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Non-identity of reaction centres for pyrophosphatase and toxic actions of cardiotoxin II: The status of cardiotoxin II as a metalloprotein

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Abstract. Cardiotoxin II of the Indian cobra (*Naja naja*) contains approximately four Mg^{2+} per mol. Complete demetallation of the toxin is achieved by three cycles of treatment with ethylenediamine tetraacetate and gel filtration. Reconstitution of toxin by treatment of the apo-protein with Mg^{2+} restores metal content and inorganic pyrophosphatase activity only to the extent of two atoms/mol and 65%, respectively. Use of Mg (II)-EDTA in the reconstitution experiment yields restoration of half the original enzyme activity. Mg^{2+} is required for the inorganic pyrophosphatase action of the toxin. A definitive statement on the non-essentiality of Mg^{2+} for the lethal toxicity of the toxin is not possible at present, although experimental observations indicate that demetallated toxin is as toxic as the native toxin. Based on this and the differing sensitivities of the enzyme and toxic activities of the toxin to heat, it is suggested that the reaction centres in the toxin for the two activities are different and that the pyrophosphatase activity is not causally connected with the lethal toxicity of the toxin.

Keywords. Cardiotoxin II; pyrophosphatase; magnesium; metalloprotein; reaction centres; cobra.

Introduction

Previous work from this laboratory had demonstrated that cardiotoxin II as isolated by chromatography of cobra (*Naja naja*) venom on CM-Sephadex C-25 columns (Achyuthan *et al.*,1980) was associated with a nearly constant inorganic pyrophosphatase activity, irrespective of whether chromatography was done in the presence or absence of the detergent Triton X-100 (Achyuthan and Ramachandran, 1980). A subsequent report (Achyuthan and Ramachandran, 1981) had recorded in detail the properties relating to the pyrophosphatase action of cardiotoxin II. It was strange that, unlike many pyrophosphatases, no metal (magnesium) requirement for enzyme activity could be demonstrated as neither added Mg^{2+} nor ethylenediamine tetraacetate exerted any pronounced action. The question whether the pyrophosphatase action has any causal relationship to the cardiotoxicity of the protein also remained to be answered. In this paper we

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report evidence showing that cardiotoxin II is indeed a Mg^{2+}-containing protein, that enzymatic and toxic actions of the protein seem causally unrelated and that cardiotoxin II has no apparent requirement for Mg^{2+} for its toxic action.

Materials and methods

Cardiotoxin II was isolated as described earlier (Achyuthan *et al.*, 1980). Mg (II)-EDTA complex (Pfeiffer and Simmons, 1943) was prepared according to Brunisholz (1957) in 94.2% yield with a Mg^{2+} content of 94.2% of theoretical and titratable carboxyl content of 1.85 groups/mol. It had a solubility of about 1 µmol/ml at 27°C. Sephadex G-10 was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. All other chemicals used were of analytical grade and water for use was double distilled/deionized.

Inorganic pyrophosphatase, protein and lethal toxicity (LD_{50}) were determined by the methods of Heppel (1955) at pH 7.2, Lowry *et al.* (1951) and Litchfield and Wilcoxon (1949), respectively. Magnesium was estimated using an atomic absorption spectrophotometer (Varian Techtron Model 1000), and processing of samples (protein, Tris-(hydroxymethyl) -amino methane (Tris), Mg^{2+}-EDTA and blanks) for analysis was done according to Allan (1971).

Demetallation of cardiotoxin II was done as follows. The protein (20 mg) was dissolved in Tris-HCl buffer (0.4 ml, 0.2 M, pH 7) containing EDTA (0.05 M) and incubated at 4°C for 10 h. It was then applied to a column of Sephadex G-10 (0.9 × 80 cm) and elution done using Tris-HCl buffer not containing EDTA. Fractions of 1 ml were collected. The protein appearing in fractions 10-16 was pooled/lyophilized and analyzed. The protein so recovered was dissolved in 0.5 ml 0.2 M sodium acetate solution (adjusted to pH 7.0) containing 0.05 M EDTA and incubated for 10 h at 4°C. The protein was again recovered after passage through a Sephadex G-10 column using 0.1 M sodium acetate solution (pH 7.0) containing no EDTA for elution. After analyses, the remainder of the protein was subjected to one more cycle of EDTA treatment and gel filtration and the demetallated protein recovered and lyophilized. Remetallation of demetallated cardiotoxin II was done by dissolving the protein (3 mg) in 0.5 ml Tris-HCl buffer containing $MgCl_2$ (50 µmol) and incubating at 37± 1°C for 4 h. The solution was then passed through the Sephadex G-10 column and the protein eluted with the Tris-HCl buffer to yield 2.1 mg protein (70%).

The efect of metal ions and metal chelates in restoring the enzymatic activity to the toxin was ascertained by the addition of known concentrations of additives to the pyrophosphatase assay tubes.

