protein A–gold-colloid conjugate, according to the instructions of the manufacturer (Bio-Rad; no. 170-6510). The rabbit anti-(bovine OCTase) IgG (0.22 mg/ml solution) was used as the antibody.

Cadmium-binding assay of 40K Cd-BP

The eluate containing 40K Cd-BP_a from a Phenyl-5PW column was concentrated to 0.5–0.8 mg of protein/ml by ultrafiltration on a PM-10 membrane (Amicon). The concentrated solution was used for the binding assay after dialysis overnight against 500 ml of 10 mM-Tris/HCl buffer (pH 7.4, 25 °C) containing 0.2 M-KCl (dialysis buffer).

The binding of cadmium to 40K Cd-BP_a was measured by equilibrium dialysis in a micro-dialysis cell (Kimura Scientific Instruments, Tokyo, Japan) [10] designed originally by Englund *et al.* [11]. This cell consisted of two chambers (200 μl) separated by a disc of the dialysis membrane (7 mm diameter; Viscase, Chicago, IL, U.S.A.). The sulphur content of this membrane was removed by boiling in 5% (w/v) Na_2CO_3 for 5 min. A 70 μl sample of the concentrated 40K Cd-BP_a solution was introduced into one chamber (side A) and 70 μl of the dialysis buffer containing $^{109}CdCl_2$ (1 μCi/ml) and 1–160 μM-$CdCl_2$ into the other (side B). The chambers were gently shaken at 23 °C for 14 h. Then a sample was

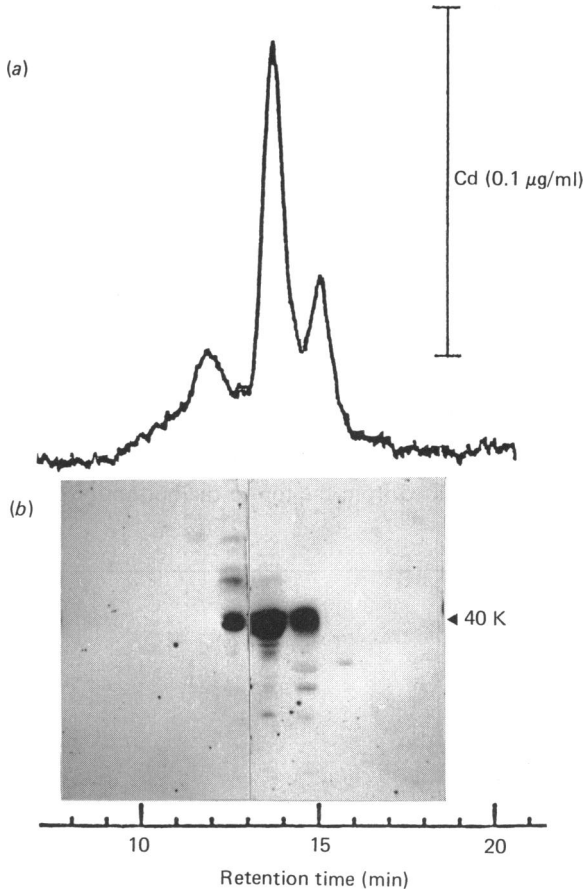


Fig. 1. Elution profile of 40K Cd-BP and cadmium in the crude extract from cadmium-treated rat liver on a GS-520 column

A 100 μl portion of the crude extract prepared from liver of a cadmium-treated rat was applied on to a GS-520 column which was subsequently eluted with 10 mM-Tris/HCl buffer containing 0.9% NaCl and 0.05% NaN_3. (*a*) Elution profile of cadmium. Cadmium concentration in the eluate was monitored continuously by an atomic-absorption spectrophotometer. The vertical bar indicates the detector level. (*b*) Elution profile of 40K Cd-BP. The eluate from the column was fractionated at 1 min intervals. Each fraction was concentrated to one-third the original volume by Amicon CS-15. A 35 μl portion of the sample was analysed for Cd-BP content by Western blotting. After SDS/polyacrylamide-gel electrophoresis, the proteins on the gel were transferred to a nitrocellulose membrane. The position of 40K Cd-BP is labelled '40K'.

