Nitric oxide induces Zn^{2+} release from metallothionein by destroying zinc–sulphur clusters without concomitant formation of S-nitrosothiol

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The reaction of nitric oxide (NO) with metallothionein (MT) has been investigated at neutral pH *under strictly anaerobic conditions*. It is observed that NO mediates zinc release from MT by destroying zinc–sulphur clusters, but that it does not by itself S-nitrosylate MT in contrast to common belief. Zinc release and loss of thiolate groups under anaerobic conditions is found to be much slower than under aerobic conditions. The observed percentage loss of Zn^{2+} and thiolate groups after 3 h of NO treatment are 62 and 39 %, respectively. The reaction of NO with cysteine is reinvestigated and it is found that cysteine is quan-

titatively converted to cystine after 5 min of NO treatment at pH 7.3. At lower pH, a much lower rate of conversion is observed confirming the base-catalysed nature of the reaction of NO with thiols. On the basis of these results, a reaction mechanism involving electrophilic attack of NO on thiolate groups and subsequent formation of a nitrogen-centred radical, $\text{MTSN}^{\bullet}\text{OH}$, as intermediate is proposed for the reaction of NO with MT that leads to zinc release.

Key words: Ellman's reaction, S-nitrosylation.

INTRODUCTION

There has been much increased interest in nitric oxide (NO) in recent years due to its diverse actions in biological systems and its natural presence in the body as a result of its formation by a variety of cell types such as endothelial cells, neutrophils, neurons and hepatocytes. Its possible involvement in vascular smooth muscle relaxation [1–4], platelet deaggregation [5–7], neuronal communication [8,9], photoreceptor signalling [10,11] and its antimicrobial activity and cytotoxicity for tumour cells [12] make NO a molecule of remarkable importance. The reaction of NO with proteins is undoubtedly of considerable interest from a biological perspective. Metallothioneins (MT) are ubiquitous low-molar-mass, sulphur-rich proteins that have a high affinity for essential, as well as toxic, trace metals. Different isoforms of MT are now known. MT1 and MT2 are generally found in the liver and kidney [13]. A third isoform, MT0, is also known to exist in human fetal liver (HFL) [14]. Other isoforms such as MT3 and MT4 are reported to be expressed in the brain [15,16] and in mouse tissues containing stratified squamous epithelial cells [17], respectively. The elucidation of the possible role of MT in the cellular response to NO is of particular importance, as MT has been shown to be involved in protecting cells from a variety of toxic substances including metal ions [18–20]. Also, MT has been postulated to play a role in scavenging hydroxyl and superoxide radicals and the appropriate reaction processes have been studied [21,22]. It is interesting to note that most of such radical reactions are associated with the destruction of metal–thiolate bonds. A recent study described the release of $\text{Zn}^{2+}/\text{Cd}^{2+}$ from MT by action of NO with concomitant formation of S-nitrosothiol [23].

There is a general misconception that NO can directly S-nitrosylate the thiol groups in proteins [23–25]. As a matter of fact, most of the bioregulatory actions of NO have been attributed to the formation of S-nitrosothiol by reaction with the thiol group of proteins and hence S-nitrosothiols have gained very

considerable attention in recent years [26]. However, the present study and report by Kharitonov et al. [27] contradict the concept of *direct* S-nitrosylation by NO. The aim of this communication is to show that NO does cause the destruction of metal–thiolate bonds but does not by itself S-nitrosylate human MT at neutral pH in contrast to an earlier report [23]. Furthermore, it is demonstrated that both Zn^{2+} release and loss of thiol groups are much slower than indicated in the previous report [23] and two distinct reaction mechanisms are proposed for the reaction of NO with MT under aerobic and anaerobic conditions, respectively, involving a decisive role for oxygen in the S-nitrosylation of MT. To our knowledge, this is the first report on the reaction of NO under strictly anaerobic conditions with a protein thiol group involved in Zn^{2+} complexation. A re-investigation of the reaction of NO with cysteine is also carried out and it is shown that NO reacts with cysteine to form cystine without the formation of S-nitrosocysteine at a much higher rate than reported earlier [28]. The base-catalysed nature of the reaction of NO with thiols is confirmed, allowing us to propose electrophilic addition of NO to thiolate groups as the reaction mode for the direct attack of NO on MT.

