Nitric oxide selectively releases metals from the amino-terminal domain of metallothioneins: potential role at inflammatory sites[1]

KLAUS ZANGGER,*,[2] GÜLIN ÖZ,† ERNST HASLINGER,* OLAF KUNERT,* AND IAN M. ARMITAGE†,[2]

*Institute of Pharmaceutical Chemistry, University of Graz, A-8010 Graz, Austria; and †Department of Biochemistry, Molecular Biology and Biophysics, University of Minnesota, Minneapolis, Minnesota 55455, USA

SPECIFIC AIMS

We examined the structural consequences of the release of metal ions from the small, cysteine-rich, metal binding protein metallothionein (MT) brought about by the interaction with nitric oxide (NO). Homonuclear (^{1}H) as well as heteronuclear (^{113}Cd) NMR spectroscopy was used to characterize the structural changes associated with this interaction of potential physiological significance.

PRINCIPAL FINDINGS

1. Nitric oxide selectively releases metals from the amino-terminal domain of metallothioneins

To monitor the structural changes upon the interaction of nitric oxide with metallothionein, a 1 mM mouse [Cd_7]-metallothionein-1 sample was titrated with an NO donor (DEA/NO) and monitored by NMR spectroscopy. The amount of DEA/NO added at each increment corresponded to 0.5 mM of NO after complete release from its donor. After the first addition, it became obvious that the presence of NO leads to the selective reduction of ^{1}H-NMR signal intensities from the amino-terminal β domain of mouse MT1, comprising residues 1–30, which binds three metals in a M_3S_9 cluster (**Fig. 1**). In contrast, the resonances belonging to the carboxyl-terminal α domain (residues 31–61), which forms a four-metal M_4S_{11} cluster, are left basically unchanged. At the highest NO concentration used (10 mM nitric oxide), ^{1}H-NMR resonances from the β domain are completely missing whereas just a few signals from the α domain are reduced in intensity or missing (Fig. 1).

Another sensitive way of observing structural and dynamical changes in metallothioneins can be achieved through observing the ^{113}Cd-NMR resonances from $^{113}Cd^{2+}$ substituted MT samples. ^{113}Cd is often used as an NMR active replacement of zinc in metal binding proteins, and equivalent structures for zinc and cadmium metallothionein have been reported. ^{113}Cd-^{1}H HMQC spectra of a sample of 0.5 mM mouse [$^{113}Cd_7$]-metallothionein-1 with varying amounts of DEA/NO are shown in **Fig. 2**. The enhanced overall flexibility of the β domain leads to the observed lower intensity of cadmium signals from this domain. With the addition of nitric oxide, the difference in signal intensity between cadmium resonances from the β and α domains becomes even more pronounced; after adding 4.5 mM NO, only the signals from the α domain remain in the ^{113}Cd-^{1}H HMQC (Fig. 2). Additions of greater amounts of NO (up to 10 mM) had negligible effects on both the ^{1}H and ^{113}Cd signals from the α domain.

2. Tertiary structure of the amino-terminal domain is lost after nitric oxide induced metal release

It has been known for a couple of years that nitric oxide releases metals from metallothionein with concomitant formation of S-nitrosothiols. These –SNO groups subsequently form intramolecular disulfide bonds between the cysteines in metallothionein. However, no information existed about the detailed structural changes involved or about which cadmium ions were released.

The complete absence of the β domain ^{1}H- and ^{113}Cd-NMR signals can only be explained by the presence of an ensemble of interconverting structures, because simple unfolding of the protein and the formation of a random coil would lead to averaged, but still observable, resonances. Therefore, a random formation of Cys-Cys disulfide bonds is suggested. The formation of specific and stable intramolecular S-S bonds is prohibited by these experimental results, since

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[1] To read the full text of this article, go to http://www.fasebj.org/cgi/doi/10.1096/fj.00–0641fje; to cite this article, use *FASEB J.* (March 20, 2001) 10.1096/fj.00–0641fje

[2] Correspondence: K.Z.: Institute of Chemistry/Organic and Bioorganic Chemistry, University of Graz, Heinrichstrasse 28, A-8010 Graz, Austria. E-mail: klaus.zangger@kfunigraz.ac.at; I.M.A.: Department of Biochemistry, Molecular Biology and Biophysics, University of Minnesota, 6–155 Jackson Hall, 321 Church St. SE, Minneapolis, MN 55455, USA. E-mail: armitage@bscl.msi.umn.edu