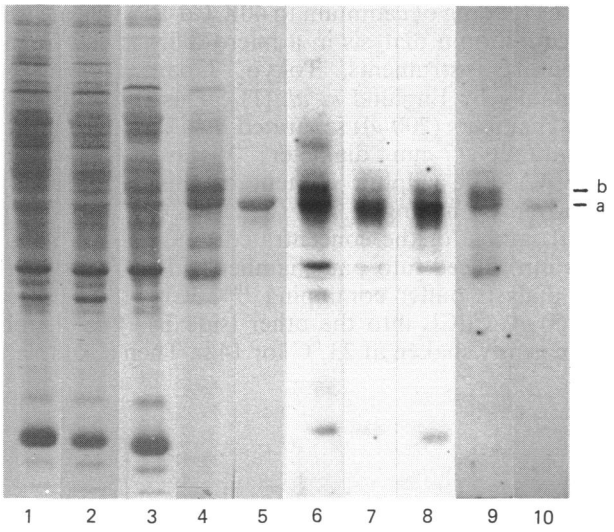


Fig. 2. Purification of 40K Cd-BP

A sample from each purification step was examined for Cd-BP content by Western blotting. After SDS/polyacrylamide-gel electrophoresis, the proteins on the gel were transferred to a Durapore membrane. Lanes 1–5, proteins on the membrane stained by Amide Black; lanes 6–10, results of the autoradiogram. Lanes 1 and 6, crude extract (Step 1, 23 μg of protein); lanes 2 and 7, $(NH_4)_2SO_4$ precipitate (Step 2, 27 μg); lanes 3 and 8, heat-treated supernatant (Step 3, 34 μg); lanes 4 and 9, eluate from the CM-cellulose column (Step 4, 13 μg); lanes 5 and 10, eluate from the Phenyl-5PW column (Step 5, 5 μg). a and b indicate 40K Cd-BP_a and 40K Cd-BP_b respectively. Proteins migrated from the top to the bottom of gels.

removed from each side of the cell and the radioactivity was counted in a γ-counter. The radioactivity for side B was regarded as free cadmium and the excess radioactivity for side A over that for side B as protein-bound cadmium.

Other methods

Protein concentration was determined as described by Lowry *et al.* [12], with bovine serum albumin as standard.

RESULTS

Properties of 40K Cd-BP on a gel-permeation h.p.l.c. column

A crude extract prepared from the liver of a cadmium-treated rat (1 mg Cd/kg body wt., intraperitoneally, and killed 30 min later) was subjected to h.p.l.c. on an Asahipak GS-520 column as previously reported [6]. Fig. 1 shows the elution profile of cadmium bound to proteins and the 40K Cd-BP content in each fraction. 40K Cd-BP was eluted primarily at a retention time between 13 and 14 min, corresponding to the Cd peak at a retention time of 13.6 min. It appears from this result that 40K Cd-BP is one of the intracellular Cd-binding components.

Purification of 40K Cd-BP

40K Cd-BP was purified in five steps. The extent of purification at each step is indicated in Fig. 2. At the step of CM-cellulose chromatography, this protein separated into two bands on SDS/polyacrylamide-gel electrophoresis, corresponding to the lower- and higher-M_r proteins designated 40K Cd-BP_a and 40K Cd-BP_b respectively. Only the former was purified and characterized. At the final stage of purification by Phenyl-5PW

