Kinetics of the anaerobic reaction of nitric oxide with cysteine, glutathione and cysteine-containing proteins: implications for *in vivo S*-nitrosation

Charuvila T. Aravindakumar,[a] Marc De Ley[a] and Jan Ceulemans*[b]

[a] *Laboratory for Biochemistry, K.U.Leuven, Celestijnenlaan 200-G, B-3001 Leuven, Belgium*
[b] *Department of Chemistry, K.U.Leuven, Celestijnenlaan 200-F, B-3001 Leuven, Belgium. E-mail: Jan.Ceulemans@chem.kuleuven.ac.be; Fax: +32-16-32.79.91*

Received (in Cambridge, UK) 10th August 2001, Accepted 11th December 2001
First published as an Advance Article on the web 24th January 2002

A study is made of the kinetics of the anaerobic reaction of nitric oxide (˙NO) with cysteine and glutathione in relation to its potential physiological importance for the *S*-nitrosation of cysteine-containing peptides and proteins. The kinetics of the reaction with cysteine (the basic reagent unit) is studied most extensively and it is found that the rate constant is directly proportional to the degree of ionization (a), $k = 0.37 \times 10^3 a$ M^{-1} s^{-1}, offering clear proof for a mechanism based on electrophilic attack of ˙NO on thiolate anions. The rate constant for glutathione is considerably lower than for cysteine at identical pH, which can be attributed to its higher pK_a; steric effects do not appear to affect the reactivity of glutathione significantly. On the basis of the rate equations obtained and of similar data for bovine serum albumin and metallothionein-1 a number of calculations were performed with the aim of determining the relative importance of the reaction of ˙NO with O_2 *vs.* the direct reaction of ˙NO with peptide and protein thiols under *in vivo* conditions. The results clearly show that in cells that as a rule contain an appreciable concentration of glutathione the autoxidation of ˙NO and thus the reaction of higher oxides of nitrogen ($^{\bullet}NO_2$, N_2O_3) with thiol groups in peptides and proteins does not play a role of any significance with respect to the formation of *S*-nitroso-thiols, as the direct reaction of ˙NO with the thiolate group in glutathione leading to the formation of GS–$^{\bullet}NO^-$ is much faster than the reaction of ˙NO with O_2. The difference in reactivity is less pronounced in the case of bovine serum albumin, but again the electrophilic attack clearly is more important than the autoxidation at most physiological ˙NO concentrations. Direct electrophilic attack of ˙NO on metallothionein is of no practical significance, as the process is very much slower than the attack on glutathione.

Introduction

The discovery of the synthesis of nitric oxide (˙NO) in the body by conversion of L-arginine to L-citrulline by nitric oxide synthase (NOS) and of the involvement of ˙NO in a wide range of biological processes including vasodilation,[1–3] platelet deaggregation,[4–6] messenger action in the central nervous system,[7,8] photoreceptor signalling[9,10] and cytotoxic action with respect to tumor cells,[11] has sparked off intense activity in the field of ˙NO research in recent years.[12–16] The chemical behavior of ˙NO in biological systems, therefore, has been a topic of increased attention.

One particularly important reaction of ˙NO from a biological perspective is its reaction with peptide and protein sulfhydryl groups. Under physiological conditions, such a reaction is now known to lead to *S*-nitrosation of biological thiols and the corresponding formation of *S*-nitrosothiols (RS–NO).[17] These compounds are known to act as potent vasodilators and inhibitors of platelet aggregation[17,18] and are capable of storing and transporting ˙NO within the body. As a matter of fact, RS–NOs may act as bioactive reservoirs for ˙NO. The transformation of ˙NO into RS–NOs is of great physiological importance, as it preserves the bioactivity of ˙NO and increases its half-life. Nitrosation is not limited to thiols in proteins, but can take place at oxygen and nitrogen atoms as well; thiols are the preferred target for nitrosation, however, and *S*-nitrosation confers greater ˙NO-related bioactivity than nitrosation of other groups.[19] An increasing number of proteins have been found to be *S*-nitrosated *in vivo* by both endogenous and exogenous ˙NO, indicating that the formation of *S*-nitroso-thiols may be quite important in a number of physiological processes and that the regulatory and cytotoxic action of ˙NO may, at least partially, be effected through *S*-nitrosation.[20–22] *S*-Nitrosation can result from reaction of ˙NO and thiols along various pathways and from *trans*-*S*-nitrosation, *e.g.* from *S*-nitrosoglutathione (GS–NO)

$$P\text{–}S^- + GS\text{–}NO \rightleftharpoons PS\text{–}NO + G\text{–}S^- \qquad (1)$$

with P representing any cysteine-containing peptide or protein. In spite of its great physiological importance, at present the mechanism of the biosynthetic pathway for the formation of *S*-nitrosothiols from ˙NO is unclear.[23]

In contrast to the earlier concept of nitrosation of thiols by ˙NO itself,[24,25] it is now known that under anaerobic conditions ˙NO can not directly *S*-nitrosate protein thiols and that it is the higher oxides of nitrogen such as N_2O_3 and $^{\bullet}NO_2$ that cause *S*-nitrosation under standard (aerobic) laboratory conditions.[26–31] Studies by Kharitonov *et al.*[29] and Goldstein and Czapski[32] have independently demonstrated the involvement of N_2O_3 and $^{\bullet}NO_2$ in the nitrosation of low molecular weight thiols and serum albumins at physiological pHs. It has further been shown that the rate-limiting step in the nitrosation of thiols is the third-order reaction of ˙NO with O_2.[29] However, both studies[29,32] have concluded that such reaction pathways do not necessarily have much significance with respect to the *in vivo* nitrosation of protein thiols. Under *in vivo* conditions, where the concentrations of O_2 and especially of ˙NO are low, one has to keep in mind that the pathways of the various reactions

DOI: 10.1039/b107273k

involving $^{\bullet}NO$, O_2 and thiols may be very different from those in standard laboratory experiments.

Direct attack of $^{\bullet}NO$ on thiolate groups leading to an intermediate RS–$^{\bullet}NO^{-}$, which on losing an electron in the presence of an electron acceptor such as oxygen can give rise to RS–NO, must be considered as an alternative mechanism for the formation of RS–NO at physiological concentrations of $^{\bullet}NO$. Such a mechanism has been proposed (in somewhat different form) by Gow *et al.* and evidence has been presented with respect to its validity.[33] It is obvious that the relative importance of the two competing mechanisms with respect to *in vivo* RS–NO formation will depend on the rate of reaction of $^{\bullet}NO$ with peptide and protein thiols, the rate of the reaction of $^{\bullet}NO$ with O_2 and the concentrations of the respective reactants.

