Dynamic structure of metallothionein

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Molecular sieve chromatography of rabbit liver metallothionein at different electrolyte concentrations revealed that this protein undergoes an increase in Stokes radius from 1.50 to 1.78 nm when the ionic strength is lowered from 0.5 to 0.015 indicating a change in molecular shape and/or hydration. The variation in ionic strength also affects the far-UV circular dichroism of metallothionein reflecting a conformational transition in the protein. The effects are attributed to changes in intramolecular repulsion between the strongly negatively charged metal-thiolate clusters of the protein. It is suggested that metallothionein exists in at least two interchangeable conformational states which differ in hydrodynamic properties and whose equilibrium concentrations are determined by the electrostatic free energy of the system.

Metallothionein *Stokes radius* *Circular dichroism* *Molecular expansion* *Flexibility* *Metal-thiolate cluster*

1. INTRODUCTION

Metallothioneins (MTs) are widely occurring proteins which are characterized by a low M_r (6500 to 6800) and an extremely high metal and sulfur content. The mammalian forms usually contain 7 diamagnetic metal ions such as Zn(II) and/or Cd(II) and 20 cysteine residues but are completely lacking in aromatic amino acid residues [1]. Their biosynthesis is induced by certain metal salts and hormones and, hence, it is believed that MT is involved in trace metal metabolism, homeostasis and detoxification [2]. Structural studies have established that MTs have a well-defined tertiary structure and that the metal ions are organized in two metal clusters built up of tetrahedral tetrathiolate units [3,4]. The two metal clusters containing 3 and 4 metal ions are believed to be located in the N- and C-terminal portions of the molecule, respectively [5]. We report here the occurrence of an intramolecular conformational transformation in MT which is effected by changes in ionic strength and which manifests itself in substantial changes in the hydrodynamic behavior and far-UV circular dichroism.

2. MATERIALS AND METHODS

Rabbit liver MT-1 was isolated from the liver of rabbits injected subcutaneously 15 times with 1 mg Cd(II) per kg body wt at 2–3-day intervals [6]. The protein was purified by a procedure adapted from [7] and [6]. The purity of the preparation was assessed by amino acid analysis and atomic absorption spectrometry. The native protein ($(Cd,Zn)_7$-MT-1) contained a total of 7 mol bivalent metal per 6100 g apoprotein (apo-MT-1) with a metal ratio of 55% Cd to 45% Zn. The form containing solely Zn (Zn_7-MT-1) was prepared from the liver of rabbits submitted to injections of Zn(II) instead of Cd(II). Cd_7-MT-1 was obtained by reconstituting apo-MT-1 with Cd(II). Apo-MT-1 was prepared by dialyzing $(Cd,Zn)_7$-MT-1 against 0.01 M HCl. Except where specified otherwise (table 1), gel filtration measurements were performed at 4°C on a 0.8 × 150 cm Sephadex G-75 (fine) column equilibrated with a buffer composed of 50 mM Tris and 12.5 mM HCl (pH 8.6). The desired ionic strength was obtained by addition of KCl to the buffer. Samples containing 1 mg protein and 10% (w/v) NaCl in 1 ml Tris–HCl

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buffer were applied to the column and 1.5-ml effluent fractions were collected at a constant flow rate of 10 ml per h. Elution distribution coefficients, K_d, were evaluated from the relationship $K_d = (V_e - V_o)/(V_t - V_o)$. The elution volumes of protein, V_e, of blue dextran, V_o, and of salt, V_t, were determined by absorbance measurements at 250 and 600 nm, and by conductivity measurements, respectively. V_o varied slightly with ionic strength ($V_{o,I=0.5}/V_{o,I=0.015} = 0.95$). V_t was independent of ionic strength. The K_d values of marker proteins were determined in separate experiments. Measurements of K_d were reproducible within 3%. Circular dichroism (CD) spectra were recorded at room temperature with a Cary 61 spectropolarimeter. Molar ellipticity, $[\theta]$, is given in units of deg · cm^2 · dmol^{-1}. Mean residue ellipticity, $[\theta]_{MRW}$, is the molar ellipticity divided by the number of residues, i.e., 61. Spectrophotometric measurements of MT concentration were made in 0.01 M HCl at 220 nm using a molar absorptivity of the apo-MT of 47300 [8].