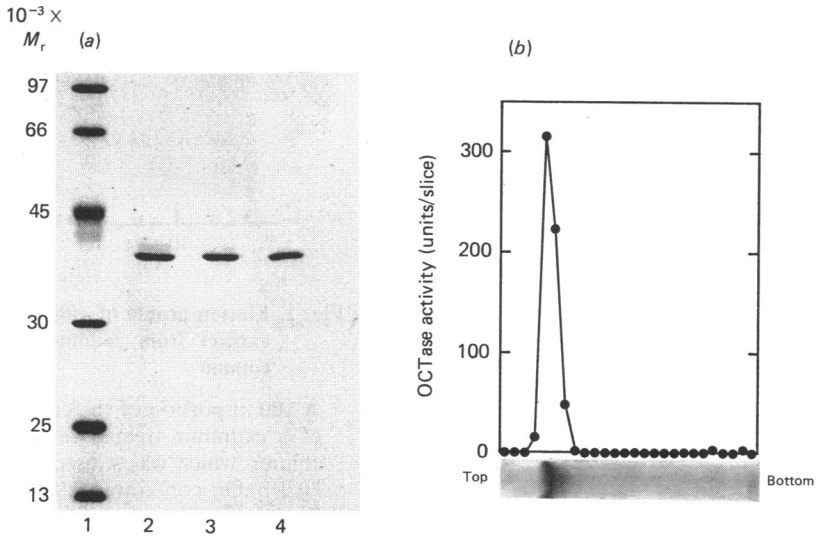


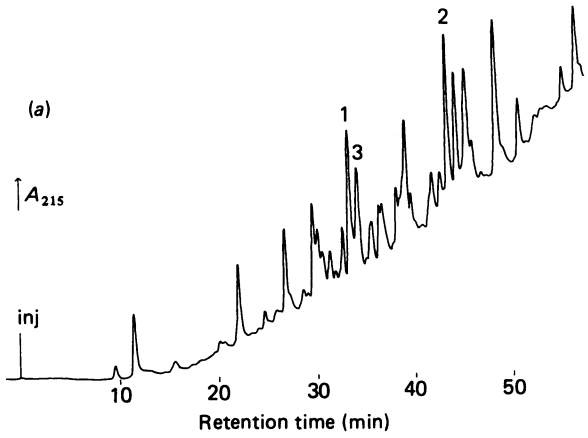
Fig. 3. Electrophoresis of 40K Cd-BP_a

(*a*) SDS/polyacrylamide-gel electrophoresis. Purified 40K Cd-BP_a (Phenyl-5PW fraction) and rat OCTase were subjected to SDS/polyacrylamide-gel electrophoresis after treatment with SDS and 2-mercaptoethanol as described by Laemmli [5]. Lane 1, M_r markers: phosphorylase *b* (M_r 97000); bovine serum albumin (66000); ovalbumin (45000); carbonic anhydrase (30000); soya-bean trypsin inhibitor (25000); lysozyme (13000). Lane 2, 40K Cd-BP_a (1 μg). Lane 3, a mixture of 40K Cd-BP_a (0.5 μg) and rat OCTase (0.5 μg). Lane 4, rat OCTase (1 μg). (*b*) Non-denaturing gel electrophoresis. Purified 40K Cd-BP_a (Phenyl-5PW fraction, 10 μg) was subjected to non-denaturing gel electrophoresis as described by Davis [9]. The lower part of the Figure shows the gel with 40K Cd-BP_a stained by Coomassie Brilliant Blue R-250. The upper part shows OCTase activity for each gel slice. Recovery of OCTase activity was 40%.

Table 1. Sequence analysis of 40K Cd-BP$_a$

Amino acid sequences were identified by using a gas-phase sequencer and on-line-connected h.p.l.c. A 40 μg sample of 40K Cd-BP$_a$ was used to determine its *N*-terminal amino acid sequence. A 60 μg sample of 40K Cd-BP$_a$ was subjected to tryptic digestion, and the amino acid sequence of each tryptic peptide (1–3 in Fig. 4*a*) was determined after separation by h.p.l.c. on a reversed-phase column. The yield is based on the results of amino acid analysis: X, not detectable.

			Tryptic digest					
	N-Terminal		1		2		3	
Cycle no.	Amino acid	Yield (pmol)	Amino acid	Yield (pmol)	Amino acid	Yield (pmol)	Amino acid	Yield (pmol)
1	Ser	16	Val	28	Lys	7	Leu	2
2	Gln	31	Ala	27	Pro	7	Val	2
3	Val	61	Ala	25	Glu	6	Phe	10
4	Gln	47	Ser	5	Glu	7	Pro	19
5	Leu	70	Asp	8	Val	4	Glu	25
6	Lys	41	Gly	5	Asp	4	Ala	21
7	Gly	50	Thr	4	Asp	5	Glu	27
8	Arg	18	Phe	10	Glu	3	Asn	14
9	Asp	39	Leu	10	Val	3		
10	Leu	70	His	1	Phe	3		
11	Leu	62			Tyr	3		
12	Thr	15						
13	Leu	64						
14	Lys	38						
15	Asn	39						
16	Phe	29						
17	Thr	14						
18	Gly	35						
19	X							
20	X							
21	Ile	28						
22	Gln	18						
23	X							
24	Met	14						



(*b*)