MATERIALS AND METHODS

Materials

All the chemicals used were of highest purity and were obtained commercially, except for MT which was isolated from HFL.

Isolation and purification of MT

MT was extracted from HFL and purified as described by Clough et al. [29]. MT was initially isolated from HFL homogenates by gel chromatography on a Sephadex G-75 column and was identified by its Zn^{2+} content and absorbance at 218 nm using atomic absorption and UV-VIS spectrometry, respectively. It was then separated from fatty acid-binding proteins using

Abbreviations used: MT, metallothionein; HFL, human fetal liver; DEAE, diethylaminoethyl; TNB^{2-} , 2-nitro-5-thiobenzoate anion.

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negative-affinity chromatography. MT1 was separated from the other isoforms of MT such as MT0 and MT2 on a DEAE-Sephacel anion-exchange column. The MT1 was then concentrated using ultrafiltration and the purity was checked by SDS/PAGE analysis. The concentration of Zn^{2+} in this solution was again determined and was found to be 1.17×10^{-4} M. The concentration of MT1 was then calculated from the Zn^{2+} content by taking a standard value of 7 Zn^{2+} per each MT1 (assuming that all the 20 cysteines are bonded to Zn^{2+}). For subsequent experiments, this sample was diluted in phosphate buffer to adequate concentrations.

Reaction set-up

It is essential for the investigation to perform the experiments with pure NO, uncontaminated by traces of higher oxides of nitrogen. This requires the experiments to be conducted under strictly anaerobic conditions. A special gas line and reaction chamber set-up were developed for this purpose. A tonometer consisting of two compartments (a reaction chamber and a pre-reaction chamber) was connected to a spectrophotometric cell containing the reactant solution (buffered aqueous solution of cysteine or MT). On the basis of previous reports [26,27,30] and of our own experience that higher oxides of nitrogen such as NO_2 and N_2O_3 readily react with cysteine over the entire pH range, the pre-reaction chamber was filled with 15 ml L-cysteine hydrochloride (0.1 M, pH 3). NO, on the other hand, has a very low reactivity towards cysteine at low pH [28]. The entire set-up including the tonometer and spectrophotometric cell was first evacuated using a vacuum pump and Ar was slowly purged into it. It was again evacuated followed by Ar purging and this procedure was repeated 3–4 times. Subsequently, high purity NO gas was slowly introduced into the tonometer keeping the reaction chamber closed. The tonometer was then disconnected from the gas line and NO was mixed with cysteine in the pre-reaction chamber by gentle shaking. The appearance of a light-brownish yellow colour indicated the formation of small amounts of S-nitrosocysteine, resulting from the reaction of higher oxides of nitrogen with cysteine. The NO thus available in the pre-reaction chamber may be expected to be quite pure and free from higher oxides and the successive experiments confirmed this. The purified NO was subsequently allowed to pass into the reaction chamber connected to the spectrophotometric cell and mixed with the reactant solution. The absorbance changes characteristic for the occurrence of S-nitrosylation were monitored immediately after the mixing of NO with the reactant solution.

Determination of Zn^{2+} release from MT1

MT1 (4–6 μM) at pH 7.0 in phosphate buffer was treated with excess NO during different time intervals and the remaining NO was removed after the reaction by evacuation from the reaction chamber. After this treatment, the solution was checked for the absence of S-nitrosylation in an oxygen atmosphere using UV-VIS spectrophotometry, thus confirming the complete removal of residual NO. Subsequently, approx. 0.5 ml of the solution was placed in a Microcon microconcentrator (Amicon Inc., U.S.A.) with a membrane cut-off of 3000 Da and filtered repeatedly after adding the buffer by centrifugation at 11000 rev./min. A blank solution of MT1 without NO treatment was also filtered under the same conditions as a reference. The remaining MTs (NO treated and untreated) after filtration and the filtrates were analysed for their Zn^{2+} content by atomic absorption spectrometry. The percentage of Zn^{2+} loss was calculated from the so-measured Zn^{2+} contents. Experiments were performed at least twice and less than 10% deviation between results was observed.

