PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 167, 40–44 (1981)

The Binding of Cadmium Metallothionein to Isolated Renal Brush Border Membranes (41122)

W. SELENKE AND E. C. FOULKES

Departments of Environmental Health, and Physiology, University of Cincinnati, College of Medicine, 3223 Eden Ave., Cincinnati, Ohio 45267

Abstract. Cadmium metallothionein (CdMT) binds to isolated renal proximal tubular brush border membranes (BBM). The characteristics of this *in vitro* binding reflect those of the process of CdMT reabsorption *in vivo* in at least three aspects: (i) two parallel processes are involved in the reabsorption of CdMT *in vivo*, and two classes of binding sites appear to exist for CdMT on BBM *in vitro*. Both *in vitro* and *in vivo*, the processes differ in their affinity for CdMT. (ii) Myoglobin inhibits the renal reabsorption of CdMT *in vivo* and the binding of CdMT to isolated BBM. (iii) Reabsorption of CdMT is depressed in rabbits chronically treated with cadmium; isolated BBM from such animals have a decreased ability to bind CdMT. We conclude that the reaction of CdMT with BBM *in vitro* is the same as that which controls rate and specificity of CdMT reabsorption *in vivo*.

Cadmium metallothionein (CdMT) is a low-molecular-weight anionic metalloprotein (p*I* around 4) of yet uncertain function. Its synthesis is induced in many tissues under the influence of cadmium, and the metal is very tightly bound by the protein. Previous work from this laboratory (1–3) showed that CdMT is (i) freely filtered, (ii) reabsorbed from tubular fluid by two processes, one of which becomes saturated at relatively low-filtered loads while the second process approaches saturation only at much higher plasma levels, (iii) is delayed relative to inulin while passing from the renal artery to ureter, (iv) appears to compete with myoglobin (p*I* 6.8) for reabsorption, and (v) is less efficiently reabsorbed in rabbits chronically treated with cadmium than in control animals.

The first step in tubular reabsorption of protein must consist of the reaction of protein with the luminal or brush border membranes (BBM). A detailed analysis of the mechanism of protein reabsorption therefore requires the study of this protein–membrane interaction. The present paper describes the characteristics of CdMT binding to isolated BBM from rabbit kidneys.

Materials and Methods. Male New Zealand white rabbits were maintained on ordinary rabbit chow (Purina) and tap water prior to CO_2 asphyxiation. BBM were obtained essentially by the method of Pockrandt–Hemstedt *et al.* (4) using STED buffer (2.5×10^{-1} *M* sucrose, 1×10^{-2} *M* triethanolamine, 5×10^{-3} *M* disodium ethylenediamine-tetraacetic acid, 5×10^{-3} *M* dithiothreitol, final pH adjusted to 7.4). The final suspension was stored on ice in MSTH buffer [10^{-1} *M* mannitol, 10^{-1} *M* sodium chloride, 2×10^{-2} *M* tris(hydroxymethyl)aminomethane, 10^{-2} *M* Hepes (*N*-2 hydroxyethylpiperazine-*N*-2 ethanesulfonic acid) pH adjusted to 7.0]. There was a fivefold enrichment of alkaline phosphatase in BBM compared to whole kidney homogenate; activity of ouabain-inhibited Na^+, K^+-ATPase increased by an average of only 30%. Other investigators reported an eightfold enrichment of alkaline phosphatase (4, 5), but this was accompanied by a fivefold enrichment of Na^+, K^+-ATPase (5). Electron micrographs of the preparation resembled those reported by earlier workers (5). ^{109}CdMT was prepared according to the method of Cherian (6) from livers of Cd-treated animals injected 48 hr prior to death with $^{109}CdCl_2$: a typical CdMT preparation contained 117 μg Cd/ml (specific activity 20 μCi/mg), equivalent to 1.67 mg of CdMT on the basis of a molecular weight of 10,000 and a cadmium content of 7 mole/mole; the zinc content was 19 μg/ml, and the absorbance at 252 n*m* was 20.9 cm^{-1}.