3. RESULTS

Native rabbit liver (Cd,Zn)$_7$-MT-1 and the monometal forms Zn$_7$-MT-1 and Cd$_7$-MT-1 have identical elution distribution coefficients, K_d, when chromatographed on Sephadex G-75 at constant temperature and ionic strength (table 1). The chromatographic behavior is also unchanged when the metal is removed by exposure to low pH. There is, however, an unusual dependence on salt concentration. While on raising the ionic strength, I, from 0.015 to 0.5 the K_d values are slightly reduced for the marker proteins, they are increased by about 15% for MT. The effect is fully reversible and is independent of the Zn:Cd ratio and of protein concentration. In all instances the peaks retain their nearly symmetrical shape and are of comparable half-width.

Since in the range of salt concentrations employed the effective pore size of the gel is nearly constant, these results indicate that the Stokes radius of MT is decreased on going from lower to higher ionic strength. Comparison with marker proteins of known Stokes radius indicates a reduction from 1.78 nm at $I = 0.015$ to 1.50 nm at $I = 0.5$ (fig.1, top). The decrease in Stokes radius is a monotonic function of salt concentration reaching

Table 1

Effect of ionic strength on gel filtration behavior of rabbit liver metallothionein and of marker proteins

Protein	Elution distribution coefficient on Sephadex G-75 (K_d)	
	$I = 0.015$	$I = 0.5$
(Cd,Zn)$_7$-MT-1[a]	0.50	0.59
Cd$_7$-MT-1[a]	0.50	0.59
Zn$_7$-MT-1[a]	0.50	n.d.
Apo-MT-1[b]	0.50[b]	n.d.
Ovalbumin	0.14	0.13
Chymotrypsinogen	0.38	0.37
Ribonuclease	0.55	0.53
Insulin, oxidized A-chain	0.83	0.82

[a] Metal compositions before and after gel filtration were identical

[b] Measured in 0.015 M HCl

n.d, not determined

a constant minimum value above $I = 0.25$ (fig.1, bottom). The reduction corresponds to a change in frictional ratio, f/f_{min}, from 1.51 at $I = 0.015$ to 1.27 at high ionic strength, indicating a transition to a more globular shape. Assuming a rigid prolate ellipsoid shape and a standard degree of hydration of 0.2, such a decrease in f/f_{min} is equivalent to a change in axial ratio, a/b, from approx. 8 to 4 [10].

The variation in ionic strength also affects the CD features of MT (fig.2). The CD spectrum of this protein arises both from the optically active metal-thiolate transitions of the metal complexes and of the secondary amide transitions of the protein moiety [8,12,13]. Above 240 nm the ellipticity is due exclusively to the metal complexes. At shorter wavelength CD contributions from both sources are superimposed but with that arising from polypeptide chain folding dominating. As shown in fig.2 (top) addition of salt to Cd$_7$-MT-1 reduces the ellipticity below 240 nm but has no effect on the biphasic ellipticity band centered about the first CD-thiolate transition near 250 nm [11]. Analogous and fully reversible ellipticity changes also occur with (Cd,Zn)$_7$-MT-1. Therefore, it is most likely that the observed effects reflect alterations in polypeptide conformation. The change in amplitude of the 227 nm ellipticity maximum from