Results and discussion

The magnesium content of the native toxin and protein obtained after twice- and thrice-repeated cycles of EDTA treatment and gel filtration were 1.47%, 0.22% and 0%, respectively. The magnesium content after remetallation of demetallated protein was 0.69%. Thus native cardiotoxin II, as isolated by ion exchange gel chromatography, contained 4.36% or approximately four atoms of Mg^{2+} per mol based on a molecular weight of 6825 calculated from the known amino acid composition (Srinivasa *et al.*, 1982), where: demetallated protein obtained after

two cycles of EDTA treatment and gel filtration contained 0.65 atoms/mol and the fully demetallated product none. Remetallation of fully demetallated cardiotoxin II restored Mg^{2+} content to the extent of only 2.05 atoms per mol. Thus the effectiveness of remetallation is only about 50%.

Naja naja atra (Formosan cobra) venom is reported to have an ash content of 2.16%, of which 6.49% is magnesium (Tu, 1977). Other values reported for venom of other cobras fall in the range 2-100 mg/g venom (Kaye, 1960; Meldrum, 1965; Henriques and Henriques, 1971). Our limited analyses, on two samples of *Naja naja* venom from two different sources (Haffkine Institute and Astik Farm), yielded magnesium contents in the range 4.5-9.2 mg/g venom. Thus, it is interesting to find that cardiotoxin II, the more basic of the two cardiotoxins in venom, which is isolated in weight yields of 15% from crude venom may account for 25-50% of the magnesium present in venom. As remetallation, under conditions used, restores the metal content only to the extent of two atoms/mol, one may infer that half the magnesium in the toxin as isolated, is in a loosely bound form perhaps just carried along with the toxin during ion exchange chromatography and not easily removed from it by desalting on Sephadex G-10 for removal of phosphate buffer salts. Nevertheless, the evidence now presented clearly establishes that cardiotoxin II is a magnesium containing metalloprotein.

The ability to demetallate cardiotoxin II enabled observations on the role of Mg^{2+} in toxicity. Table 1 presents data on LD_{50} values for native cardiotoxin II and demetallated toxin. The latter is only 7% less toxic. On first appearance this would mean that Mg^{2+} is not essential for the display of toxicity. But as the route of administration for LD_{50} measurements in mice is intraperitoneal and deaths occur or are observed over a period of 24 h the possibility is there for remetallation *in vivo*

Table 1. Effect of demetallation and of heating (60°C) on the toxicity of cardiotoxin II.

Time (min)	LD_{50} (mg/kg mouse)	Slope function	Potency ratio**
Demetallated Cardiotoxin II: B	2.8 (3.33-2.35)*	1.28	0.93
Cardiotoxin II:			
0 (Control)	2.6 (3.09-2.18)*	1.36	1.00**
40	3.2 (4.16-2.46)*	1.50	0.80
60	3.4 (4.08-2.83)*	1.34	0.77
80	4.0 (4.96-3.23)*	1.47	0.65
100	5.0 (6.65-3.76)*	1.56	0.52

« The values in parentheses represent 19/20 confidence limits

« « The potency of native cardiotoxin II is taken as 1.00

caused by Mg^{2+} in the body pool. Observations on the pyrophosphatase activity of native and demetallated cardiotoxin II under various experimental conditions are

given in table 2. The effect of varying concentration of Mg^{2+} on the enzyme activity of cardiotoxin II put through only one cycle of demetallation shows that maximal activity (about 65%) is restored at a Mg^{2+} concentration of 12.5 µmol/ml,

Table 2. Effect of metal ions, EDTA and metal chelates on the pyrophosphatase activity of native and demetallated cardiotoxin II.

Sample	Additions	Concentration of additive in assay medium (µmol/ml)	Relative pyrophosphatase activity
Cardiotoxin II-control	None	—	100
Cardiotoxin II	Na_2-EDTA	100	73
Demetallated Cardiotoxin IIA*	None	—	15
Demetallated Cardiotoxin IIB*	None	—	7
Demetallated Cardiotoxin IIC*	None	—	0
Demetallated Cardiotoxin IIB	Mn^{2+}	1-20	0
Demetallated Cardiotoxin IIB	Co^{2+}	1-20	0
Demetallated Cardiotoxin IIB	Mg^{2+}	20	75
Demetallated Cardiotoxin IIB	Mg^{2+}+EDTA	20	
		50	53
Demetallated Cardiotoxin IIB	Mg^{2+} (II)-EDTA	1	44
Demetallated Cardiotoxin IIB	Na_2-EDTA	10	5
Demetallated Cardiotoxin IIB	Mg^{2+} (II)-EDTA	1	
	+Na_2-EDTA	9	17

Protein concentration in all experiments was 0.5 mg/ml

« Demetallated Cardiotoxins II: A, B and C refer to protein recovered after one, two and three cycles, respectively, of treatment with EDTA followed by gel-filtration on Sephadex G-10.