Table 1 Percentage decrease of total thiol(ate) content upon reaction of nitric oxide with L-cysteine and MT

Deoxygenated L-cysteine (various pH values) and MT1 (pH 7.0) were treated with excess NO during different time intervals and the remaining NO was removed after the reaction by evacuation from the reaction chamber. The thiol(ate) contents before (C_0) and after (C_t) NO treatment were determined by Ellman's method.

Reactant	pH	Time/min	C_0/mM	C_t/mM	% Decrease
L-Cysteine	3.4	60	0.467	0.481	0
	5.8	18	0.488	0.130	73
	5.8	20	0.483	0.128	74
	6.7	15	0.467	0	100
	7.3	5	0.556	0	100
	7.3	11	0.540	0	100
Metallothionein	7.0	120	0.115	0.080	30
	7.0	180	0.085	0.052	39

Determination of loss of thiol(ate) groups

MT1 (4–6 μM , pH 7.0) in phosphate buffer and L-cysteine (0.5 mM, various pH values) were treated with excess NO during different time intervals and the remaining NO was removed after the reaction by evacuation from the reaction chamber. The thiol(ate) contents before and after NO treatment were determined spectrophotometrically by reaction with Ellman's reagent [5,5-thiobis-(2-nitrobenzoic acid)] in the presence of EDTA (1 mM) in phosphate buffer (0.1 M, pH 7.3) as described [31]. In the case of MT1, guanidine hydrochloride (6.4 M) was also present. Cysteine was used as standard. Repetitive experiments with L-cysteine showed that the experimentally determined concentration deviated in general less than 10% from the actual (nominal) concentration (see for instance Table 1). In contrast, the measured thiol content of the original solution of MT1 was always approx. 30–40% less than the expected value as calculated from the concentration and thiol content of MT1. This discrepancy is dealt with in detail below (see Results section).

Amino acid analysis

Amino acid analysis of a sample containing L-cysteine (0.5 mM) at pH 7.3 in phosphate buffer was carried out after NO treatment for 5 min on a 4150 Alpha amino acid analyser (LKB). The amount of cystine formed after the reaction was determined by comparison with a chromatogram of a standard containing 2.5 nmol cystine per 50 μl of solution.

RESULTS

Spectroscopic search for S-nitrosothiol formation upon reaction of L-cysteine and MT with NO

In an initial set of experiments, we carefully introduced commercially available high-purity NO gas, after repeated evacuation of the gas lines and degassing of the sample followed by purging with Ar, into a tonometer connected to a spectrophotometric cell containing 1 mM L-cysteine at pH 9.3 and monitored the absorbance changes in the region 195–600 nm. A time-dependent absorption in the 300–400 nm region with a distinct peak around 335 nm was observed in accordance with previous investigations [23,26], clearly indicating the formation of S-nitrosocysteine. However, in the course of repetitive experiments the time dependence appeared to fluctuate randomly and in a way unconnected to experimental parameters, arousing the suspicion of the presence of trace amount of higher oxides of nitrogen, despite the

Figure 1 Spectroscopic search for S-nitrosothiol formation upon reaction of MT with NO

Electronic absorption spectra recorded in a solution containing deoxygenated MT1 (6 μM) at pH 7.0 in phosphate buffer (1) before, (2) immediately after and (3) 90 min after NO treatment; (4–6) spectra obtained after subsequent introduction of different concentrations of oxygen in the presence of NO (measured immediately after the introduction).

care exercised in the deoxygenation process. A previous report [27] also supports this observation. Therefore, the entire experimental set-up was redesigned as mentioned in the Materials and methods section.

NO, after purification by gentle shaking with the cysteine-containing solution in the pre-reaction chamber, was introduced in excess to deoxygenated solutions of L-cysteine (pH 9.3) and MT1 (pH 7.0), respectively, and the absorbance changes between 200 and 600 nm were monitored immediately. The distinct peak in the region 330–350 nm indicative for the formation of S-nitrosothiol was not observed in either case, even up to 90 min after the introduction of NO (Figure 1). The small increase in the absorption spectrum recorded immediately after NO treatment [Figure 1 (2)] compared to the initial spectrum [Figure 1 (1)] may represent a very small amount of S-nitrosothiol formation, resulting from the reaction of NO with trace amounts of oxygen still present in the reaction chamber after the evacuation procedure. However, when oxygen was subsequently introduced into the reaction chamber a fast build-up of absorption around 350 nm was observed for MT1 as shown in Figure 1. Excess oxygen was avoided in such experiments to prevent nitrite formation in solution. This observation contradicts the earlier work on the NO–MT reaction, where a time-dependent formation of S-nitroso MT was reported [23]. Similar results were obtained when using a mixture of MTs (MT0, MT1 and MT2) and fatty acid-binding proteins. The S-nitrosylated MT was found to have decayed fully after about 20 h.

