observations of Pocker and Deits that at least certain anions, such as SCN^- [3], OCN^-, and N_3^-, give rise to uncompetitive inhibition patterns of CO_2 hydration at high pH. This is illustrated for N_3^- in Fig. 1. However, we offer an alternative interpretation to that of Pocker and Deits. We will show that the uncompetitive pattern is a direct consequence of the kinetic mechanism which we have proposed previously [3, 4]. The crucial point is that a strongly anion binding enzyme species, His–E–Zn^{2+}–OH_2 (see ref. [5]), precedes the rate-limiting step in catalysis.

Recently, some organic compounds that bind at or near the metal ion have received attention. One of these is phenol which has been shown to act as a competitive inhibitor of CO_2 hydration and as a mixed noncompetitive inhibitor of HCO_3^- dehydration [6]. We have also studied *o*-nitrophenol, 1,2,4-triazole and tetrazole. However, these inhibitors behave kinetically like anions and give rise to mixed uncompetitive-noncompetitive patterns in the CO_2 hydration reaction at high pH.

1 S. Lindskog, *Advan. Inorg. Biochem., 4,* 115 (1982).
2 Y. Pocker and T. L. Deits, *J. Am. Chem. Soc., 104,* 2424 (1982).
3 S. Lindskog, S. A. Ibrahim, B.-H. Jonsson and I. Simonsson, in 'Coordination Chemistry of Metalloenzymes', I. Bertini, R. S. Drago and C. Luchinat, (eds.), Reidel, Dordrecht, in press.
4 H. Steiner, B.-H. Jonsson and S. Lindskog, *Eur. J. Biochem., 59,* 253 (1975).
5 S. Lindskog, these proceedings.
6 I. Simonsson, B.-H. Jonsson and S. Lindskog, *Biochem. Biophys. Res. Commun., 108,* 1406 (1982).

O12

Spectroscopic Studies of Mercury Binding to Metallothionein

JADWIGA A. SZYMAŃSKA+, ANNIE Y. C. LAW, ANDRZEJ J. ZELAZOWSKI+ and MARTIN J. STILLMAN*

Department of Chemistry, University of Western Ontario, London, Ont., N6A 5B7, Canada

Metallothioneins (MT) are a class of low-molecular weight, sulfur-rich metalloproteins that have been isolated from a wide variety of organisms [1]. Exposure to metal ions, such as Cd, Zn, Cu, Hg, Ag and Bi, result in tissues, especially the liver and kidneys, that contain much higher levels of these proteins. The most widely studied metallothioneins have been hepatic proteins isolated following induction with $CdCl_2$ [2–4]. In these proteins there is usually a mix of cadmium and zinc with a very little copper. Early reports have described some similar induction properties for mercury from which a metallothionein-like protein was obtained [5]. Despite this very wide interest in the metallothioneins there are very few papers describing mercury binding *in vitro* [6, 7].

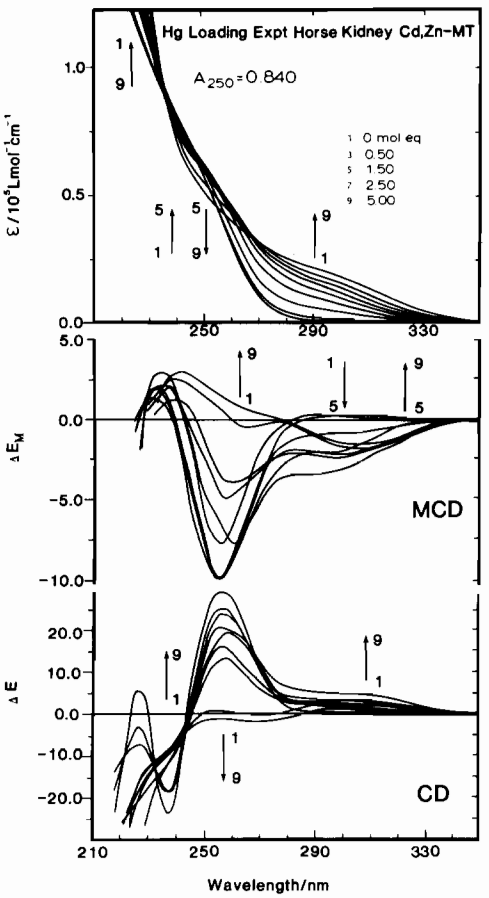

Fig. 1. Conversion of horse kidney Cd,Zn-MT 1 into Hg-MT.

Mercury has been shown to displace zinc and cadmium from metallothionein *in vitro* [6, 7]. Changes in the metal composition within Cd,Zn-MT are readily followed in the UV absorption, CD and MCD spectra as the prominent shoulder at 250 nm is a good indicator of the presence of cadmium [2, 3]. In titrations with mercury the 250 nm band is replaced by a broad band near 300 nm [6]. In this paper we present a detailed description of mercury binding to metallothionein. Titrations were carried out using horse kidney and rat liver Cd,Zn-MT and rat liver Bi,Zn-MT. Metal binding was followed by monitoring the absorption, CD and MCD spectra in the 210–350 nm region.

Rat liver Cd,Zn-MT and Bi,Zn-MT 2, and rat kidney Hg,Cu-MT 1 were induced in rat tissues by

+Permanent address: Department of Toxicological Chemistry, Medican Academy, 90145 Lodź, Poland.

injections of $CdCl_2$, $BiCl_3$ or $HgCl_2$, respectively [8]. Equine metallothionein was isolated from horse kidney [9]. Optical measurements were carried out as previously reported [2]. Figure 1 shows an example of the optical changes that occur when mercury is added *in vitro* to metallothionein. In these experiments horse kidney Cd,Zn-MT 1 was titrated with aliquots of an aqueous solution of $Hg(NO_3)_2$. The metal concentrations in the Cd, Zn-MT 1 were determined by atomic absorption spectroscopy; the values measured were Cd: 4.0, Zn: 1,2 and Cu: 0.2 (each expressed as moles of metal/mole of protein).

