The binding of cadmium to crab cadmium metallothionein

A polarographic investigation

Alfred-Martin SPARLA* and Julian OVERNELL†

The Dunstaffnage Marine Laboratory, P.O. Box 3, Oban, Argyll PA34 4AD, Scotland, U.K.

Differential pulse polarography was used to monitor the titration of crab (*Cancer pagurus*) cadmium metallothionein with additional cadmium. Scatchard analysis was used to calculate an apparent second-order dissociation constant. A mean value of 3.60×10^{-7} M was calculated at 20 °C in 0.016 M-NaClO₄, pH 8.0.

INTRODUCTION

Metallothioneins are a family of low- M_r proteins that bind the metals cadmium, copper and zinc through cysteine thiolate-metal bonds [1]. Metallothionein isolated from the crab *Scylla serrata* has been shown [2] to bind six cadmium atoms in an arrangement in which each cadmium atom is tetrahedrally bound to four cysteine thiolate ligands. The cadmium atoms are arranged in two clusters of three, whereby each atom in a cluster is linked to each of its two neighbours by a bridging thiolate. Cadmium metallothionein from the crab *Cancer pagurus* also has six cadmium atoms per molecule [3] and is apparently very similar to the *Scylla serrata* protein. Overnell *et al.* [4] showed that *Cancer pagurus* cadmium metallothionein binds one additional cadmium atom, which has a profound effect on the c.d. spectrum. The c.d. arises from the individual metal clusters, and this profound change implies that the seventh cadmium atom may link the two clusters and hence alter the conformation of the protein. In order to investigate this site further, we wished to determine the apparent dissociation constant of the seventh cadmium atom.

MATERIALS AND METHODS

Cadmium metallothionein was prepared as described previously [3]. Electrophoresis in non-denaturing buffer on 10% polyacrylamide gel showed only a single band. Metallothionein concentrations were determined by using a molar absorption coefficient of $50\,000\text{ M}^{-1}\cdot\text{cm}^{-1}$ at 220 nm at pH 1.5 [3]. All reagents were of AnalaR grade from BDH Chemicals. Polarographic analysis was carried out by using a Metrohm type Polarecord 626 polarograph with a Metrohm type 663 mercury electrode. Metal analysis was performed by using a Pye-Unicam type SP192 atomic absorption spectrometer. Buffer solutions were rendered cadmium-free by passage through a column of Chelex-100.

The polarograph was operated in the differential pulse mode with a pulse height of 50 mV and a pulse duration of 60 ms. Potential was scanned from -0.40 V to -0.95 V at a scan rate of 5 mV/s and sensitivity was 0.1 nA/mm. The electrode was operated in the static mercury-drop mode at a dropping rate of 1/s. Measurements were carried out at room temperature (20 °C).

The supporting electrolyte was 0.016 M-NaClO₄ in 0.04 mM-Na₂HPO₄/NaH₂PO₄ buffer. The pH was adjusted to 8.0 with HClO₄. Cadmium for titration was made up as a stock solution of CdCl₂ in the supporting electrolyte. Cadmium metallothionein was dissolved in buffered supporting electrolyte and added to the electrode vessel to give a final volume of 20 ml. This was

deoxygenated with O₂-free N₂ for 12 min and the polarogram was recorded. The first and subsequent additions of cadmium were mixed by gassing the solution with N₂ for a further 5 min and the new polarograms were recorded.

RESULTS AND DISCUSSION

Titration curves were carried out at metallothionein concentrations of 0.75, 0.77, 1.72 and 2.32 μM . Fig. 1 illustrates the titration of 2.32 μM -metallothionein. It can be seen that in the native protein

Fig. 1. Polarograms of titration points for the addition of CdCl₂ to 2.32 μM crab cadmium metallothionein, pH 8.0, at 20 °C

Curve a, buffer only; curve b, metallothionein only; curves c–j, CdCl₂ added to the following concentrations: curve c, 0.50 μM ; curve d, 1.02 μM ; curve e, 2.55 μM ; curve f, 3.05 μM ; curve g, 5.05 μM ; curve h, 5.55 μM ; curve i, 6.54 μM ; curve j, 6.80 μM .