Decrease of thiol(ate) content and formation of cystine upon reaction of L-cysteine with NO; effect of pH

A solution of L-cysteine (0.5 mM) at pH 7.3 in phosphate buffer was treated with excess NO for 5 min and the thiol(ate) content before and after NO treatment was determined by Ellman's reaction. It was found that cysteine had fully reacted with NO within this short time period which was the minimum time required to introduce NO and then evacuate it fully from cysteine after the reaction using our set-up (the reaction could

Figure 2 Comparison of the electronic absorption spectrum of the product of the reaction of NO with L-cysteine with the spectrum of cystine

A solution of deoxygenated L-cysteine (0.5 mM) at pH 7.3 in phosphate buffer was treated with excess NO for 5 min and the resulting electronic absorption was compared with that of cystine. Electronic absorption spectra (1) before, (2) after NO treatment and (3) spectrum of cystine (0.5 mM) at pH 7.5 in phosphate buffer.

Figure 3 Concentration of L-cysteine at pH 5.8 as a function of time after treatment with excess NO

A solution of deoxygenated L-cysteine (0.5 mM) at pH 5.8 was treated with excess NO during different time intervals and the thiol(ate) content was determined by Ellman's reaction. The exponential reaction between cysteine concentration and reaction time clearly points to first-order kinetics with respect to cysteine.

well be complete, however, in a much shorter time period). This is considerably faster than reported earlier [28], where complete reaction was observed only after 15 min at pH 9 and 2.5 h between pH 5 and 7. Comparison of the UV-VIS spectrum of the solution after NO treatment with that of cystine gave clear indication of the formation of this product (Figure 2) and subsequent amino acid analysis of the solution confirmed its quantitative formation. A cystine concentration of 0.248 mM was obtained from 0.5 mM cysteine, corresponding to essentially full conversion. A number of experiments were subsequently carried out at different pH values to investigate the pH dependence of the NO–cysteine reaction. The relevant data at various pH values are given in Table 1. From these data it is clear

Figure 4 Percentage loss of Zn²⁺ from MT1 as a function of time upon NO treatment (A) and zinc content in MT1 as a function of time after treatment with excess NO (B)

(A) Deoxygenated MT1 (4–6 μ M) at pH 7.0 in phosphate buffer was treated with excess NO during different time intervals. After filtration, the remaining MT1 (NO treated and untreated) and the filtrates were analysed for their Zn²⁺ content by atomic absorption spectrometry. The percentage of Zn²⁺ loss was calculated from the so-measured Zn²⁺ contents. (B) The Zn²⁺ content for an MT1 sample with a nominal Zn²⁺ concentration of 30 μ M is calculated from the measured percentage loss on NO treatment and shown on a logarithmic scale as a function of time. The strictly exponential reaction between Zn²⁺ content and reaction time clearly indicates first-order kinetics with respect to the (residual) Zn²⁺ content in MT1.

that when the reaction was carried out at pH 3.4, no change in the cysteine concentration was observed even after 1 h, whereas at pH 6.7 and 7.3 cysteine had fully reacted with NO after a short time interval (≤ 5 min at pH 7.3). The experiments thus clearly confirm the pH dependence of the NO–cysteine reaction. At pH 5.8 partial conversions are obtained in convenient time intervals, allowing the study of the kinetics of the reaction. The decrease of the concentration of L-cysteine [thiol(ate) content of the solution] after treatment with excess NO is shown on a logarithmic scale as a function of time in Figure 3. Obviously, an exponential relation is observed between cysteine concentration and reaction time, clearly pointing to first-order kinetics with respect to cysteine.