In spite of its great potential physiological importance, the anaerobic reaction of $^{\bullet}NO$ with cysteine and cysteine-containing peptides and proteins and especially the kinetics of the process have been studied only rudimentarily.[26,30,31,34] We embarked therefore on a study of the kinetics of the anaerobic reaction of $^{\bullet}NO$ with cysteine (Cys) and glutathione (GSH), in order to obtain some fundamental data on this process. Cysteine is the basic reactant unit for the reaction of $^{\bullet}NO$ with peptide and protein thiols and is therefore ideal to study the process in a fundamental way. Glutathione is the most abundant sulfur-containing (non-protein) intracellular entity (cellular concentration 0.5–10 mM), so study of this compound can provide realistic and directly applicable results from a physiological point of view. On the basis of the results and rate equations obtained for these compounds and of similar data for bovine serum albumin (BSA) and metallothionein-1 (MT1) derived from earlier experimental results,[30,31] a number of calculations are performed with the aim of determining the relative importance of the reaction of $^{\bullet}NO$ with O_2 *vs.* the direct reaction of $^{\bullet}NO$ with peptide and protein thiols under *in vivo* conditions. BSA is a high molecular weight protein ($M_r \approx 68000$) that has only one free thiol group. It is used as a model protein to study the effect of steric hindrance on the rate of the reaction with $^{\bullet}NO$. Its inclusion in the study is also important from a direct physiological point of view, as *S*-nitrosoalbumin is the most abundant *S*-nitrosated protein and is present in micromolar concentrations *in vivo*.[35] Metallothioneins (MT) are ubiquitous low-molecular mass ($M_r \approx 6000$), sulfur-rich proteins and metallothionein-1 (MT1) is one of the isoforms of MT. It has 20 cysteine residues which are coordinated to Zn^{2+}. MT1 is used as a model protein to study the effect of Zn^{2+}-coordination and associated steric effects on the rate of the reaction with $^{\bullet}NO$.

Experimental

Materials

Commercially available, highest-purity cysteine–HCl (>98.5%), glutathione (>99%), Ellman's reagent [5,5′-thiobis(2-nitrobenzoic acid)], and EDTA (ethylenediaminetetraacetic acid) were used without further purification.

Purification of $^{\bullet}NO$

In order to carry out the experiments under strictly anaerobic conditions, the $^{\bullet}NO$ used must be completely free of traces of higher oxides of nitrogen. Therefore, a special gas line and reaction chamber set-up were developed for this purpose. This set-up involves a modified tonometer consisting of a reaction chamber and a pre-reaction chamber that can be connected to a main gas line through which commercially available high purity $^{\bullet}NO$ can be introduced. The pre-reaction chamber contained 15 ml L-cysteine–HCl (0.1 M, pH 3) in order to scavenge all the higher oxides of nitrogen such as N_2O_3 and $^{\bullet}NO_2$. These higher oxides have a very high reactivity with cysteine over the entire pH range in contrast to $^{\bullet}NO$ which has a very low reactivity with cysteine at low pH.[26] The entire set-up including the gas line and reaction chamber (with sample solution) was first evacuated using a vacuum pump and high purity argon was introduced thereafter. It was again evacuated followed by argon purging and this procedure was repeated 3–4 times. $^{\bullet}NO$ was subsequently introduced into the pre-reaction chamber and thoroughly mixed with the L-cysteine–HCl by gentle shaking. A light-brown–yellow color in the L-cysteine–HCl solution indicated the formation of low quantities of *S*-nitrosocysteine due to the reaction of N_2O_3 or $^{\bullet}NO_2$ with cysteine. The successive experiments confirmed that the $^{\bullet}NO$ available in the pre-reaction chamber by this procedure is very pure and actually completely free of N_2O_3 and $^{\bullet}NO_2$ as it induced no *S*-nitrosation in the reaction chamber which could easily be checked spectroscopically.

Reaction with $^{\bullet}NO$ and determination of the extent of the reaction

$^{\bullet}NO$ was introduced at atmospheric pressure in the pre-reaction chamber; expansion over the combined volume of the pre-reaction and reaction chamber resulted in a small pressure reduction and an $^{\bullet}NO$ concentration in the aqueous solution of the reaction chamber of 1.52 mM. The concentration of cysteine and glutathione in the reaction chamber was 0.1 mM. Quantitative determinations of the free thiol groups were carried out spectrophotometrically before and (at different time periods) after $^{\bullet}NO$ treatment by their reaction with Ellman's reagent[36] in the presence of EDTA (1 mM) in phosphate buffer (0.1 M, pH 7.3). The Ellman reaction with cysteine and glutathione, untreated with $^{\bullet}NO$, gave results that corresponded well to the expected free thiol(ate) content. Before analysis, $^{\bullet}NO$ was completely removed from the reaction chamber after the required time interval in order to preclude any further reaction of $^{\bullet}NO$ and higher oxides of nitrogen, which would otherwise be formed by the reaction of $^{\bullet}NO$ with O_2 upon exposure to air. The absence of reactions with higher oxides of nitrogen was confirmed by measuring the absorbance in the region around 350 nm (absorption maximum of *S*-nitrosothiols). The temperature of the reaction chamber was controlled by a thermostat and kept at 25 °C during the reaction with $^{\bullet}NO$. The full experimental cycle including $^{\bullet}NO$ purification and $^{\bullet}NO$ removal before analysis was performed separately for each individual result (*i.e.*, each individual data point on a concentration–time graph).

Results

In order to determine the rate constant (and its pH dependence) for the reaction of $^{\bullet}NO$ with cysteine, the loss of thiol(ate) content was investigated at different times of $^{\bullet}NO$ treatment and at different pH values using Ellman's reaction under strictly anaerobic conditions in a solution containing 0.1 mM cysteine. The reaction is very pH dependent and initial experiments showed a fast disappearance of thiol(ate) content at pH > 6.5 and virtually no reaction at pH < 5.0. A workable pH range for monitoring the reaction using our set-up was selected from these initial experiments. A typical plot obtained for the decrease in concentration of cysteine as a function of time of $^{\bullet}NO$ treatment is given in Fig. 1. The figure shows the pseudo first-order nature of the reaction, indicating first-order dependence on cysteine concentration. Pseudo first-order rate constants were determined from these and similar results at different pH values. In a similar way the anaerobic reaction of $^{\bullet}NO$ with glutathione was investigated. A plot showing the thiol(ate) loss with respect to time of $^{\bullet}NO$ treatment at pH 6.1 and the first-order dependence on glutathione concentration of the GSH with $^{\bullet}NO$ reaction is shown in Fig. 2. The first-order dependence on nitric oxide concentration has been established

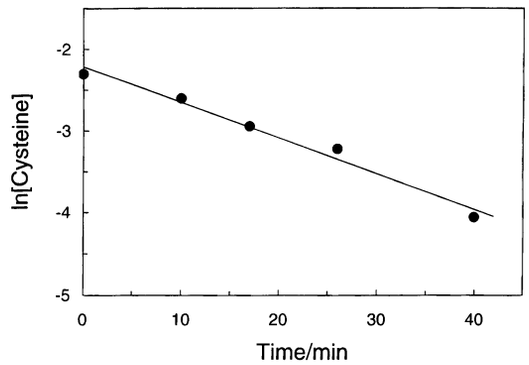

Fig. 1 Kinetics of the reaction of $^{\bullet}$NO with cysteine under anaerobic conditions at pH 5.7 and a temperature of 25 °C. Initial reactant concentrations were $C_{Cys} = 0.1$ mM and [$^{\bullet}$NO] = 1.52 mM.