* Present address: Vincent-Lübeck-Schule, Glückstädterstrasse 4, 2160 Stade, Federal Republic of Germany.

† To whom correspondence should be addressed.

Fig. 2. Scatchard-type analysis of titration data at different metallothionein concentrations

Metallothionein concentrations: ■, 2.32 μM ; ▼, 0.77 μM ; ●, 1.72 μM ; ★, 0.75 μM .

reduction is occurring at potentials of -0.48 and -0.905 V. These reductions are not present in native BSA (A.-M. Sparla & J. Overnell, unpublished work) and presumably represent reduction of cadmium thiolate clusters in the dissolved protein. Cyclic voltammetry of *Scylla serrata* cadmium metallothionein [5] in Hepes buffer, pH 7.3, showed a peak at 0.59 V in the negative-going direction, which was interpreted as being due to reduction of thiol groups of the protein adsorbed on the mercury surface. Adsorption on the mercury drop was not investigated in the present work, but lack of agreement with the cyclic-voltammetry results may indicate that adsorption was not significant in this work. The peak at -0.665 V in Fig. 1 coincides with the reduction potential of free cadmium in the absence of protein but in the same matrix.

The height of this peak at -0.665 V was used to calculate the concentration of free Cd^{2+} ion in the presence of the metallothionein and hence, by difference, the amount of the added cadmium that had been bound to the metallothionein. Fig. 2 is a plot of the concentration of bound cadmium versus the ratios of concentrations of bound to free cadmium for the four metallothionein concentrations used. The dissociation constants were determined from the slopes by using the expression [6]:

$$[\text{Bound Cd}] = n - K_{\text{diss.}} \cdot [\text{bound Cd}]/[\text{free Cd}]$$

where n is the concentration of protein binding sites. Deviations from the straight line produced by the first three points were found in three of the four sets of measurements at higher cadmium concentrations. The straight lines have been drawn through the first three points only in these cases, although the first four points have been plotted. (The observed deviations may be due to increasing errors associated with the calculation of the

bound cadmium concentrations.) The results of the measurements were as follows: for 0.75 μM -protein $K_{\text{diss.}} = 2.61 \times 10^{-7}$ M and $m = 1.17$, for 0.77 μM -protein $K_{\text{diss.}} = 1.48 \times 10^{-7}$ M and $m = 1.70$, for 1.72 μM -protein $K_{\text{diss.}} = 2.11 \times 10^{-7}$ M and $m = 0.76$, and for 2.32 μM -protein $K_{\text{diss.}} = 8.19 \times 10^{-7}$ M and $m = 1.35$, where m is the concentration of binding sites divided by the metallothionein concentration.

Binding of cadmium to the buffered supporting electrolyte was assumed to be negligible, and no correction for this has been applied. (In all cases the concentrations of added cadmium and phosphate were below their solubility product.) The slopes give a mean value for the apparent second-order dissociation constant of 3.6×10^{-7} M. This may be compared with the estimated [7] apparent dissociation constant of 5×10^{-20} M for horse metallothionein at pH 8.0 (which is based on an assumption of equal binding strengths for all sites). The intercepts of the lines with the ordinate give the concentration of binding site(s) studied by this method. From these values the number of binding sites per molecule was calculated by dividing by the metallothionein concentration used. This gave a mean value of 1.25 sites per molecule.

Clearly the binding strength for this additional seventh cadmium-binding site is very much weaker than for the other six, but it could represent a temporary 'safe home' for additional cadmium before the induction of synthesis of further metallothionein. Indeed, if a large conformational change were elicited [4] the molecule with the seventh binding site occupied could be the putative binding protein recognizing gene metal regulatory elements [8]. If mammalian metallothioneins similarly bound additional metal, then binding to DNA could explain the presence of metallothionein in the cell nucleus [9], as detected immuno-histochemically.

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