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Letter

Steric effect and effect of metal coordination on the reactivity of nitric oxide with cysteine-containing proteins under anaerobic conditions

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Abstract

A comparison is made between the reactivity of nitric oxide (NO) with cysteine, bovine serum albumin (BSA) and metallothionein-1 (MT1) at pH 7 under strictly anaerobic conditions. The rate of reaction of NO with these amino acid/proteins was found to be of the order: cysteine > BSA ≫ MT1, in clear disparity with the size of the reactants. The difference in the reaction rates is attributed to steric effects due to the high molecular size in the case of BSA and to effects of metal coordination proper as well as to steric effects associated with the closed dual shell-like structure resulting from the tight coordination of the thiolate groups with Zn^{2+} in MT1. The mechanisms of the reaction of NO with cysteine, BSA and MT and its possible implication for the rate of the respective reactions are discussed.

Keywords: Steric effect; Metal coordination; Nitric oxide; Cysteine; Bovine serum albumin; Metallothionein

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1. Introduction

The chemical behaviour of nitric oxide (NO) in biological systems has been under intense investigation in recent years due to its possible role in various physiological processes [1]. NO is produced in the body by a variety of cell types such as endothelial cells, neutrophils, neurons and hepatocytes. The increased interest in this area came from the experimental evidence that NO is involved in smooth muscle relaxation [2,3], platelet deaggregation [4–6], neuronal communication [7], photoreceptor signalling [8,9] and cytotoxic action with respect to tumour cells [10]. To a great extent, the bioregulatory actions of NO have been attributed to the formation of *S*-nitrosothiols, which result from its reaction with protein thiols [11]. Therefore, the mechanism by which *S*-nitrosothiols are formed from the reaction of NO with protein thiols is a subject of remarkable importance.

NO is one of the simplest odd electron species and its physiological action always takes place in aqueous solution, where its solubility is 1.8×10^{-3} mol dm^{-3} (at 25°C and 1 atm pressure) within the pH range 2–13. It has frequently been observed that NO does not cause nitrosation under anaerobic conditions [12–16] and it is, therefore, believed that it is the higher oxides such as N_2O_3 and NO_2 that are responsible for the process [12,15,17]. However, in a report by Gow et al. [18], a reaction mechanism for the formation of *S*-nitrosothiol in vivo has been proposed based on the formation of a radical intermediate, RSNOH, from the initial attack of NO under anaerobic conditions, which on loosing an electron in the presence of an electron acceptor such as oxygen leads to the formation of RSNO. This reaction mechanism emphasises the need to study the kinetics of the reaction of NO with protein thiols under strict anaerobic conditions. In a recent paper we have reported on research related to the reaction of NO with cysteine and MT1 [one of the isoforms of metallothionein (MT)] under strict anaerobic conditions [16]. In that investigation, it was observed that the reaction of NO with MT1 is very much slower than with cysteine. This may be attributed to steric effects and to metal coordination of the thiolate groups in MT1 with Zn^{2+}. In order to differentiate between these two possibilities, we now report on a comparative study of the reaction of NO with cysteine, bovine serum albumin (BSA) and MT1 at pH 7. BSA is a protein in which steric effects, undoubtedly, are quite extensive but in which there is no metal coordination with thiolate groups.

2. Materials and methods

Commercially available cysteine (98.5%) and BSA (96–99%) were used without further purification. MT was extracted from human fetal liver by a method described by Clough et al. [19]. MT1 was separated from other isoforms of MT, such as MT0 and MT2, by anion exchange chromatography. The purity of MT1 was checked by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) analysis after concentration using ultrafiltration. The concentration of MT1 was calculated from the total Zn^{2+} contents (measured by atomic absorption spectrometry) in the solution by assuming seven Zn^{2+} per each MT1.

Commercially available high purity NO ($\geq$ 99.5%) was used. It is essential to the present research that the NO utilised is free of traces of higher oxides of nitrogen and that experiments be performed under strict anaerobic conditions. Therefore, a special reaction set-up was developed using a modified tonometer that can be connected to a gas line and that consists of both a reaction chamber and a pre-reaction chamber containing 15 ml L-cysteine-HCl (0.1 M, pH 3) to scavenge NO_2 and N_2O_3 present in commercially available NO. These higher oxides have a very high reactivity towards cysteine over the entire pH range [15,17,20]. A detailed description of the set-up and operational procedures has been given elsewhere [16].

All the solutions were prepared in phosphate buffer at pH 7. The extent of the reaction with NO was determined by measuring the thiol(ate) content before and after the reaction using Ellman's method [21]. The Ellman's reaction with BSA was carried out immediately after the preparation of solutions and for samples untreated with

NO gave results that corresponded well with the expected free thiol(ate) content (> 90%).