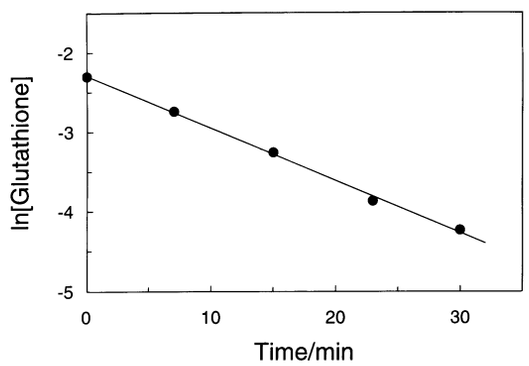

Fig. 2 Kinetics of the reaction of $^{\bullet}$NO with glutathione under anaerobic conditions at pH 6.1 and a temperature of 25 °C. Initial reactant concentrations were $C_{GSH} = 0.1$ mM and [$^{\bullet}$NO] = 1.52 mM.

previously.[34] Second-order rate constants for the reaction of $^{\bullet}$NO with cysteine and glutathione were calculated from these experimental results, taking into consideration that the concentration of $^{\bullet}$NO in the reaction chamber equals 1.52 mM. In Fig. 3 rate constants for the reaction of cysteine with $^{\bullet}$NO are

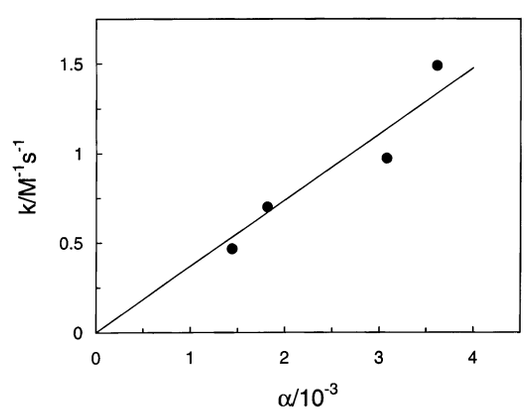

Fig. 3 Dependence of the rate constant for the anaerobic reaction of cysteine with $^{\bullet}$NO on its degree of ionization. The solid line corresponds to the equation $k = 0.37 \times 10^3 a$ M^{-1} s^{-1}.

plotted as a function of the degree of ionization (a) as calculated from the pK_a of cysteine, *viz.* 8.54, corresponding to –SH $\rightleftharpoons$ –S$^-$ + H$^+$ as reported in the in-depth study of the microscopic proton dissociation constants of this amino acid by Kallen.[37] These results allow one to conclude that *the rate constant is directly proportional to the degree of ionization*. The pH dependence of the anaerobic reaction of $^{\bullet}$NO with cysteine can thus be represented by

$$k(\mathrm{pH}) = ak \qquad (2)$$

with k being the rate constant at full dissociation, *i.e.*, for $a = 1$. From the experimental data, a value for k of 0.37×10^3 M^{-1} s^{-1} is obtained for the anaerobic reaction of $^{\bullet}$NO with cysteine at 25 °C. The corresponding value for GSH is 0.26×10^3 or 0.49×10^3 M^{-1} s^{-1} based on pK_a values of 8.66 and 8.93, respectively, for the dissociation into thiolate ions.[38] The arithmetic mean of these two values corresponds almost exactly to the k value for cysteine. As the expected encounter rate in aqueous solution at 25 °C is *ca.* 7×10^9 M^{-1} s^{-1},[39] the anaerobic reaction of $^{\bullet}$NO with cysteine and glutathione is clearly far from diffusion controlled.

The anaerobic reaction of $^{\bullet}$NO with MT1 and BSA has recently been studied by us[30,31] and it has been concluded that the rate of reaction of $^{\bullet}$NO with these proteins (relative to cysteine) is in the order: Cys > BSA > MT1. Based on these experimental data pseudo first-order rate constants can be determined (from plots of the loss of thiol(ate) in the case of BSA and of Zn^{2+} in the case of MT1 *vs.* time of $^{\bullet}$NO treatment), allowing the derivation of corresponding second-order rate constants, again taking into consideration that the concentration of $^{\bullet}$NO in the reaction chamber amounted to 1.52 mM. The rate constants, k, are respectively equal to 0.57 M^{-1} s^{-1} (BSA) and 0.06 M^{-1} s^{-1} (MT1). No effect of a (and thus of pH) was considered for these proteins. The thiol group in BSA appears to have an abnormally low pK_a of less than 5[38b] and thus primarily exists as thiolate anion at physiological pH (a > 0.992 at pH 7.1). Though such a state of affairs may be somewhat surprising and has been disputed recently,[40] it appears to be not uncommon; the cysteine residue (Cys-149) at the active site in glyceraldehyde-3-phosphate dehydrogenase (GAPDH), for instance, has a pK_a of 5.5, well below that of the other cysteine residues in the enzyme.[20] In the case of MT1 all the thiol groups in the cysteine residues are bonded to Zn^{2+} as deprotonated thiolate, irrespective of pH.[41]

Discussion

Effect of pH and mechanism of the reaction

In the present study, the rate of the reaction of $^{\bullet}$NO with Cys is found to be *directly proportional to the degree of ionization* (Fig. 3). This offers clear and unambiguous proof that the reaction of $^{\bullet}$NO with thiols is in essence an electrophilic attack of $^{\bullet}$NO on thiolate anions.

$$\mathrm{RSH} + \mathrm{H_2O} \rightleftharpoons \mathrm{RS^-} + \mathrm{H_3O^+} \qquad (3)$$

$$\mathrm{RS^-} + {}^{\bullet}\mathrm{NO} \longrightarrow \mathrm{RS\text{–}{}^{\bullet}NO^-} \qquad (4)$$