0037-9727/81/050040-05$01.00/0

Proteins were measured using the Bradford Coomassie Brilliant blue G-250 dye binding method (7) (Bio-Rad) with bovine serum albumin as the standard. Alkaline phosphatase was analyzed using a commercial method (Hycel). Ouabain-sensitive Na^+,K^+-ATPase was measured by standard techniques. The phosphate released was analyzed by the method of Fiske and SubbaRow (8). Enzyme activities were calculated as micromoles of phosphate liberated per hour per milligram of protein. Osmolarity was measured with a freezing-point depression system (Advanced Instruments).

All metallic divalent cations were of reagent grade and were added as the chloride. The following chemicals were used: triethanolamine hydrochloride (Eastman), DL-dithiothreitol (Sigma), disodium ethylenediamine-tetraacetate (Fisher), tris-(hydroxymethyl)aminomethane (Fisher), Hepes (ICN Life Sci. Group) imidazole (Eastman), horse muscle adenosine 5′-triphosphate (vanadium free, Sigma), whale muscle myoglobin (Sigma), and lysozyme (p*I* 11.0) (ICN Life Sci. Group), $^{109}CdCl_2$ and [^{3}H]methoxyinulin were purchased from New England Nuclear. Other common chemicals were reagent grade.

Unless otherwise noted, all experiments were carried out as follows: 0.9 ml pH 7.0 MSTH buffer containing ^{109}CdMT, [^{3}H]-inulin and other experimental substances including a divalent cation (either 0.03 m*M* Zn or 1.2 m*M* Ca) was incubated for 3 min at 37° in a shaking water bath. Addition of divalent cation was mandated by the observation that interaction between CdMT and BBM was minimal in their absence. Ca was used at its approximate concentration in glomerular filtrate; the concentration of Zn is that producing maximal binding, as determined in preliminary studies. Then 0.6-ml suspension of 2.5 mg BBM protein/ml MSTH buffer was added and incubation continued for an additional 3 min. Four 0.2-ml samples were taken from the incubation mixture. One was left unfiltered to obtain the original ratio of ^{109}Cd/^{3}H; the other three were filtered through a Millipore EH 0.5-μm filter, prewashed with 1 ml MSTH buffer. The filter was then placed in counting vials using Biofluor (New England Nuclear). ^{3}H and ^{109}Cd were counted with suitable quench and crossover corrections on a Packard Tricarb liquid scintillation spectrometer with automatic external standardization. Variation between replicate assays did not differ by more than 7.5 ± 5.3% (SD). The presence of contaminating medium was evaluated from ^{3}H counts. Results are expressed as micrograms of ^{109}CdMT bound per milligram of BBM protein. The Scatchard plot of the data was analyzed by a conventional procedure (9).

Results. Figure 1 shows a Scatchard plot of the binding of CdMT to BBM. The results suggest the existence of at least two classes of binding sites with different affinities for CdMT. The binding capacity of the higher-affinity sites (sites A) is 0.13 μg/mg BBM protein, with an affinity constant of $2.2 \times 10^7\ M^{-1}$. The corresponding values for the lower-affinity binding sites (sites B) are 1.07 μg and $6.5 \times 10^5\ M^{-1}$.

The time course of binding at sites A and B is shown in Fig. 2. Binding at site A was complete after the shortest measurable contact time (5 sec). In contrast, at sites A and B about 55% of maximum binding was reached in 15 sec and about 80% after 1 min; it continued to increase slowly for at least another 3 min. The slow binding may be taken to reflect activity at sites B. In any case, the usual-3 min incubation period clearly is adequate for maximum reaction between CdMT and BBM.

To determine whether CdMT retention by BBM represents binding or intravesicular trapping, effects of altering vesicular volume on CdMT uptake were determined. For this purpose CdMT was incubated with BBM for the standard 3 min. At that time either distilled water, standard buffer, or hypertonic mannitol was added and the incubation was continued for an additional 3 min. No differences in the amount of CdMT retained by BBM were apparent after osmotic changes likely to alter greatly vesicular volumes (see Table I).