Zn²⁺ release and decrease of thiolate content upon reaction of MT1 with NO; interference of Zn²⁺ with Ellman's method

MT1 (4–6 μ M) at pH 7.0 in phosphate buffer was treated with excess NO during different time intervals and the remaining NO was removed after the reaction by evacuation from the reaction chamber. Figure 4A shows the time dependence of Zn²⁺ loss from MT1 on such treatment. As can be seen, only about 62% of Zn²⁺ is lost after 3 h. This is a much slower rate compared to earlier observations, in which formation of S-nitrosothiol was also reported [23]. The Zn²⁺ content for a sample with a nominal Zn²⁺ concentration of 30 μ M is shown on a logarithmic scale as a function of time in Figure 4B. Clearly, a strictly exponential relation is observed between Zn²⁺ content and time, indicating

Figure 5 Dependence of the absorbance of TNB²⁻ at 412 nm on the ratio of Zn²⁺/cysteine concentration after Ellman's reaction of L-cysteine

Ellman's reaction was carried out in a solution containing cysteine (3.5×10^{-5} M) and monitored by measuring the absorbance at 412 nm. Zn²⁺ was subsequently added to the solution at different concentrations and the absorbance noted in each case.

first-order kinetics with respect to the (residual) Zn²⁺ content in MT. The thiolate contents before and after NO treatment were determined spectrophotometrically by reaction with Ellman's reagent in the presence of guanidine hydrochloride (6.4 M) and EDTA (1 mM) in phosphate buffer (0.1 M, pH 7.3). The loss of thiolate groups was 30 and 39% for a reaction time of 2 and 3 h, respectively, as indicated in Table 1.

The measured percentage loss of thiolate groups by Ellman's method is lower than the observed Zn²⁺ release, whereas intuitively at low and intermediate conversion the opposite (or about equal conversion) would be expected. Also, the measured thiolate content of the original solution of MT1 using Ellman's method was always approx. 30–40% less than the expected value as calculated from the concentration and thiolate content of MT1. In contrast, quite accurate (i.e. within less than 10%) values were obtained with other proteins such as β -lactoglobulin. It is thus clear that there are problems with quantitative determinations with Ellman's method in the case of MT, problems which do not appear to be recognized in the literature [23]. Obviously, the problem can be associated with the presence of Zn²⁺ in the protein and both the effect on Ellman's reaction of Zn²⁺ complexation with the thiolate groups in MT and the effect of Zn²⁺ on the electronic absorption of the 2-nitro-5-thiobenzoate anion (TNB²⁻) must be taken into consideration. In order to shed some light on this, the latter effect, which is easily accessible experimentally, was investigated in some detail. To this end, Ellman's reaction was carried out in a solution containing cysteine (3.5×10^{-5} M) and monitored by measuring the absorbance at 412 nm. Zn²⁺ was subsequently added to the solution at different concentrations and the absorbance was noted in each case. The absorbance of TNB²⁻ and 412 nm plotted against the ratio of the concentrations of Zn²⁺ and cysteine is shown in Figure 5. This plot clearly shows that there is an effect of the presence of Zn²⁺ on the electronic absorption of TNB²⁻. In order to assess this effect more quantitatively, the absorption coefficient of TNB²⁻ was determined from the plot of the absorbance of TNB²⁻ vs. cysteine concentration at a very high Zn²⁺/cysteine ratio and a value of $12910 \text{ M}^{-1} \cdot \text{cm}^{-1}$ was calculated. This value is lower than the reported value (in the absence of guanidinium chloride) which is $14150 \text{ M}^{-1} \cdot \text{cm}^{-1}$ [31]. When this difference in absorption coefficient is taken into account in the determination of the thiolate content in MT, which undoubtedly is an overcorrection

because of the presence of EDTA in this determination, the discrepancy between the measured and expected value is lowered but not fully removed. Also, the effect of Zn^{2+} on the absorption coefficient cannot affect the measured *percentage* loss of thiolate in MT. The remaining discrepancy is most likely due to the tight co-ordination between the thiolate groups and Zn^{2+} . In conclusion, the determination of the decrease of the thiolate content upon treatment of MT1 with NO gives qualitative indication of the reaction of NO with MT1, but from a quantitative point of view the determination of Zn^{2+} release must be considered the most accurate.