Under strictly anaerobic conditions, as are prevalent in the present study, the reaction process will result in the formation of disulfides if it proceeds to its full conclusion. This is the case in the anaerobic reaction of $^{\bullet}$NO with cysteine and glutathione, resulting in the formation of cystine and glutathione disulfide, respectively.[26,30] According to Pryor *et al.*,[26] the intermediary acid–base equilibrium

$$\mathrm{RS\text{–}{}^{\bullet}NO^-} + \mathrm{H_3O^+} \rightleftharpoons \mathrm{RS\text{–}{}^{\bullet}NOH} + \mathrm{H_2O} \qquad (5)$$

results in the formation of RS–$^{\bullet}$NOH, which by radical coupling

$$2\ \mathrm{RS\text{–}{}^{\bullet}NOH} \longrightarrow \mathrm{RSN(OH)\text{–}N(OH)SR} \qquad (6)$$

and elimination of hyponitrous acid results in the formation of the disulfide.

$$\mathrm{RSN(OH)\text{–}N(OH)SR} \longrightarrow \mathrm{RSSR} + \mathrm{HON{=}NOH} \qquad (7)$$

The hyponitrous acid may decompose into N_2O and H_2O, so N_2O and disulfides (homodimeric disulfides if only one type of thiol is present) are the expected products of the reaction process. An alternative pathway consisting of reaction of RS–$^{\bullet}$NOH with $^{\bullet}$NO resulting in the formation of an *S*-(*N*-nitroso)hydroxyamino intermediate, which by solvolytic disproportionation results in the formation of sulfenic acid and N_2O,

$$RS–{}^{\bullet}NOH + {}^{\bullet}NO \longrightarrow RS–OH + N_2O \qquad (8)$$

has been proposed by DeMaster *et al.*[28] Due to the electrophilic character of the sulfenyl sulfur, the so-formed sulfenic acids react with thiols to produce the disulfides.

$$RS–OH + RSH \longrightarrow RSSR + H_2O \qquad (9)$$

The fact that the rate of the reaction is directly proportional to the degree of ionization is a clear indication that the initial electrophilic attack of $^{\bullet}$NO on thiolate anions is the rate-determining step in the kinetics studied. The subsequent formation of RS–$^{\bullet}$NOH by the intermediary acid–base equilibrium is affected by pH in the opposite direction and further processes do not involve acid–base equilibria. The observation that the electrophilic attack is rate determining is not at all surprising as the subsequent processes can all be expected to be quite rapid. Protonation in acid–base equilibria as in all proton-exchange reactions occurs very swiftly (for the proton exchange of the free radical $^{\bullet}CH_2OH$ in acidified aqueous solution, for instance, a rate constant of 1.76×10^8 M^{-1} s^{-1} has been determined by EPR spectroscopy)[42] and radical combination reactions are very nearly diffusion controlled. The resulting combination product appears unstable and its decomposition can be expected to be almost 'instantaneous' (*i.e.*, on the time scale of bond vibrations) as *in statu nascendi* it contains substantial excitation energy as a result of the N–N bond formation. The alternative (and additional) pathway consisting of reaction of RS–$^{\bullet}$NOH with $^{\bullet}$NO can only have a positive effect on the kinetics of the processes subsequent to the initial electrophilic attack. From the bimolecularity of the rate-determining step (reaction 4) second-order kinetics are expected, partial first order with respect to each reactant ($^{\bullet}$NO and thiolate), as fast elementary steps that *follow* the rate-determining step have no effect on the rate law of a reaction. These expectations on the rate law of the reaction clearly are in agreement with the experimental observations on the order (and partial orders) of the process.

Steric effect, effect of pK_a and effect of metal coordination on rate constants

The rate constants for the reaction of $^{\bullet}$NO with cysteine, glutathione and the different cysteine-containing proteins considered clearly follow the order: Cys > GSH ⩾ BSA > MT1. The rate constant of the anaerobic reaction of glutathione with $^{\bullet}$NO is considerably lower than that of cysteine at identical pH, which, in principle, can be attributed to both steric hindrance and difference in acid strength of the thiol group in these two compounds. Rate constants at complete ionization cannot be differentiated and thus appear essentially identical, however, taking into consideration experimental uncertainties with respect to the pK_a of GSH. As a result, the lower reactivity of GSH at a particular pH must thus (largely) be attributed to its higher pK_a relative to that of cysteine. The reduction in rate from Cys to BSA has, quite reasonably, been attributed to steric hindrance due to the high molecular size of the BSA protein.[31] The steric effect is larger, however, than would be deduced from a simple comparison of reactivities, as differences in acid strength and (associated) degree of ionization of the thiol group also affect the difference in reactivity between Cys and BSA. Actually, the steric effect is given by the ratio of rate constants at full ionization and thus amounts to about 640. Steric effects have been invoked previously with respect to the reaction of $^{\bullet}$NO with thiols in proteins. To cite one particularly interesting example, in a study of the interaction of $^{\bullet}$NO with the *ras* oncogene product p21 it was observed that single *S*-nitrosation occurs selectively at Cys 118, in line with modelling studies which show that Cys 118 is most exposed whereas Cys 51 and Cys 80 are fairly well shielded.[43] The reduction in rate from Cys to MT1 has been 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+} in MT1.[31] The global effect is given by the ratio of rate constants at full ionization and thus amounts to about 6470. Comparison of rate constants of GSH and MT with respectively free hydroxyl ($^{\bullet}$OH), superoxide ($O_2^{\bullet-}$) and $^{\bullet}$NO radicals allows differentiation between these two effects and rationalization of the effects observed. The rate constant for the reaction of $^{\bullet}$OH radicals with metallothionein is very high, $k_{^{\bullet}OH/MT} \approx 10^{12}$ M^{-1} s^{-1}, suggesting that all 20 cysteine sulfur atoms are involved in the reaction and that they all act at the diffusion control limit.[44] The comparable rate constant for glutathione $k_{^{\bullet}OH/GSH} = 8 \times 10^9$ M^{-1} s^{-1}. From these data, it can be deduced that there is essentially no steric effect on the reaction of GSH *and* MT with $^{\bullet}$OH. The rate constant for reaction of superoxide (SO) with metallothionein, $k_{SO/MT} \approx 5 \times 10^5$ M^{-1} s^{-1}, is about an order of magnitude lower than that for glutathione, $k_{SO/GSH} = 6.7 \times 10^6$ M^{-1} s^{-1}. This lowering in reactivity is possibly (but not necessarily) partly due to steric effects, which might start to become effective as $O_2^{\bullet-}$ is considerably larger than $^{\bullet}$OH. As is evident from the present study, the rate constant for the reaction of $^{\bullet}$NO with metallothionein, $k_{^{\bullet}NO/MT} = 0.06$ M^{-1} s^{-1}, is very much lower than that for glutathione, $k_{^{\bullet}NO/GSH} = 0.49 \times 10^3$ M^{-1} s^{-1}. The much larger decrease in the case of $^{\bullet}$NO (~10^4 *vs.* ~10) cannot be attributed to steric effects as $^{\bullet}$NO is certainly not a larger entity than $O_2^{\bullet-}$. It is thus clear that the lowering of the reactivity from GSH to MT in the case of $^{\bullet}$NO is largely due to the direct effect of Zn^{2+}–thiolate complexation and that this effect is much larger for $^{\bullet}$NO than for $O_2^{\bullet-}$ and especially for $^{\bullet}$OH. This can be rationalized on the basis of the rate constants for reaction of these entities with glutathione. These rate constants are in the order $k_{^{\bullet}OH/GSH} > k_{SO/GSH} > k_{^{\bullet}NO/GSH}$, *i.e.*, $^{\bullet}$OH radicals are much more reactive towards the thiol group than $O_2^{\bullet-}$ radicals and these are in turn much more reactive than $^{\bullet}$NO. The effect of metal coordination on the reactivity (when comparing MT with GSH) clearly goes in the opposite direction, with essentially no effect for $^{\bullet}$OH, a quite considerable effect for $O_2^{\bullet-}$ and a very large effect for $^{\bullet}$NO. This clearly makes sense as it is in line with expectation that the rate constant of a less reactive entity will be much more affected by the stabilizing effect of metal coordination on the thiolate groups in MT than the rate constant of a highly reactive one.