To obtain a measure of the tightness of binding of CdMT to BBM, the exchangeability of bound CdMT with CdMT in solution was determined. Figure 3 shows that when ^{109}CdMT, 1 μg/ml, was mixed with carrier CdMT in the ratios of 1/1, 1/3.16,

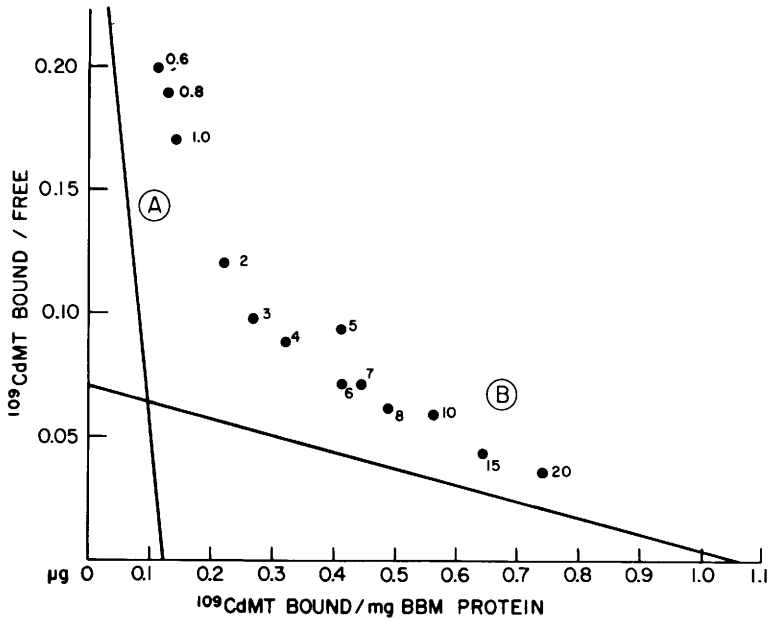

FIG. 1. Scatchard plot of binding of ^{109}CdMT to BBM. Each flask contained CdMT in amounts shown as μg/ml in numbers next to experimental points, 1 mg BBM protein/ml, and 0.03 mM Zn. Nearly identical results were obtained in a duplicate experiment (not shown). Compartment A: Affinity constant $2.2 \times 10^7\ M^{-1}$; binding capacity 0.13 μg ^{109}CdMT/mg BBM protein. Compartment B: Affinity constant $6.5 \times 10^5\ M^{-1}$; binding capacity 1.07 μg ^{109}CdMT/mg BBM protein.

1/10, 1/31.6, and 1/100, the amounts of ^{109}CdMT retained by the BBM reflected the decreased specific activity of CdMT. In contrast, if the ^{109}CdMT reacted with the BBM for 3 min prior to the addition of the carrier, the amount of ^{109}CdMT bound was not reduced to 50% of the control until a 100-fold excess of carrier CdMT was

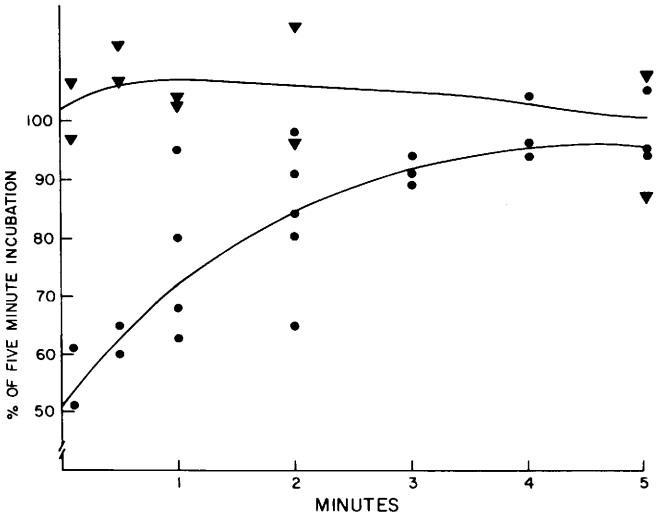

FIG. 2. Time course of binding of CdMT to BBM. 1 μg CdMT added; site A, ▲. 20 μg CdMT added; sites A and B, ●. Each point represents the mean of triplicate determinations.