DISCUSSION

The results obtained clearly show the absence of S-nitrosothiol formation upon reaction of L-cysteine and metallothionein with NO under strictly anaerobic conditions, in stark contrast to the general belief that NO can directly S-nitrosylate the thiol groups in proteins [23–25]. The absence of a S-nitrosothiol band between 330 and 350 nm after NO treatment of cysteine and MT up to about 90 min and the sudden build-up of this band on subsequent introduction of oxygen indicate that oxygen plays a decisive role in the outcome of the reaction. The reaction set-up used in an earlier work [23] on MT must have contained traces of oxygen that converted NO to NO_2 and N_2O_3 as is evident from the presence of the S-nitrosothiol band in their experiments. Both NO_2 and N_2O_3 are known to nitrosylate thiol groups [27,28,30]. A number of data in the literature support our conclusion that S-nitrosothiol formation depends on the presence of oxygen and/or higher oxides ($\text{NO}_2/\text{N}_2\text{O}_3$) and does not take place by direct NO attack. Foremost, a recent report by Kharitonov et al. [27] also contradicts the concept of direct S-nitrosylation of cysteine-containing proteins by NO. In addition, evidence from the field of organic chemistry further supports the view of the absence of direct nitrosylation. With both thiols and secondary amines no nitrosylation occurs by NO when oxygen is rigorously excluded from the system, but S-nitrosothiol/N-nitrosamine formation takes place rapidly when air is admitted [28,32]. On the basis of such observations it has been stated that when pure, and, in particular, when totally free from oxygen, nitric oxide is *not* an electrophilic nitrosylating species [33]. The present observations clearly support this view.

The strong dependence on pH (and base-catalysed nature) of the reactivity of NO with regard to L-cysteine indicates that NO participates in an ionic reaction with cysteine, namely by electrophilic addition to thiolate groups with corresponding formation of the radical anion, $\text{RSN}^{\bullet}\text{O}^-$, which can easily be protonated by water to form $\text{RSN}^{\bullet}\text{OH}$, leading to the formation of the disulphide by mutual combination as reported earlier [28]. In cysteine, the ratio of SH vs. S^- groups is strongly pH dependent, resulting in a strong pH dependence of the electrophilic attack by NO. A radical intermediate, $\text{RSN}^{\bullet}\text{OH}$, in the reaction of NO with cysteine has also been proposed in a recent study in which the mechanism of S-nitrosothiol formation was investigated under strictly anaerobic conditions in the presence of electron acceptors [34]. The concentration of cysteine decreases exponentially with time upon treatment with excess NO, indicating that the NO–cysteine reaction is characterized by first-order kinetics with respect to cysteine.

The release of Zn^{2+} from MT1 and the concomitant loss of thiolate groups observed clearly indicate that NO reacts with MT1, but at a much lower rate than with L-cysteine. The lower rate can be attributed to steric hindrance and to Zn^{2+} complexation with the thiolate groups in MT, factors which undoubtedly play an important role in resisting NO attack. A further

Scheme 1. Reaction pathway for attack of NO on MT under aerobic and anaerobic conditions.

observation is that Zn^{2+} release and loss of thiolate groups on reaction of NO with MT1 under strictly anaerobic conditions (i.e. the present work) takes place at a much lower rate, compared to earlier observations in which also formation of S-nitrosothiol was reported (i.e. non-strict anaerobic conditions) [23]. Experimentally, under strictly anaerobic conditions the Zn^{2+} content in MT decreases with time according to a relation that is strictly exponential, indicating first-order kinetics with respect to the (residual) Zn^{2+} content in MT. These are surprisingly simple kinetics considering the complexity of the process. Indeed, the rate of attack by NO on thiolates in a particular MT domain is intuitively expected to increase with the extent of reaction (at the level of this domain), by a decrease in steric hindrance as a result of gradual unfolding of the protein as Zn^{2+} –thiolate bonds are broken. A further increase with respect to Zn^{2+} release may be expected to result from the fact that such Zn^{2+} release probably only occurs efficiently when several Zn^{2+} –thiolate bonds are broken. The physical meaning of the first-order kinetics cannot be pinpointed with absolute certainty, but a highly plausible explanation is that the first attack of NO on a thiolate group in a particular MT domain is the rate-determining step and that further attacks on thiolate groups and Zn^{2+} release in that domain then occur much more rapidly. In other words, once ‘wounded’ the protein will quickly be destroyed completely. Such an outcome is completely in line with the observed first-order kinetics.