Implications for *in vivo S*-nitrosation

The determination of absolute rate constants for the anaerobic reaction of $^{\bullet}$NO with cysteine, glutathione and cysteine-containing proteins allows some conclusions to be drawn with respect to *S*-nitrosation of peptide and protein thiols by $^{\bullet}$NO under physiological conditions. Two major reaction processes that have at some point been proposed in this regard will be kinetically compared, *viz.* the transformation of $^{\bullet}$NO into $^{\bullet}NO_2/N_2O_3$ by reaction with oxygen and subsequent reaction of these higher oxides with peptide and protein thiols on the one hand, and the direct reaction of $^{\bullet}$NO with such thiols followed by transformation of the reaction intermediates thus formed to nitrosothiols by reaction with oxygen on the other hand.

The first process is initiated by reaction of $^{\bullet}$NO with O_2 resulting in an intermediary ONOO$^{\bullet}$ (or $^{\bullet}NO \cdots O_2$), which on

further reaction with $^{\bullet}NO$ results in the formation of $^{\bullet}NO_2$ (through an intermediary trimolecular complex, *i.e.* ONOONO or some variant), the overall reaction equation of this initial process being

$$2\ ^{\bullet}NO + O_2 \longrightarrow 2\ ^{\bullet}NO_2 \qquad (10)$$

The rate-determining step of the autoxidation of $^{\bullet}NO$ appears to be the formation of the trimolecular complex. The kinetics of nitric oxide autoxidation in aerated aqueous solution, which ultimately results fully in the formation of nitrite in the absence of thiols and other compounds that can be nitrosated (see below), has been studied repeatedly in recent years because of the physiological importance of $^{\bullet}NO$.[45–50] The reaction is second order in $^{\bullet}NO$ concentration and first order in O_2 concentration; the overall third-order rate constant is about 1000-fold larger than that for the gas phase reaction. The kinetics and mechanism of the overall process, *i.e.*, the nitrosation of thiols by oxygenated $^{\bullet}NO$ solutions and the nature of the nitrosating intermediates has been studied in detail by Kharitonov *et al.*[29] and by Goldstein and Czapski.[32] It has been demonstrated that $^{\bullet}NO_2$ initiates the nitrosation process

$$^{\bullet}NO_2 + RS^- \longrightarrow NO_2^- + RS^{\bullet} \qquad (11)$$

$$RS^{\bullet} + {}^{\bullet}NO \longrightarrow RS\text{–}NO \qquad (12)$$

unless it is scavenged by $^{\bullet}NO$ to form N_2O_3.

$$^{\bullet}NO_2 + {}^{\bullet}NO \rightleftharpoons N_2O_3 \qquad (13)$$

This reaction has a high rate constant ($k_{13,f} = 1.1 \times 10^9\ M^{-1}\ s^{-1}$). Also, N_2O_3 is considerably more stable in aqueous solution ($k_{13,b} = 8.0 \times 10^4\ s^{-1}$) than in the gas phase with respect to homolytic dissociation due to more effective heat dissipation and as a result of the cage effect which keeps the $^{\bullet}NO$ and $^{\bullet}NO_2$ fragments together, resulting quite frequently in mutual recombination.[51] N_2O_3 itself is a strong nitrosating agent that can directly nitrosate thiols *via* reaction 14.

$$N_2O_3 + RS^- \longrightarrow RS\text{–}NO + NO_2^- \qquad (14)$$

Full nitrosation yields are obtained either when $^{\bullet}NO_2$ rapidly oxidizes the thiolate or when the nitrosation by N_2O_3 competes efficiently with the hydrolysis of this reagent to nitrite.

$$N_2O_3 + 3\ H_2O \longrightarrow 2\ NO_2^- + 2\ H_3O^+ \qquad (15)$$

Kharitonov *et al.*[29] suggest that at physiological concentrations of GSH, BSA and human serum albumin (HSA) the main nitrosating species is N_2O_3. They conclude that at physiological concentrations of GSH nitrosoglutathione formation represents a significant metabolic fate of N_2O_3 (*i.e.*, the reaction rate of N_2O_3 with GSH is competitive with its rate of hydrolysis) and that at glutathione concentrations of 5 mM or higher almost all of the N_2O_3 formed is consumed in nitrosation of glutathione. In contrast, HSA and BSA compete less successfully with water for reaction with N_2O_3 and nitrosation of HSA and BSA is thus certainly less significant by this reaction path. Goldstein and Czapski also conclude that under limiting concentrations of $^{\bullet}NO$ or O_2 full nitrosation yields are obtained at sufficiently high glutathione concentration, but they attribute the nitrosation process fully to $^{\bullet}NO_2$–$^{\bullet}NO$ (reactions 11 and 12) under these conditions.[32] Both research groups have found that the kinetic results for the nitrosation of thiols by oxygenated $^{\bullet}NO$ solutions are identical to those obtained for the autoxidation of $^{\bullet}NO$ and it has been concluded that the rate-determining step of the nitrosation by $^{\bullet}NO/O_2$ *by this pathway* is the formation of the trimolecular complex (ONOONO or some variant), which is the precursor of $^{\bullet}NO_2$. It is to be stressed that these conclusions apply to 'laboratory' (10^{-3}–10^{-4} M) $^{\bullet}NO$ concentrations.