TABLE I. EFFECT OF OSMOLARITY ON RETENTION OF ^{109}CdMT BY BRUSH BORDER MEMBRANES

	μg ^{109}CdMT (bound/mg BBM protein)	Final osmolarity (mosm)
Controls (6-min incubation)	0.30	
Distilled water added	0.29	192
MSTH buffer added	0.29	367
Hypertonic mannitol added	0.31	634

Note. 1 μg ^{109}CdMT/ml + 1.25 m*M* Ca; each point represents mean of triplicate determination from two separate experiments. ^{109}CdMT was allowed to react for standard 3-min time. Then 1.5 ml of solution containing 1.25×10^{-3} *M* $CaCl_2$ was added to the standard 1.5 ml solution and incubation continued for 3 more min. Neither the dilution nor the changes in osmolarity affected the amounts ^{109}CdMT retained by BBM.

added. Clearly, CdMT bound to sites A does not readily exchange. This tight binding is also shown by the fact that CdMT at site A does not appreciably dissociate upon 10-fold dilution of the incubation medium after binding was complete (results not shown). In contrast, similar experiments involving both sites A and B showed ready exchange of CdMT bound at sites B with carrier CdMT. Indeed, essentially identical amounts of ^{109}CdMT remained bound

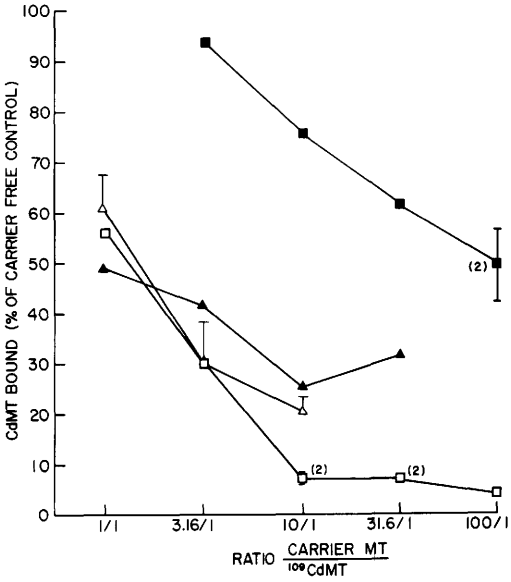

FIG. 3. Exchangeability of ^{109}CdMT bound to isolated brush border membranes. 1 μg ^{109}CdMT (sites A): Unlabeled CdMT added either with tracer (□) or after 3 min incubation (■). 20 μg ^{109}CdMT (sites A and B): Unlabeled CdMT added either with tracer (△) or after 3 min incubation (▲). Numbers inside parentheses indicate separate determinations with range; each determination was carried out in triplicate. Where bars are shown without (2), they represent mean ± (SD) of four determinations.

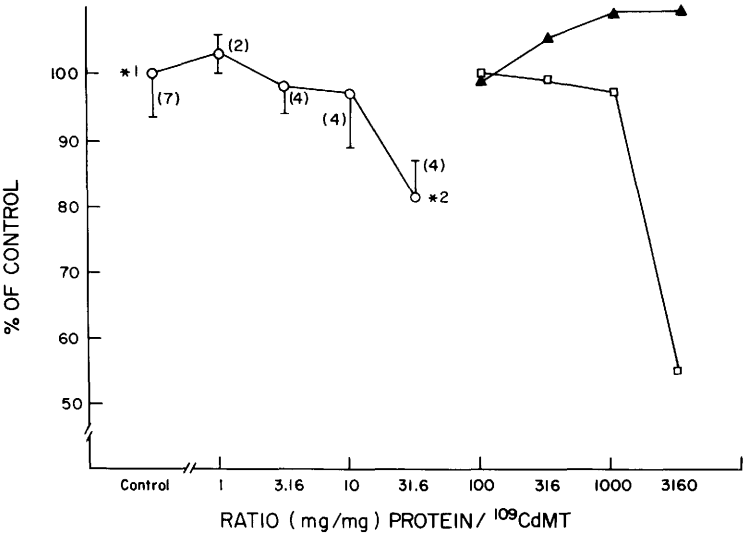

FIG. 4. Competition of myoglobin (p*I* 6.8) or lysozyme (p*I* 11.0) mixed with ^{109}CdMT (p*I* about 4.0) prior to incubation with brush border membranes. (○) 20 μg ^{109}CdMT and myoglobin. Parentheses enclose number of experimental samples. Bars are SD, but range only is indicated where $n = 2$. (□) 1 μg ^{109}CdMT and myoglobin; (▲) 1 μg ^{109}CdMT and lysozyme; typical of repeated experiments. *1 and 2 differ by 18%. ($P = 0.003$) Mann–Whitney *U* test.

whether excess carrier was added at time 0 or at 3 min (Fig. 3).