3. Results

Deoxygenated L-cysteine (0.5 mM), BSA (0.4 mM) and MT1 (4–6 μM), prepared in phosphate buffer solution at pH 7, were treated with NO in excess. The measurement of thiol(ate) content before and 5 min after NO treatment showed a complete loss of thiol(ate) in the case of L-cysteine, which is presented as *percentage* loss in Fig. 1. It is to be noted that the time required for the NO-cysteine reaction could be much less than 5 min at pH 7, as it was impossible to analyse the reaction in a shorter time scale with our set-up. When a similar experiment was carried out with BSA at different time intervals, it was found that complete loss of thiol(ate) content was obtained after approximately 30 min of NO treatment (Fig. 1). However, when MT1 was treated with NO, the *percentage* loss of both thiolate and Zn^{2+} contents was much lower compared to the thiol(ate) loss in BSA and L-cysteine as is clearly evident from Fig. 1. Since we observed some discrepancy between the measured and expected thiolate contents using Ellman's reaction in the case of MT1, most plausibly due to tight coordination between Zn^{2+} and cysteine residues and the effect of Zn^{2+} on the electronic absorption of the 2-nitro-5-thiobenzoate anion (TNB^{2-}), the loss of Zn^{2+} was taken as the more accurate indication for the reaction of NO with MT1. Therefore, the extent of the reaction was assessed in terms of Zn^{2+} release from MT1. As can be seen from Fig. 1, the reaction is not complete even 3 h after NO treatment.

In order to investigate whether there is any dimer formation in the case of the BSA-NO reaction, SDS-PAGE analysis was carried out with and without the involvement of mercaptoethanol on a BSA solution treated with NO for 30 min. BSA dimer could not be detected in either case.

4. Discussion

The results obtained clearly point to strong differences between the rate of reaction of NO with L-cysteine, BSA and MT1. The reaction of NO with cysteine is quite fast and is complete in 5 min, which was the minimum time required to introduce NO and then evacuate it fully from cysteine after the reaction using our set-up. On the other hand, the reaction of NO with BSA

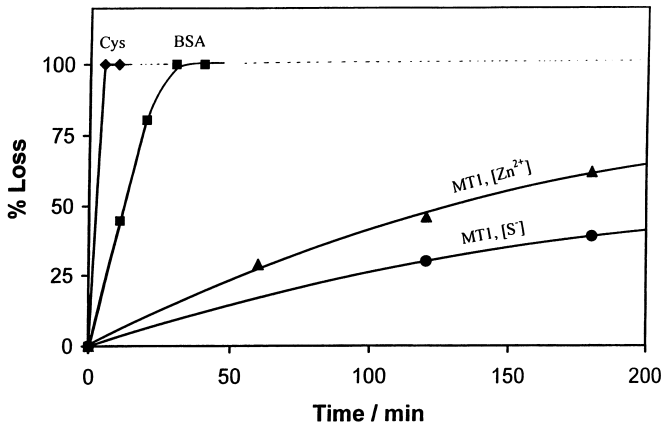

Fig. 1. Comparison of the percentage loss of thiol(ate) contents in cysteine, BSA and MT1 (and Zn^{2+} content in MT1) as a function of time upon NO treatment under anaerobic conditions. Deoxygenated cysteine (0.5 mM), BSA (0.4 mM) and MT1 (4–6 μM) at pH 7 in phosphate buffer were treated with excess NO during different time intervals. The percentage loss of thiol(ate) contents in cysteine, BSA and MT1 was determined by measuring the thiol(ate) contents before and after NO treatment using Ellman's reaction. (The percentage loss of zinc vs. time plot is taken from Aravindakumar et al. [16].)

could be monitored on a convenient time scale. It was found that the reaction was approximately complete after 30 min of NO treatment. BSA is a high molecular weight protein (M_r 68 000) and has only one free thiol group. It is, therefore, logical to attribute the longer time scale and lower reactivity as compared to cysteine to steric effects.