The second process is initiated by electrophilic attack of $^{\bullet}NO$ on thiolate anions (reaction 4). In aerated aqueous solutions, RS–$^{\bullet}NO^-$ formed may react with O_2 by electron transfer as proposed (in a somewhat different form) by Gow *et al.*,[33] resulting in the formation of *S*-nitrosothiols.

$$RS\text{–}{}^{\bullet}NO^- + O_2 \longrightarrow RS\text{–}NO + O_2^{\bullet -} \qquad (16)$$

Evidence presented for this mechanism includes the observation that (i) cysteine accelerates the decomposition of $^{\bullet}NO$ under physiological conditions (low $^{\bullet}NO$ concentration), (ii) cysteine also accelerates the consumption of O_2 (in the presence of $^{\bullet}NO$) and its reaction with $^{\bullet}NO$ results in the formation of hydrogen peroxide when Cu,Zn-superoxide dismutase is present in the system indicating that the reaction generates $O_2^{\bullet -}$ and (iii) *S*-nitrosothiols can be formed in an anaerobic environment in the presence of an electron acceptor.[33]

In order to assess the relative importance of these two reaction processes for *in vivo* nitrosation, the rate of the processes is compared at physiological concentrations of O_2, $^{\bullet}NO$ and the cysteine-containing peptide/proteins GSH, BSA and MT1. Oxygen concentrations in cytosol are 20–50 μM.[52] Physiological concentrations of $^{\bullet}NO$ appear to be around 0.1–1 μM. *in situ* $^{\bullet}NO$ levels have been measured by electrochemical $^{\bullet}NO$ probes in cerebellar slices and increases in $^{\bullet}NO$ concentration of up to 75 nM were observed to accompany electrical stimulation of the tissue.[53] To our knowledge, the highest reported level for endogenous $^{\bullet}NO$ *in vivo* is 4 μM observed in rat brain after transient middle cerebral artery occlusion.[54] The intracellular glutathione concentration in mammalian cells varies between 0.5 and 10 mM.[55] Normal concentrations of glutathione and serum albumin in human plasma are 3.4 μM and 0.6 mM, respectively.[56] The basal concentration of metallothionein in platelets amounts to about 40 μM, as can be derived from the published value of 39.4 μg per 10^{10} cells;[57] no significant amounts of MT are present in plasma or serum. MT may be induced by the presence of heavy metals, however, resulting in considerably higher concentrations than the basal one; upon induction, for instance in liver cells, the MT concentration can reach approximately 0.2 mM, which corresponds to a cysteine residue concentration of 4 mM.[44]

The kinetics of the autoxidation of $^{\bullet}NO$ in aqueous solution has been studied repeatedly in recent years.[45–50] The rate of the autoxidation is given by

$$-\mathrm{d}[^{\bullet}NO]/\mathrm{d}t = k_{10}[^{\bullet}NO]^2[O_2] \qquad (17)$$

Experimental data in the literature on the third-order rate constant, k_{10}, are in the range $6.3\text{–}11.5 \times 10^6\ M^{-2}\ s^{-1}$. The rate of the electrophilic attack of $^{\bullet}NO$ on thiolate is given by

$$-\mathrm{d}[^{\bullet}NO]/\mathrm{d}t = k_4[^{\bullet}NO][RS^-] = k_4[^{\bullet}NO]\alpha C_{RSH} \qquad (18)$$

with $[RS^-]$ being the concentration of thiolate ions and C_{RSH} the formal concentration of thiol in the system. The rate constants, k_4, for GSH, BSA and MT1 respectively are equal to $0.49 \times 10^3\ M^{-1}\ s^{-1}$ (for $pK_a = 8.93$), $0.57\ M^{-1}\ s^{-1}$ and $0.06\ M^{-1}\ s^{-1}$ as indicated above. The value of α can be taken as 1 in the case of BSA (at physiological pH) and MT1. For GSH α equals 0.007, 0.015 and 0.029 at pH 6.8, 7.1 and 7.4, respectively, so the degree of ionization and thus the rate of the electrophilic attack by $^{\bullet}NO$ roughly doubles for an increase in pH of 0.3 in the physiological pH range. In Fig. 4, $\log(k_{10}[^{\bullet}NO]^2[O_2]/k_4[^{\bullet}NO]\alpha C_{RSH})$ is plotted against $\log[^{\bullet}NO]$ at typical physiological concentrations of O_2 (35 μM), GSH (2.5 mM), BSA (0.6 mM) and MT1 (0.2 mM, cysteine residue 4 mM) and using a rate constant for the autoxidation of $^{\bullet}NO$ of $10 \times 10^6\ M^{-2}\ s^{-1}$.

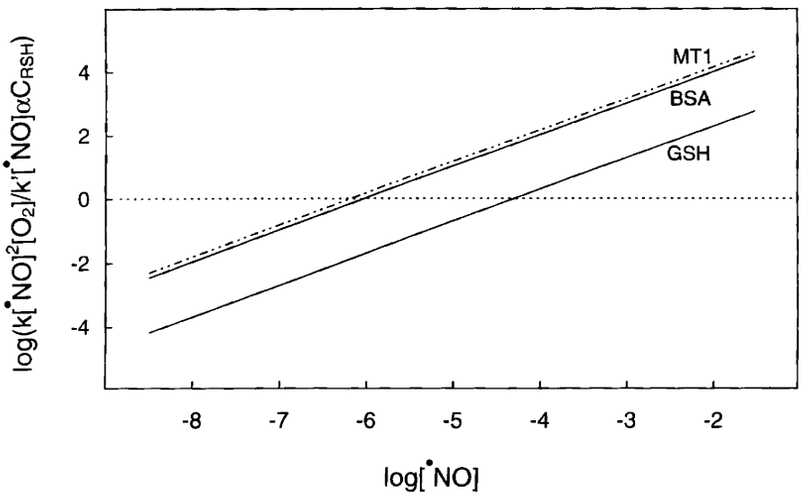

Fig. 4 Comparative kinetics of the reaction of $^{\bullet}NO$ with O_2 *vs.* the direct reaction of $^{\bullet}NO$ with GSH, BSA and MT1 as a function of $^{\bullet}NO$ concentration. The logarithm of the rate of these two reactions, *i.e.* $\log(k_{10}[^{\bullet}NO]^2[O_2]/k_4[^{\bullet}NO]\alpha C_{RSH})$, is plotted *vs.* $\log[^{\bullet}NO]$ at typical physiological concentrations of O_2 (35 μM), GSH (2.5 mM), BSA (0.6 mM) and MT1 (0.2 mM, cysteine residue 4 mM) and using a rate constant for the autoxidation of $^{\bullet}NO$, k_{10}, of 10^7 M^{-2} s^{-1}. The rate constants, k_4, for GSH, BSA and MT1 respectively are equal to 0.49 10^3 M^{-1} s^{-1} (for $pK_a = 8.93$), 0.57 M^{-1} s^{-1} and 0.06 M^{-1} s^{-1}. The graph is for a pH of 7.1.