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SQVQLKGRDL LTLKNFTGEE IQYMLWLSAD LKFRIKQKGE  40
YLPLLQGKSL GMIFEKRSTR TRLSTETGFA LLGGHPSFLT  80
TQDIHLGVNE SLTDTARVLS SMTDAVLARV YKQSDLDILA 120
KEATIPIVNG LSDLYHPIQI LADYLTLQEH YGSLKGLTLS 160
WIGDGNNILH SIMMSAAKFG MHLQAATPKG YEPDPNIVKL 200
AEQYAKENGT RLSMTNDPLE AARGGNVLIT DTWISMGQED 240
EKKKRLQAFQ GYQVTMKTAK VAASDWTFLH CLPRKPEEVD 280
DEVFYSPRSL VFPEAENRKW TIMAVMVSLL TDYSPVLQKP 320
KF
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chromatography, 40K Cd-BP$_a$ was purified as a single protein on the gel corresponding to the radioactive band (Fig. 2, lanes 5 and 10). It showed essentially one protein band on each of the polyacrylamide gels in either the presence or the absence of SDS (Fig. 3*a*, lane 2, and Fig. 3*b*). Some 2.6 mg of purified 40K Cd-BP$_a$ was obtained from 2 g of protein in the crude extract (livers of three to four rats).

Identification of amino acid sequences

Tryptic digests of 40K Cd-BP$_a$ were separated by h.p.l.c. on a reversed-phase column (Fig. 4*a*). The sequences of the *N*-terminal amino acids and three tryptic fragments were determined with a gas-phase

Fig. 4. Determination of amino acid sequence of 40K Cd-BP$_a$

(*a*) Reversed-phase h.p.l.c. of the tryptic digest of 40K Cd-BP$_a$. This digest was loaded directly (inj) on to a SynChropak RP-P column, which was subsequently eluted by a linear gradient of acetonitrile. The A_{215} was monitored. Fractions corresponding to the relatively large absorbance peaks were collected. The amino acid sequences of peptides 1–3 were determined. (*b*) Amino acid sequence (one-letter code) of rat OCTase [30]. Amino acid sequences in boxes indicate the *N*-terminal and three tryptic digests of 40K Cd-BP$_a$. The numbers 1–3 indicate the tryptic peptides in panel (*a*) and Table 1.

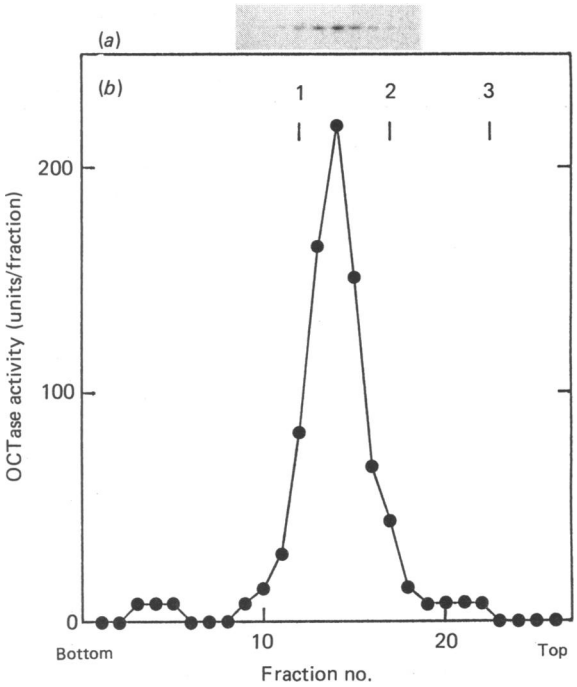

Fig. 5. Measurement of M_r of native 40K Cd-BP$_a$ by sucrose-density-gradient centrifugation

Purified 40K Cd-BP$_a$ (Phenyl-5PW fraction, 13 μg) was analysed by sucrose-density-gradient centrifugation as described in the Materials and methods section. A 20 μl portion of each fraction (200 μl) was subjected to SDS/polyacrylamide-gel electrophoresis after SDS treatment in the absence of 2-mercaptoethanol, and assayed for OCTase activity. (*a*) The 40K Cd-BP$_a$ in each fraction stained by Coomassie Brilliant Blue on the gel. (*b*) Sedimentation of OCTase activity and M_r markers: 1, rabbit IgG (M_r 160000); 2, bovine serum albumin (66000); 3, lysozyme (14000). Recovery of OCTase activity was about 60%.

sequencer (Table 1) and compared with those of other proteins by using an NBRF (National Biomedical Research Foundation) data base and IDEAS software [13]. The amino acid sequence at the *N*-terminal of 40K Cd-BP$_a$ was identical with the corresponding sequence of rat OCTase. The amino acid sequences of the tryptic fragments of 40K Cd-BP$_a$ were also the same as those of rat OCTase (residues 261–270, 275–285 and 290–297), except for residue 266 (Fig. 4*b*). Thus 40K Cd-BP$_a$ appears to be OCTase.