Figure 4 shows that when 1 μg/ml of CdMT was mixed with myoglobin a 1 to 1000 or a 1 to 3160 (weight/weight) ratio of myoglobin to CdMT was required to decrease the binding of CdMT (site A) to any significant degree. Lysozyme at the doses tested had no effect on this binding. In contrast, at sites A and B (20 μg CdMT/ml) at 31.6-fold excess (weight/weight) of myoglobin reduced CdMT binding by 18%.

Pretreatment of rabbits for 6 months with 1 m*M* $CdCl_2$ in drinking water was previously shown to reduce renal CdMT reabsorption (1). Similarily, in five such rabbits, in which mean cadmium values of 337 μg/g fresh weight cortex were found, BBM bound 36% less CdMT than did control BBM ($P = 0.036$). These membranes contained 620 μg Cd/g protein.

Discussion. Results reported in this paper show that CdMT is retained by BBM. This retention results not from trapping of protein inside vesicles, but apparently from actual binding. Both *in vivo* and *in vitro* evidence was obtained for two kinds of binding sites of CdMT, one of which (A) becomes saturated at relatively low CdMT concentrations. Further, the binding of CdMT to site B is inhibited by a moderate excess of myoglobin. This recalls the myoglobin inhibition of CdMT *in vivo* (3). The observation that CdMT bound to sites B *in vitro* can dissociate (Fig. 3) recalls the observation *in vivo* that a bolus of nonreabsorbed CdMT is delayed behind inulin during tubular transit. This delay could be readily explained by a transient reaction of CdMT with binding sites in the brush border. A fourth point of similarity between tubular handling of CdMT *in vivo* and its binding to BBM *in vitro* is that CdMT reabsorption is depressed in rabbits (2) chronically treated with CdMT, while BBM fragments from such rabbits have a decreased affinity for CdMT.

The significant similarities between tubular reactions of CdMT *in vivo* and the characteristics of its binding to BBM *in vitro* lead to the conclusion that binding *in vitro* reflects the first step in CdMT reabsorption, i.e., the one determining rate and specificity of the process. Work is continuing on the role of cations and of electric charge in the reaction between proteins and BBM.

This research was supported by NIH Grant ES 01462 and Grant ES 00159. The technical assistance of Mrs. Sheila Blanck is greatly appreciated. This work formed part of a dissertation submitted by W.S. for the PhD Degree to the University of Cincinnati, Department of Environmental Health.

1. Foulkes, E. C., Toxicol. Appl. Pharmacol. **45**, 505 (1978).
2. Nomiyama, K., Foulkes, E. C., Proc. Soc. Exp. Biol. Med. **156**, 97 (1977).
3. Foulkes, E. C., Proc. Soc. Exp. Biol. Med. **159**, 321 (1978).
4. Pockrandt-Hemstedt, H., Schmitz, J., Kinne-Saffran, E., and Kinne, R., Pflügers Arch. **333**, 297 (1972).
5. Just, M., and Habermann, E., Naunyn-Schmiedebergs Arch. Pharmacol. **280**, 161 (1973).
6. Cherian, G., Biochem. Biophys. Res. Commun. **61**, 920 (1974).
7. Bradford, M., Anal. Biochem. **72**, 248 (1976).
8. Fiske, C., and SubbaRow, Y., J. Biol. Chem. **66**, 375 (1925).
9. Thakur, A., and Rodbard, D., J. Theoret. Biol. **80**, 383 (1979).

Received October 28, 1980. P.S.E.B.M. 1981, Vol. 167.