From these results, it is obvious that at physiological concentrations of $^{\bullet}NO$ (0.1–1 μM) the direct electrophilic attack of $^{\bullet}NO$ on GSH is much faster than the autoxidation of $^{\bullet}NO$. This provides clear and irrefutable evidence that in cells that as a rule contain an appreciable concentration of GSH the autoxidation of $^{\bullet}NO$ and thus the reaction of higher oxides of nitrogen with thiol groups in peptides and proteins do not play a role of any significance with respect to the formation of *S*-nitrosothiols. From Fig. 4 it can easily be seen that it would be required to reduce the GSH concentration by a factor of almost 100 from the nominal value used in the calculations (*i.e.*, to 25 μM) to bring the autoxidation at the same level as the direct electrophilic attack at an $^{\bullet}NO$ concentration of 10^{-6} M; at lower $^{\bullet}NO$ concentrations the autoxidation would even then not be competitive. The difference in reactivity is less pronounced in the case of BSA, but again the electrophilic attack clearly is more important than the autoxidation at most physiological $^{\bullet}NO$ concentrations, dominating it by a factor of about 10 at 10^{-7} M $^{\bullet}NO$, a factor which reduces to about 1 (the two processes about equally important) at 10^{-6} M $^{\bullet}NO$. As for MT1, the dominance of the electrophilic attack over the autoxidation is not really clear cut; it should be remembered that the MT1 concentration used in the calculation is not the basal one but the higher limit by induction of heavy metals. It is more meaningful in this case to compare the rate of the electrophilic attack of $^{\bullet}NO$ on MT1 with that on GSH. Such a comparison yields the irrefutable conclusion that the direct electrophilic attack of $^{\bullet}NO$ on metallothionein is of no practical significance, as the process is very much slower than the attack on GSH. It is to be remarked that *trans*-nitrosation of MT by GS–NO also is an unlikely process for both thermodynamic and kinetic reasons. The process of *trans*-nitrosation between uncomplexed thiols can be expected to be rather close to thermoneutral, so the complexation energy associated with Zn^{2+}–thiolate coordination in MT is likely to adversely affect the equilibrium for this protein quite strongly. Also, the closed dual shell-like structure of MT resulting from the tight coordination of the thiolate groups with Zn^{2+} will make these thiolate groups quite inaccessible to the rather bulky GS–NO molecules, thus adversely affecting the kinetics of the *trans*-nitrosation.

It is interesting to repeat the calculations for standard 'laboratory' conditions. Taking typical (laboratory) GSH, O_2 and $^{\bullet}NO$ concentrations (as in ref. 29) of 5.4, 0.31 and 0.1 mM, respectively, the ratio of the rate of $^{\bullet}NO$ autoxidation to that of electrophilic attack by $^{\bullet}NO$ on thiolate amounts to 7.8 at pH 7.1; for an $^{\bullet}NO$ concentration of 1.72 mM (as in ref. 32) the ratio even amounts to 134 for the same GSH and O_2 concentrations. It is thus clear that under such conditions the dominant reaction mechanism is the autoxidation of $^{\bullet}NO$ followed by *S*-nitrosation of glutathione by $^{\bullet}NO_2/N_2O_3$, in accordance with the conclusions of Kharitonov *et al.*[29] and Goldstein and Czapski.[32] At physiological concentrations of $^{\bullet}NO$ (*i.e.*, 0.1–1 μM), direct attack of $^{\bullet}NO$ on thiolate is, however, for glutathione (and to a lesser extent for BSA) substantially faster than the autoxidation of $^{\bullet}NO$, thus (largely) precluding the occurrence of the latter process. This promotes the idea that the electrophilic attack of $^{\bullet}NO$ on thiolate (especially in the case of GSH) might be important for *S*-nitrosothiol formation *in vivo*. The electron transfer to oxygen from RS–$^{\bullet}NO^-$ would be in competition with the radical coupling of RS–$^{\bullet}NOH$ and the reaction of this reactive intermediate with $^{\bullet}NO$, two processes that result in disulfide formation (see above). Essential data for assessment of the relative importance of these two distinct pathways are the rate constant of the electron transfer process, the pK_a of the intermediary acid RS–$^{\bullet}NOH$ and the rate of the reaction of $^{\bullet}NO$ with RS–$^{\bullet}NOH$. These data are unavailable at present.

It should be noted that alternative mechanisms for *in vivo* nitrosation of peptide and protein thiols have been proposed, *viz.* by reaction of $^{\bullet}NO$ with copper–thiolate complexes[58] and by the action of dinitrosyl–iron complexes.[59] The first mechanism is not applicable to glutathione, however, as glutathione rapidly dimerizes in the presence of Cu^{2+}; the dimerization efficiently competes with *S*-nitrosation and as such prevents the formation of *S*-nitrosoglutathione. In more general terms, no solid data on the kinetics of the processes proposed and on the cellular concentrations of the complexes involved appear available. Demonstrating the occurrence of a particular reaction process *in vitro* is one thing, determining the importance for *in vivo S*-nitrosation is quite a different matter. Determination of the absolute rate constant of the different processes and of the cellular concentration of the respective reactants involved appears crucial to resolving such matters definitively. The present research is an important step in that direction.

In conclusion, data on the kinetics of the anaerobic reaction of $^{\bullet}NO$ with cysteine and cysteine-containing peptides and proteins are highly relevant with respect to the elucidation of the mechanism of *in vivo S*-nitrosation by $^{\bullet}NO$. In conjunction with kinetic data on the autoxidation of $^{\bullet}NO$, they allow us to state unequivocally that higher oxides of nitrogen (*viz.* $^{\bullet}NO_2$ and N_2O_3) do not play a significant role in intracellular

S-nitrosation despite their prominent role under laboratory conditions. Under physiological conditions, nitrosation by higher oxides of nitrogen does not significantly contribute to the formation of *S*-nitrosoglutathione and other cellular RS–NOs, as the direct reaction of $^{\bullet}NO$ with thiolate in glutathione leading to the formation of GS–$^{\bullet}NO^{-}$ is much faster than the reaction of $^{\bullet}NO$ with O_2. Comparative kinetics clearly also is the way forward with respect to demonstration of the actual physiological importance of other proposed mechanisms, *viz.* by reaction of $^{\bullet}NO$ with copper–thiolate complexes[58] and by the action of dinitrosyl–iron complexes.[59]

Acknowledgements

This work was supported by a Research Fellowship of the Research Council of K.U.Leuven (to C.T.A.) and by Research Grant G.0410.98 from 'Fonds voor Wetenschappelijk Onderzoek-Vlaanderen' (to M.D.L.).