Characterization of 40K Cd-BP$_a$ as OCTase

The physicochemical and enzymological properties of 40K Cd-BP$_a$ were compared with those of rat OCTase. The specific activity of the purified 40K Cd-BP$_a$ (Phenyl-5PW fraction) as OCTase was 230 units/mg of protein, and K_m for ornithine was 0.6 mM as determined by the method of Lusty *et al.* [7].

The M_r of 40K Cd-BP$_a$ was estimated to be 39000 from SDS/polyacrylamide-gel electrophoresis, based on the electrophoretic mobility of the polypeptide (Fig. 3*a*, lane 2). 40K Cd-BP$_a$ migrated at the same position as rat OCTase on the gel (Fig. 3*a*, lanes 2 and 4), and their mixture showed a single band (Fig. 3*a*, lane 3). The native form of 40K Cd-BP$_a$ migrated on a non-denaturing

Table 2. Physicochemical and enzymological features of 40K Cd-BP$_a$ and rat OCTase

M_r values of the denatured form and the native form of OCTase were determined by Lusty *et al.* [7] by SDS/polyacrylamide-gel electrophoresis and sedimentation-equilibrium analysis respectively. OCTase specific activity was reported by Hoogenraad *et al.* [29] (258 units/mg) and Lusty *et al.* (885 units/mg). K_m of OCTase was also given by Lusty *et al.* [7].

	40K Cd-BP$_a$	OCTase
M_r of denatured form	39000	39600
M_r of native form	110000	112000
OCTase specific activity (units/mg)	230	258 885
K_m for ornithine (mM)	0.6	0.4

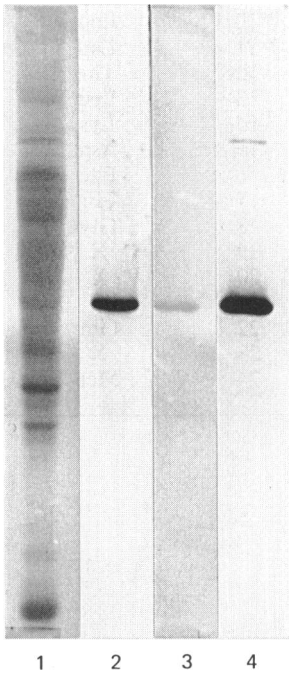

Fig. 6. Immune blotting of the crude extract from rat liver and 40K Cd-BP$_a$ with anti-OCTase antibody

The crude extract from rat liver (50 μg of protein, lanes 1 and 2) and 40K Cd-BP$_a$ (1 μg of protein, lanes 3 and 4) were separated on an SDS/polyacrylamide gel and electrophoretically transferred to a nitrocellulose membrane. The membrane was cut into pieces, and incubated with anti-OCTase rabbit IgG, and then with a Protein A–gold-colloid conjugate (lanes 2 and 4). The strips were also stained with Amido Black (lanes 1 and 3). Proteins migrated from the top to the bottom of gels.

polyacrylamide gel as a single band corresponding to OCTase activity (Fig. 3*b*). This indicates that purified 40K Cd-BP$_a$ possesses OCTase activity. To determine the M_r of native 40K Cd-BP$_a$, it was centrifuged in a sucrose density gradient. 40K Cd-BP$_a$ sedimented between bovine serum albumin and IgG (Fig. 5*a*), this position coinciding with that of OCTase activity (Fig.

5*b*). The M_r of native 40K Cd-BP_a was thus estimated as 110000. From the M_r values of the native and dissociated forms, the former was found to be trimeric, as has also been reported for rat OCTase [7].

The physicochemical and enzymological parameters of 40K Cd-BP_a, i.e. M_r values of the native and denaturing forms, specific activity and K_m, were basically the same as those of OCTase (Table 2).