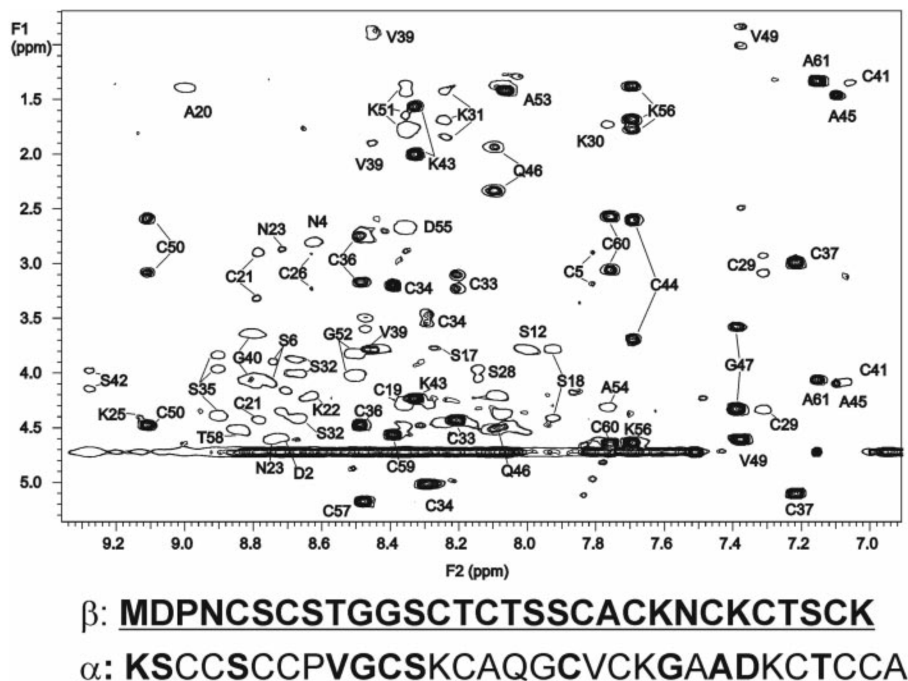

Figure 1. Overlay of the fingerprint region of TOCSY spectra of a 1 mM solution of mouse MT1 at pH 6.5, 25°C before the addition of nitric oxide donor (drawn with two closely spaced contours) and after the addition of DEA/NO giving 10 mM NO (drawn in multiple contours). Signals disappearing during the NO titration can easily be identified by open circles (two contours). Both spectra were acquired and processed under identical conditions. 32 transients were recorded for each of the 256 increments. After multiplication with a 60° phase-shifted squared cosine window function in both dimensions, the data matrix was zero filled to 2k·1k complex points and subsequently Fourier transformed. Also shown is the amino acid sequence of mouse MT1, where signals that disappeared during the titration are shown in boldface and underlined and ones with decreased intensity appear as boldface letters.

such a defined structure would again give rise to an observable NMR spectrum. Only the existence of a multitude of interconverting conformations could lead to the complete absence of all signals from the β domain.

3. The tertiary structure of the carboxyl-terminal domain is unaffected by nitric oxide

In both the ^{1}H- and ^{113}Cd-NMR spectra, the signals arising from the carboxyl-terminal α domain are basically unchanged in the presence of nitric oxide up to the highest concentrations tested. Even minor structural changes would very likely have led to changes in chemical shift in these sensitive experiments and the absence of such changes can only be explained by the structural integrity of the α domain with exposure to high concentrations of NO.

CONCLUSIONS AND SIGNIFICANCE

Although the function(s) of metallothioneins remain elusive some 40 years after the discovery of this class of proteins, various studies point to a participation of MTs in the detoxification of heavy metals and in the meta-