Line #1 in Fig. 1 shows the spectra obtained by all three techniques for the native protein; it is characteristic of Cd,Zn-MT data that derivative-shaped CD and MCD envelopes are observed under the 250 nm shoulder in the absorption spectrum [2, 3]. As mercury is slowly added, we observe first a slight increase in absorbance at 250 nm, however, after the addition of 1.50 mol eq (line #5) the absorbance at 250 nm gradually decreases. Throughout the whole titration the absorbance at 300 nm, and both the CD and MCD spectra show continuous changes that mark the loss of the cadmium and the binding of mercury to the protein. The final traces in Fig. 1 (line #9) show that the cadmium has been displaced by the mercury: there is no longer a shoulder at 250 nm in the absorption spectrum, and the derivative-shaped envelopes in both CD and MCD spectra have collapsed. The new absorption, CD and MCD intensity at 300 nm is assigned as the sulfur to mercury charge transfer band. We observe similar spectra for the Hg,Cu-MT from rat kidney and Hg complexes of BAL that serve as models of the binding sites in metallothioneins.

Acknowledgements. We thank the NSERC of Canada for financial support.

1 J. H. R. Kägi and M. Nordberg, *Experientia Supp.*, 34 (1979).
2 A. Y. C. Law and M. J. Stillman, *Biochem. Biophys. Res. Commun., 102,* 397 (1981).
3 U. Weser and H. Rupp 'The Chemistry, Biochemistry and biology of Cadmium', Ch. 7, M. Webb, ed., Elsevier/North Holland Biochemical Press, Amsterdam, 1979.
4 J. D. Otvos and I. M. Armitage, 'Biochemical Structure Determination by NMR', Ch. 4, B. D. Sykes, J. Glickson, A. A. Bothner-By, eds., Dekker, New York, 1981.
5 A. J. Żelazowski and J. K. Piotrowski, *Biochim. Biophys. Acta, 625,* 89 (1980).
6 M. Vašák, J. H. R. Kägi and H. A. O. Hill, *Biochemistry, 20,* 2852 (1981).
7 G. Sokolowski, W. Pilz and U. Weser, *FEBS-Letters, 48,* 222 (1974).
8 A. J. Żelazowski, J. A. Szymańska and H. Witas, *Prep. Biochem., 10,* 95 (1980).
9 A. J. Żelazowski, J. K. Piotrowski, E. M. Mogilnicka, J. A. Szymańska and B. W. Kaszper, *Bromat. Chem. Toksykol., 11,* 51 (1978).

O13

Isolation of Three Peptidases from *Bacillus subtilis* 168 ind⁻ with Different Specificity and Metal-Ion Activation

R. K. VAITKEVIČIUS and G. B. BABARSKIENĖ

All-Union Research Institute of Applied Enzymology, Vilnius, Lithuanian SSR, U.S.S.R.

Three metal-dependent peptidases (I, II, III) were isolated from the cells of *B. subtilis* 168 ind⁻. Phosphate buffers of pH 6.5 were used for all purification steps. Precautions were taken to avoid the contamination of enzymes by heavy metal-ions [1] in the course of the purification procedure. I and III were separated from II on DE-52 cellulose by salt gradient and I from III on hydroxylapatite with phosphate buffer concentration gradient. In addition all 3 enzymes were purified subsequently on Sephadex G-150 and DE-52 cellulose.

Leu-β-NA, Leu-Gly-Gly and Val-Val were used as specific substrates for I, II and III respectively after activation by selected metal-ions. All 3 enzymes are metalloenzymes with a comparatively weak metal-ion–enzyme bonding, thus the enzymes were isolated as inactive apoenzymes. The molecular weights of these apoenzymes are 52000, 87000 and 44000 (by gel-chromatography), isoelectric points 4.9, 4.4 and 4.7.

The mutually-independent pH-metal-ion buffers on the basis of diethylbarbiturate (phosphate or tris in some cases) for pH fixation and citrate for fixation of metal-ion concentration were used for activity determination in the course of purification. The concentration of free aqua-ions of corresponding metals in the metal-ion buffers were calculated according to Raaflaub [2], *i.e.*, without approximations when high excess of a complexing agent is present [3]. Stability constants for citrate complexes are from Li [4].

Peptidase I was activated only by Co^{++} at the concentration of free ion $1.5 \cdot 10^{-4}$ *M* for maximal enzyme activity. Leu-β-NA, Leu-NH_2, Leu-Gly-Gly, some other tripeptides and few dipeptides were hydrolyzed. II was activated by Zn^{++}, Mn^{++} and Co^{++} at the concentrations of free ions 10^{-5}, $6 \cdot 10^{-5}$ and $6 \cdot 10^{-5}$ *M* respectively for maximal activity, the maximal values of activity for Mn^{++} and Co^{++} being approx. 60 and 35 per cent as compared with Zn^{++}. Only tripeptides were hydrolyzed by enzyme II, including those that are hydrolyzed by I. III was activated by Co^{++} and Mn^{++} to the maximal degree of activity at the concentrations of free ions $3.3 \cdot 10^{-7}$ and $1.4 \cdot 10^{-6}$ *M* respectively. The enzyme hydrolyses only a large number of dipeptides.

The other metal-ions did not show any activation of all three apoenzymes in the range of concentration