Immunological identification of 40K Cd-BP_a as OCTase

The crude extract of rat liver and 40K Cd-BP_a were subjected to immune blotting using the anti-OCTase antibody and Protein A–gold-colloid conjugate. In this experiment, rabbit anti-(bovine OCTase) IgG served as the anti-OCTase antibody, in view of its cross-reactivity with rat OCTase [14]. In the crude extract from rat liver, OCTase was immunologically detected as a single band (Fig. 6, lane 2). 40K Cd-BP_a migrated at the same position as OCTase on the gel (Fig. 6, lane 3), and its band was detected as an OCTase antigen (Fig. 6, lane 4). It thus follows that anti-OCTase antibody reacts with 40K Cd-BP_a.

Binding of cadmium to 40K Cd-BP_a

The number of sites at which cadmium bound to 40K Cd-BP_a and the dissociation constant (K_d) for this metal were determined by equilibrium dialysis. The results obtained were analysed by the method of Scatchard [15] (results not shown). The number of binding sites was estimated as 0.8 mol of cadmium/mol of native form of 40K Cd-BP_a (trimer), and K_d as 7.6×10^{-6} M.

DISCUSSION

Intracellular Cd-BPs, possibly related to cadmium toxicity, have yet to be detected. Recently, three Cd-BPs (40K, 29K and 24K Cd-BPs) were found in rat liver by Western blotting. All had the ability to bind cadmium in the presence of excess zinc [3]. It was also observed, however, that cadmium taken up by the liver bound primarily to certain zinc proteins before the induction of a sufficient amount of metallothionein [6,16]. Fig. 1 shows 40K Cd-BP to be an initial cadmium-binding site in the liver.

40K Cd-BP was found to comprise at least two proteins (40K Cd-BP_a and 40K Cd-BP_b), by SDS/polyacrylamide-gel electrophoresis (Fig. 2). The protein corresponding to the lower-M_r radioactive band (40K Cd-BP_a) was purified. The linearity between the amount of protein and intensity of the radioactive band was low, thus precluding a quantitative measurement of Cd-binding activity by Western blotting. This non-linearity may be accounted for by a very small amount of contaminating cadmium from zinc acetate. The identity between a stained band on a membrane and a radioactive band in an autoradiogram thus served as the criterion for judging the purity of Cd-BP. The sequences of the *N*-terminal and three tryptic digests indicated 40K Cd-BP_a to be OCTase. On the basis of physicochemical and enzymological comparisons between the two proteins, 40K Cd-BP_a was identified as OCTase (Table 2). This enzyme catalyses the formation of citrulline from ornithine and carbamoyl phosphate, the second step in the route of urea synthesis. Thus 40K Cd-BP_a is the first Cd-BP whose function has been clarified.

One mol of the native 40K Cd-BP_a (trimer) bound to 0.8 mol of cadmium ion, and thus the native form appears to have one cadmium-binding site. The K_d of 40K Cd-BP_a for cadmium was also estimated as 7.6×10^{-6} M, this exceeding the apparent K_d of crude 40K Cd-BP (1.2×10^{-8} M) [4]. This may be due to the difference in the experimental conditions. Although the K_d of 40K Cd-BP_a is much higher than that of metallothionein ($K_d < 10^{-11}$ M), it is essentially the same as those of other metal-binding proteins [17]; the K_d values of calmodulin for calcium and cadmium were 1.1×10^{-6} M [18] and 4.5×10^{-6} M [19] respectively, and that of albumin, a cadmium carrier in serum, was 1.7×10^{-5} M [20].

OCTase is present in the matrix of mitochondria in the liver cells. Cadmium taken up by the liver parenchymal cells is known to be concentrated primarily in the mitochondria [21,22] and to disturb some mitochondrial functions. For instance, some components in the respiratory chain on the mitochondrial inner membranes and enzymes in the tricarboxylic acid cycle in the mitochondrial matrix have been observed to be inhibited by cadmium [23–26]. Such effects possibly result from the uptake of cadmium by mitochondria through the calcium-transport system [27,28]. The effect of cadmium on nitrogen metabolism has not been reported. However, urea synthesis is presumed to be disturbed by cadmium, because OCTase activity is specifically inhibited by cadmium *in vitro* (Y. Aoki & K. T. Suzuki, unpublished work). Although cadmium is a toxic metal, the specific intracellular targets for its toxic effects have not yet been determined. The present data demonstrate that OCTase is a possible target site for hepatotoxicity by cadmium. The various toxic effects of this metal are likely to be attributable to disturbances of basic metabolic pathways, such as the urea cycle and oxidative phosphorylation.

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