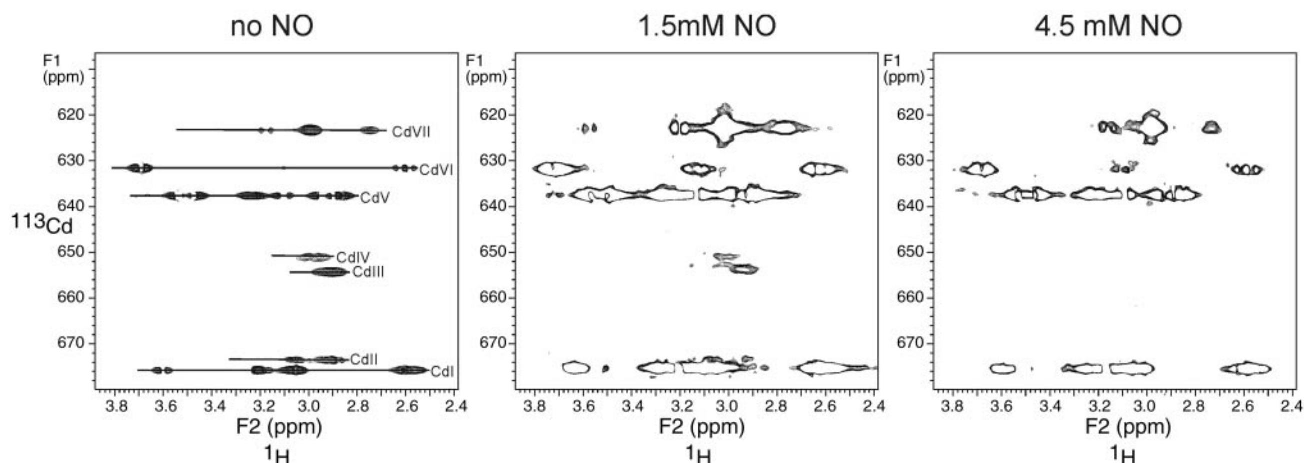

Figure 2. ^{113}Cd-^{1}H HMQC spectra of 0.5 mM ^{113}Cd-labeled mouse MT1, pH 6.5, 25°C with varying concentrations of nitric oxide as indicated. All three spectra were acquired and processed under identical conditions except that the vertical scale is increased 10-fold in the last two spectra to show the dramatic decrease in signal intensity in signals from the β domain. 320 transients were recorded for each of the 128 increments. After multiplication with a 60° phase-shifted squared cosine window function in both dimensions, the data matrix was zero filled to 2k·1k complex points before Fourier transformation. Cd I, V, VI, and VII belong to the carboxyl-terminal α domain and Cd II, III, and IV to the amino-terminal β domain.

bolic regulation of the essential metals, zinc, and copper. The tight metal binding in the α domain has been hypothesized to be important for the detoxification of heavy metals, whereas the function of MTs in the homeostasis of zinc and copper has been attributed to the β domain. The observation of nitric oxide induced release of bound metals from MT points to another possible physiological function involving metallothioneins. In our efforts to further characterize the structural consequences of the NO induced metal release in metallothioneins, we found that only the metals from the amino-terminal domain were released, whereas all the cadmium in the carboxyl-terminal domain remained bound to the protein. Metallothionein isolated from natural sources after induction by cadmium administration shows a non-uniform distribution of zinc and cadmium between the two domains. Zinc is more preferentially bound in the amino-terminal three-metal cluster, whereas cadmium can be found enriched in the carboxyl-terminal four-metal cluster. Since we have shown that cadmium bound in the carboxyl-terminal domain is not released by NO, our results contradict the hypothesis by Misra et al. that described the displacement of cadmium from [Cd_7]-metallothionein by NO as evidence for enhanced cadmium toxicity, especially at inflammatory sites.

A protective role of metallothionein against the cytotoxic and DNA damaging effects of nitric oxide was described by Schwarz et al. It is interesting that at inflammatory sites, the expression of inducible NO synthase (iNOS) is induced by some of the same stimuli which induce MT expression (e.g. interleukin 1, type α tumor necrosis factor, and lipopolysaccharide), and it has been reported that overexpression of MT reduces the sensitivity of eukaryotic cells to oxidant injury and the cytotoxic effects of NO.

Even more interesting is accumulating evidence for the anti-inflammatory role of zinc in skin disorders, as shown in the treatment of acne, alopecia, and zinc deficiency. An explanation for this physiological function might be the inhibition of iNOS activity through zinc and/or the recently reported suppression of iNOS expression, and therefore NO production by the presence of small amounts of zinc.

Integrating the above observations leads to the following hypothesis for an anti-inflammatory role of metallothionein: MTs are induced at inflammatory sites where they scavenge nitric oxide, which is produced by iNOS, which is also induced at these sites via the formation of S-nitrosothiols, and that leads to the release of bound zinc that suppresses further expression of iNOS (**Fig. 3**). Fine-tuning of the amount of zinc released and therefore the interplay between iNOS and MT could possibly be achieved via the redox state dependence of the amount of metal bound to the thiol groups in metallothioneins.

Figure 3. Schematic depiction of the evolving potential function of metallothioneins at inflammatory sites. Nitric oxide produced by inducible NO-synthase (iNOS) is scavenged by metallothionein with a concomitant release of zinc from the β domain. The released zinc then suppresses further expression and the activity of iNOS (see text for details). α-TNF: α-tumor necrosis factor; IL-1: interleukin-1; LPS: lipopolysaccharide.

In conclusion, we have found by ^{1}H- and ^{113}Cd-NMR spectroscopy that the exposure of metallothioneins to nitric oxide leads to a selective release of all three metals from the amino-terminal β domain while leaving the four metals in the α domain untouched. This finding of the selective, NO-induced release of some specific metals from a protein containing multiple bound metals has possible regulatory implications for NO via the distribution of metals from the β domain of MT. The postulated function of MTs in heavy metal detoxification that has been attributed to the α domain remains unmodified by nitric oxide. Using this information and the high preference for zinc in the β domain, we suggest a mechanism for metallothionein as an anti-inflammatory agent through both the scavenging of nitric oxide by the formation of S-nitrosothiols and the suppression of iNOS activity and expression via the metal released from the β domain. FJ