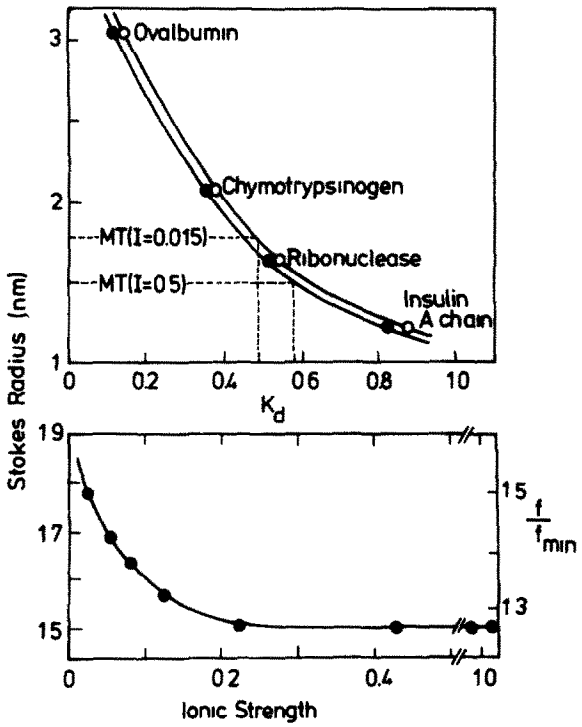

Fig.1. Effect of ionic strength (I) on Stokes radius (R_e) of MT. (Top) Evaluation of R_e of $(Cd,Zn)_7$-MT-1 from gel filtration (Sephadex G-75) distribution coefficient (K_d) at $I = 0.015$ and $I = 0.5$. Calibration curves were prepared by plotting R_e of marker proteins *vs* their K_d values (table 1) determined at $I = 0.015$ (○) and $I = 0.5$ (●). The R_e values employed were: ovalbumin, 2.81 nm; chymotrypsinogen, 2.25 nm; ribonuclease, 1.79 nm; oxidized insulin A-chain, 1.22 nm [7]. (Bottom) Plot of R_e (left ordinate) and of frictional ratio, f/f_{min} (right ordinate) of (Cd,Zn)-MT-1 *vs* ionic strength. R_e was determined from gel filtration data (top); $f/f_{min} = R_e/R_{min}$ [9]. The minimum radius, R_{min}, is 1.19 nm as given by the relationship

$$R_{min} = (3/4 \frac{M_r \bar{v}}{N})^{1/3},$$

where M_r is 6700 and $\bar{v}$ is the partial specific volume (0.635) of MT [7] and N Avogadro's number.

+65000 to +23000° upon raising the ionic strength from 0.015 to 3.0, respectively, corresponds to a decrease in mean residue ellipticity, $[\theta]_{MRW,227}$ of the protein by 690° (fig.2, bottom). The existence of an isodichroic point near 220 nm suggests that the CD changes reflect an interconversion of only two conformational forms of MT.

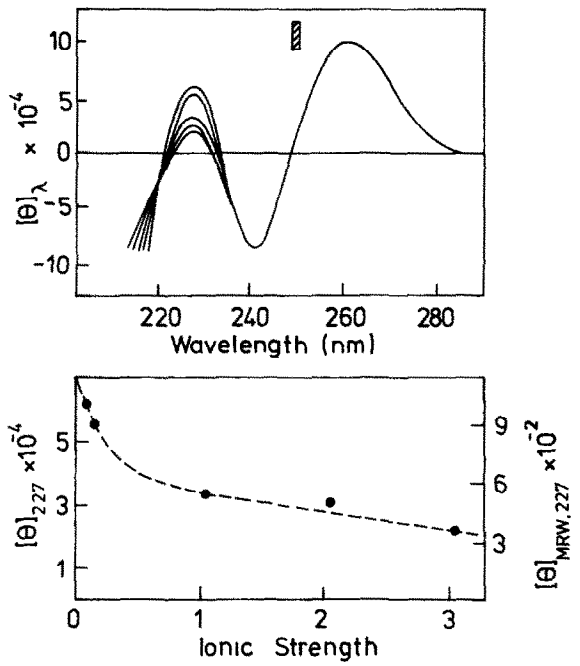

Fig.2. Effect of ionic strength on CD spectrum of MT. (Top) CD spectra of Cd_7-MT-1 at I ranging from 0.015 (top) to 3.0 (bottom). Molar ellipticity, $[\theta]$, is plotted *vs* wavelength. Conditions: protein concentration, 1.2×10^{-4} M in 0.02 M Tris–0.005 M HCl (pH 8.6), path length 0.1 cm. I was adjusted by the addition of appropriate amounts of KCl. The bar indicates the position of the first metal-thiolate transition of Cd_7-MT-1 [11]. (Bottom) Plot of $[\theta]_{227}$ of Cd_7-MT-1 *vs* ionic strength. The right ordinate is in units of mean residue ellipticity, $[\theta]_{MRW,227}$.