The reactivity of BSA towards NO is intermediate between that of cysteine and MT1, cysteine > BSA ≫ MT1, in clear disparity with the size of these reactants. MT1 is a comparatively small protein (M_r 6000) and in it all the 20 cysteine residues are coordinated to Zn^{2+}. It could be argued that both steric effects and Zn^{2+}-thiolate complexation affect the speed of the reaction of NO with MT1 and that the metal coordination is the predominant factor that affects the rate of the reaction. Zn^{2+} complexation will certainly affect the rate of the reaction because of the reduced electron density on the thiolate group and electrophilic nature of the NO attack (see below) and because of the lowering in energy associated with the complexation. The previously reported first-order kinetics with respect to residual Zn^{2+} content [16] throws some additional light on the subject, however, and leads to a more sophisticated picture. As indicated before [16], the most plausible explanation for the first-order kinetics 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 readily. *It may, therefore, be concluded that the low reactivity of NO towards MT1 vs. BSA is due to effects of metal coordination proper as well as to steric effects, caused by the closed dual shell-like structure of MT resulting from the tight coordination of the thiolate groups with* Zn^{2+}. The MT1 protein folds itself around the Zn^{2+} in two separate domains [22] and thereby protects the thiolate groups from attack by NO; once this tight structure is broken as a result of the first attack of NO on a thiolate group in a particular MT domain (a slow process), the other thiolate groups in that domain react much more readily. The structure of MT and suggested (not proven) points of initial attack leading to much increased exposure to NO are depicted in Fig. 2.

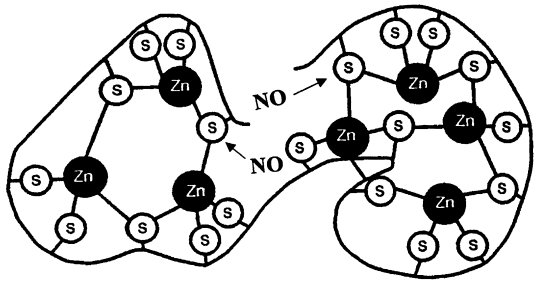

Fig. 2. Structure of MT and suggested (not proven) points of initial attack leading to much increased exposure to NO; (structure of MT adapted from Kägi [23]).

A final remark concerns the mechanism of the reaction of NO with cysteine, BSA and MT1, and the possible implication for the rate of the respective reactions. Based on an earlier report [20] and on our own recent finding [16] that there is a strong pH dependence of the NO-cysteine reaction, an ionic reaction by electrophilic addition of NO to cysteine seems to be the most plausible reaction mechanism for this process. The intermediate resulting from the electrophilic attack of NO on cysteine in the presence of water is understood to be an N-centered radical, −SNOH, which by mutual combination leads to disulfide formation [20]. In agreement with this, the analysis of the end product of the NO-cysteine reaction indicated a quantitative formation of cystine at pH 7.3 [16]. In the case of MT1, a similar reaction mechanism via formation of MT-SNOH leading to intramolecular disulfide bonds was proposed [16]. In the case of BSA, formation of an intermediate radical, BSA-SNOH may also be expected as the initial step but mutual combination leading to disulfide formation appears rather unlikely in this case as a result of steric effects. The experimental results obtained clearly confirm this. If mutual combination of radicals would take place leading to disulfide formation, it would be between radical sites on two adjacent proteins. According to this logic, formation of a dimer of BSA would be expected. However, the SDS-PAGE analysis clearly rules out dimer formation. The BSA-

SNOH radicals thus, do not mutually combine but disappear most probably by reaction with another NO, because of its radical nature and ease of penetration of BSA protein. Other investigators have come to the same conclusion. In a study by DeMaster et al. [14], investigating the reaction of NO with human serum albumin (HSA), it was found that sulfenic acid and nitrous oxide are the main end products. The proposed mechanism for the formation of sulfenic acid involves the formation of an *S*-(*N*-nitroso)-hydroxylamino intermediate from the reaction of initially formed HSA-SNOH with another NO, followed by solvolytic disproportionation [14]. Our observation with BSA clearly supports the possibility of the formation of sulfenic acid rather than disulfide as is evident from the absence of dimer in the SDS-PAGE analysis. *On the other hand and most importantly, all the above observations support the conclusion that cysteine, BSA and MT1 undergo a similar reaction with NO in the initial step,* i.e. an electrophilic addition of NO leading to the formation of R-SNOH. The end products are, however, different and this is caused by the fact that the steric effect due to the high molecular size of BSA restricts the formation of disulfide unlike the case of cysteine and MT1. The absence of mutual radical combination in the case of BSA in all likelihood does not affect the rate of the initial step of the reaction of NO with BSA leading to the thiol(ate) loss and it can most certainly not be responsible for the higher rate of the reaction of NO with BSA than with MT1 as such an effect would affect the rate in the opposite direction.

5. Conclusion

The reactivity of NO with cysteine, bovine serum albumin and metallothionein-1 is in the order: cysteine > BSA ≫ MT1, i.e. in clear disparity with the size of the reactants. The low reactivity of NO towards MT1 is attributed to effects of metal coordination proper as well as to steric effects associated with the closed dual shell-like structure resulting from the tight coordination of the thiolate groups with Zn^{2+}.

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