From an overall view of the experimental evidence, it can be concluded that N_2O_3 and/or NO_2 can mediate Zn^{2+} release and loss of thiolate groups from MT via formation of S-nitroso MT, whereas NO does so without S-nitrosylation. The rate via the S-nitroso MT pathway clearly is much higher than via the pathway without S-nitrosylation. Undoubtedly, the reaction mechanism is quite different in both cases. An outline of the reaction pathways is presented in Scheme 1. In analogy with the reaction of NO with cysteine, we invoke for the reaction with MT an electrophilic addition of NO to MTS^- with corresponding formation of the radical anion, $\text{MTSN}^{\bullet}\text{O}^-$, which can easily be protonated by water to form $\text{MTSN}^{\bullet}\text{OH}$. The mutual coupling of these radicals can give rise to a disulphide, along with N_2 and N_2O resulting from the hyponitrous acid formed. Since MT is a cysteine-rich protein, the coupling of two adjacent $-\text{SN}^{\bullet}\text{OH}$ radicals is most likely. This leads to a high probability for the formation of an intramolecular rather than an intermolecular disulphide bond. It is to be remarked that in MT all thiol groups are bound to Zn^{2+} as thiolates, making the ratio of SH vs. S^-

groups at moderate pH values essentially pH independent, which certainly will reflect itself in the rate of the electrophilic substitution. The electrophilic nature of the reaction is, however, clearly suggested by the pH dependence (and base-catalysed nature) of the reaction of NO with cysteine and by the obvious analogy between the cysteine and MT reactions, in that the rate of NO attack in both cases is much higher under aerobic than under anaerobic conditions. At present, there is no direct evidence for the formation of MTSN[•]OH and hence the mechanism proposed is only hypothetical, but it can be considered highly plausible. A detailed investigation of the exact nature of the intermediate radical is currently in progress in our laboratory.

As to the alternative pathway via S-nitrosylation, different reaction mechanisms have been proposed for the decay of S-nitrosothiols and formation of disulphides [28,35,36]. Disulphides can be formed via a simple radical pathway by combination of two sulphur-centred radicals (RS[•]), resulting from the initial decay of S-nitrosothiols with release of NO, and by reaction of RS[•] with RSNO [28]. Recently, it has been shown that the decomposition of S-nitrosothiols in aqueous solution is brought about by Cu⁺, generated by reduction of Cu²⁺ by thiolate ion [35]. Thiols (as thiolate) have a dual effect on the decomposition of S-nitrosothiols, (i) as a reducing agent generating Cu⁺ and (ii) as a complexing agent for Cu²⁺ which is then less available for reduction, the balance of these effects depending on the structure and concentration of the thiol [36]. It has clearly been shown [35] that in the absence of light no reaction occurs, except for a very slow thermal decomposition, when metal ions are removed (e.g. by EDTA) or when Cu⁺ is removed specifically with neocuproine. In the presence of light, photochemical decomposition also takes place, in all likelihood via the simple radical pathway cited above. The decay of S-nitroso MT in the present study quite plausibly takes place via both pathways (i.e. simple radical and Cu⁺-mediated), as the sample was not shielded from light and Cu²⁺ was likely to be present as a trace impurity. According to an earlier report [23], S-nitroso MT upon decomposition mostly forms intramolecular disulphide bonds.

In conclusion, it is demonstrated for the first time that NO does cause Zn²⁺ release and loss of thiolate groups from sulphur-containing proteins involved in Zn²⁺ complexation without formation of S-nitrosothiol. In all likelihood, the reaction takes place by electrophilic addition of NO to the thiolate anion groups. Under strictly anaerobic conditions, this is the only mode of attack. In the presence of oxygen, NO is transformed into N₂O₃ and/or NO₂ and the attack of these species results in S-nitrosylation and in Zn²⁺ release and loss of thiolate groups at a much higher rate than through attack by NO itself.

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