4. DISCUSSION

MTs have long been known to be nonglobular proteins. Their M_r, ranging from 6500 to 6800 depending on metal composition, and their reported Stokes radius of 1.61 nm, measured in 0.1 M Tris–acetate buffer (pH 7.0), are consistent with a molecular form approximating that of a prolate ellipsoid with an axial ratio of 6 [7]. Recently, this nonspherical model has received independent support from the suggestion that the metals are located in two separate domains involving the C- and N-terminal halves of the polypeptide chain [5].

The present data extend the previous hydrodynamic studies [7] and reveal as a new find-

ing that the Stokes radius of this protein is highly sensitive to ionic strength. Thus, in contrast to the globular marker proteins employed, MT undergoes a reversible 16% decrease in Stokes radius when the ionic strength is changed from 0.015 to 0.5 (table 1, fig.1). Since the effect is independent of protein concentration, the possibility that the change in Stokes radius arises from an association-dissociation process is excluded. Hence, the change in Stokes radius must derive either from an alteration of molecular shape or of hydration of the monomeric species or both.

While over a wide pH region many globular proteins are insensitive to changes in ionic strength [9], it is of interest that a reversible hydrodynamic volume change analogous to that seen with MT occurs with bovine serum albumin in moderately acidic solution. Thus, at pH 3, this protein undergoes a nearly 50% increase in equivalent sphere radius when the ionic strength is lowered from 0.15 to 0.01 [14]. The volume change is also accompanied by a change in optical activity indicative of alterations in protein conformation [15]. Compared to these structural changes the ionic strength-dependent effects on the conformation of MT are less extensive (fig.2). The salt-induced reduction in ellipticity of MT below 240 nm could indicate a small increase in α-helix content or a decrease in β-structure [16,17]. The latter seems particularly plausible since a computer analysis of the far-UV CD spectrum of apo-MT and a structure prediction calculation from sequence data [13] indicate a total of about 40% β-sheet and β-bend conformation. The absence of absorption and ellipticity changes in the region of Cd(II)-thiolate excitation shows that the coordination geometry of the metal-thiolate complexes remains unaffected by the conformational transformation attending the molecular expansion.

As in bovine serum albumin, the increase in Stokes radius at low ionic strength is probably caused by an increase in intramolecular repulsion of like electric charges due to the loss of the ion atmosphere [9]. In the pH range studied rabbit liver MT-1 has an overall charge of -2 (in preparation). However, an assessment of the charge distribution within the molecule indicates that each of the two metal-thiolate clusters carries 3 negative charges [13] which locally may be balanced only incompletely by the positive charges of the lysine residues of the polypeptide chain. Hence, at low ionic strength mutual electrostatic repulsion of the strongly negatively charged cluster domains may be the cause of the observed molecular expansion.

The change in Stokes radius in response to the modulation of electrostatic forces by variations in ionic strength implies that native MT has a flexible structure with several accessible conformational options. In the simplest model the measured hydrodynamic effects could reflect changes in the populations of just two dynamically interchangeable conformers which differ widely in shape or hydration or both and whose equilibrium concentrations are determined by the electrostatic free energy of the system. Such a model of two principal interchangeable conformational states obtains some support from the existence of a well-defined isodichroic point in the CD spectrum (fig.2, top). The occurrence of at least two coexistent isomeric forms could account for the well-known difficulties in crystallizing MT. It may also be responsible for the presence of supernumerary resonances underneath the ^{113}Cd-NMR signals of $^{113}Cd_7$-MT recently resolved by two-dimensional ^{113}Cd-NMR spectroscopy [18].

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