at a protein concentration of 0.5 mg/ml. Mg (II)-EDTA at a concentration of 1 µmol/ml restores 44% of the enzyme activity. Variations in the ratio of Mg (II)-EDTA to the substrate, namely pyrophosphate, from 1:1 to 1: 5 did not affect level of activity regained. Reconstitution of the metal-enzyme at pH 6.5 or 7.5, and assay of enzyme at pH 7.2 as usual, did not lead to better recovery of activity than that gained by reconstitution and assay at pH7.2. A 53 % regain of activity is seen when reconstitution for enzyme assay is effected using 50 µmol/ml of EDTA and 20 µmol/ml Mg^{2+}, conditions in which virtually all Mg^{2+} would exist as Mg (II)-EDTA. That Mg. (II) -EDTA can to a large extent be effective in taking on the role of free Mg^{2+} is interesting. This observation explains our earlier finding (Achyuthan and Ramachandran, 1981) that the activity of cardiotoxin II as a pyrophosphatase is diminished by only 25% on treatment with EDTA, The experimental difficulty observed in not being able to restore the original level of enzyme activity in the reconstitution experiments may mean that either ideal conditions for reconstitution are yet to be established or that the protein on demetallation has

undergone some irreversible change. However, the data amply reveal that the magnesium present in cardiotoxin II, at least about half that originally present, is essential for its pyrophosphatase activity. Neither Co^{2+} nor Mn^{2+} are able to replace Mg^{2+} in this regard.

The data on the effect of heat ($60^{o}C$) on the pyrophosphatase activity and toxicity of cardiotoxin are presented in table 1 and figure 1. In contrast to toxicity, the enzyme activity is more susceptible to heat. Half of it disappears in less than 1 h and almost all of it in 1.5 h (figure 1). The two activities decay at vastly different rates, with the relative sturdiness of the toxic activity of cardiotoxin II attesting further to the rather well-known heat resistance of snake neurotoxins and

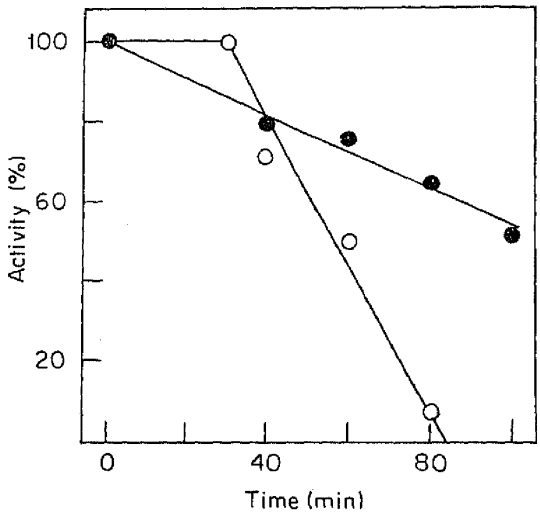

Figure 1. Effect of temperature ($60_{o}C$) on toxicity and pyrophosphatase activity of Cardiotoxin II. The native protein in water solution was heated in a water bath for different durations. The samples were removed, cooled rapidly and assayed for pyrophosphatase activity (O) and lethal toxicity (LD_{50}) (=).

cardiotoxins in general. That a single polypeptide, cardiotoxin II, should be invested with two apparently different reaction centres (active sites) need not occasion surprise. One could certainly visualise situations in biologically active proteins where more than one function (active site) is fortuitously present, of which the one conformationally more mobile is more susceptible to heat denaturation. Since samples of toxin in which all enzymatic activity has been destroyed still retain about half the original toxicity, one must conclude that the pyrophosphatase activity is not causally related to the toxic action of cardiotoxin II. That is not to say that pyrophosphatases cannot be toxic *per se.*

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References

Allan,J. E. (1971) *The Preparation of Samples for Analysis by Atomic Absorption Spectroscopy* (California: Varian Techtron Ltd).

Achyuthan, K. E., Ranganatha Rao, K. and Ramachandran, L. K. (1980) *Indian J. Biochem. Biophys.*, **17,** 228.

Achyuthan, K. E. and Ramachandran, L. K. (1980) *Proc. Indian Natl. Sci. Acad.*, **B46,** 603.

Achyuthan, K. E, and Ramachandran, L. K.(1981) *J. Biosci.*, **3,** 149.

Brunisholz, G. (1957) *Chimia (Switz.),* **11,** 363; C. A. **52,** 6048g.

Henriques, S. B. and Henriques, O. B. (1971) *Int. Encycl. Pharmacol. Ther.*, **1,** 225.

Heppel, L. A. (1955) *Methods Enzymol.*, **2,** 570.

Kaye, M. A. G. (I960) *Biochim. Biophys. Acta,* **38,** 34.

Litchfield, J. T., Jr. and Wilcoxon, F. (1949) *J. Pharm. Exp. Therap.*, **96,** 99.

Lowry, O. H., Rosebrough, N.J., Farr, A. L. and Randall, R.J. (1951) *J. Biol. Chem.*, **198,** 265.

Meldrum, B. S. (1965) *Pharmacol. Rev.*, **17,** 402.

Pfeiffer, P. and Simmons, H. (1943) **B76,** 847; C.A. **38,** 2931[1]

Srinivasa, B. R., Achyuthan, K. E. and Ramachandran, L. K. (1982) *Indian J.Biochem. Biophys.,* **19,**52.

Tu, A. T. (1977) *Venoms: Chemistry and Molecular Biology,* (New York: John Wiley and Sons) p. 8.