References

1 L. J. Ignarro, *Semin. Hematol.*, 1989, **26**, 63.
2 T. G. Taylor and V. S. Sharma, *Biochemistry*, 1992, **31**, 2847.
3 S. Moncada and A. Higgs, *N. Engl. J. Med.*, 1993, **329**, 2002.
4 H. Azuma, M. Ishikawa and S. Sekizaki, *Br. J. Pharmacol.*, 1986, **88**, 411.
5 B. Furlong, A. H. Henderson, M. J. Lewis and J. A. Smith, *Br. J. Pharmacol.*, 1987, **90**, 687.
6 M. W. Rodomski, R. M. J. Palmer and S. Moncada, *Br. J. Pharmacol.*, 1987, **92**, 639.
7 J. Garthwaite, *Trends Neurosci.*, 1991, **14**, 60.
8 J. Zhang and S. H. Snyder, *Annu. Rev. Pharmacol. Toxicol.*, 1995, **35**, 213.
9 L. Stryer, *Annu. Rev. Neurosci.*, 1986, **9**, 87.
10 Y. Horio and F. Murad, *J. Biol. Chem.*, 1991, **266**, 3411.
11 P. J. Barnes, *Ann. Med.*, 1995, **27**, 389.
12 S. Moncada, R. M. Palmer and E. A. Higgs, *Pharmacol. Rev.*, 1991, **43**, 109.
13 A. R. Butler and D. L. H. Williams, *Chem. Soc. Rev.*, 1993, **22**, 233.
14 R. A. Robbinson and M. B. Grisham, *Int. J. Biochem. Cell Biol.*, 1997, **29**, 857.
15 D. A. Wink and J. B. Mitchell, *Free Radical Biol. Med.*, 1998, **25**, 434.
16 B. Gaston, *Biochim. Biophys. Acta*, 1999, **1411**, 323.
17 J. S. Stamler, D. I. Simon, J. A. Osborne, M. E. Mullins, O. Jaraki, T. Michel, D. J. Singel and J. Loscalzo, *Proc. Natl. Acad. Sci. U. S. A.*, 1992, **89**, 444.
18 J. S. Stamler, *Curr. Top. Microbiol. Immunol.*, 1995, **196**, 19.
19 D. I. Simon, M. E. Mullins, L. Jia, B. Gaston, D. J. Singel and J. S. Stamler, *Proc. Natl. Acad. Sci. U. S. A.*, 1996, **93**, 4736.
20 C. M. Padgett and A. R. Whorton, *Am. J. Physiol.*, 1995, **269**, C739.
21 (*a*) L. Jia, C. Bonaventura, J. Bonaventura and J. S. Stamler, *Nature*, 1996, **380**, 221; (*b*) J. S. Stamler, L. Jia, J. P. Eu, T. J. McMahon, I. T. Demchenko, J. Bonaventura, K. Gernert and C. A. Piantadosi, *Science*, 1997, **276**, 2034.
22 (*a*) A. R. Butler and P. Rhodes, *Anal. Biochem.*, 1997, **249**, 1; (*b*) D. L. H. Williams, *Acc. Chem. Res.*, 1999, **32**, 869.
23 M. F. Perutz, *Nature*, 1996, **380**, 205.
24 P. Girad and P. Potier, *FEBS Lett.*, 1993, **320**, 7.
25 A. F. Vanil and I. A. Malenkova, *Portland Press Proc.*, 1996, **10**, 107.
26 W. A. Pryor, D. F. Church, C. K. Govindan and G. Gank, *J. Org. Chem.*, 1982, **47**, 156.
27 D. A. Wink, R. W. Nims, J. F. Darbyshire, D. Christodoulou, I. Hanbauer, G. W. Cox, F. Laval, J. Laval, J. A. Cook, M. C. Krishna, W. G. DeGraff and J. B. Mitchell, *Chem. Res. Toxicol.*, 1994, **7**, 519.
28 E. G. DeMaster, B. J. Quast, B. Redfern and H. T. Nagasawa, *Biochemistry*, 1995, **34**, 11494.
29 V. G. Kharitonov, A. R. Sundquist and V. S. Sharma, *J. Biol. Chem.*, 1995, **270**, 28158.
30 C. T. Aravindakumar, J. Ceulemans and M. De Ley, *Biochem. J.*, 1999, **344**, 253.
31 C. T. Aravindakumar, J. Ceulemans and M. De Ley, *Biophys. Chem.*, 2000, **85**, 1.
32 S. Goldstein and G. Czapski, *J. Am. Chem. Soc.*, 1996, **118**, 3419.
33 A. J. Gow, D. G. Buerk and H. Ischiropoulos, *J. Biol. Chem.*, 1997, **272**, 2841.
34 N. Hogg, R. J. Singh and B. Kalyanaraman, *FEBS Lett.*, 1996, **382**, 223.
35 J. S. Stamler, D. J. Singel and J. Loscalzo, *Science*, 1992, **258**, 1898.
36 P. W. Riddles, R. L. Blakeley and B. Zerner, *Methods Enzymol.*, 1983, **91**, 49.
37 R. G. Kallen, *J. Am. Chem. Soc.*, 1971, **93**, 6227.
38 (*a*) *Lange's Handbook of Chemistry*, 15th edn., ed. J. A. Dean, McGraw-Hill, New York, 1999, p. 8.49; (*b*) S. D. Lewis, D. C. Misra and J. A. Shafer, *Biochemistry*, 1980, **19**, 6129.
39 B. C. Challis and S. A. Kyrtopoulos, *J. Chem. Soc., Perkin Trans. 1*, 1979, 299.
40 Lewis *et al.* (ref. 38*b*) report an abnormally high acidity ($pK_a < 5$) for the thiol in native BSA and a normal acidity ($pK_a = 8.88$) for the thiol in the urea-denatured protein. The low pK_a of BSA has recently been disputed and a pK_a value of 8.32 reported. See: D. R. Noble and D. L. H. Williams, *J. Chem. Soc., Perkin Trans. 2*, 2001, 13 . Without expressing any preference for either of these values, the 'old' value for native BSA has been adopted in this paper. The extent to which the choice of pK_a affects the results and conclusions is as follows. With a pK_a equal to 8.32 the rate constant for the reaction of BSA with $^{\bullet}NO$ is given by $k = 12.48\alpha\ M^{-1}\ s^{-1}$ and the steric factor (relative to cysteine) amounts to 30. The conclusions with respect to *in vivo S*-nitrosation of BSA are essentially unaffected by the choice of pK_a, as both the experimental measurements and the calculations were carried out at physiological pH.
41 D. H. Hamer, *Annu. Rev. Biochem.*, 1986, **55**, 913.
42 H. Fischer, *Mol. Phys.*, 1965, **9**, 149.
43 H. M. Lander, A. J. Milbank, J. M. Tauras, D. P. Hajjar, B. L. Hempstead, G. D. Schwartz, R. T. Kraemer, U. A. Mirza, B. T. Chait, S. C. Burk and L. A. Quilliam, *Nature*, 1996, **381**, 380.
44 P. J. Thornalley and M. Vašák, *Biochim. Biophys. Acta*, 1985, **827**, 36.
45 D. A. Wink, J. F. Darbyshire, R. W. Nims, J. E. Saavedra and P. C. Ford, *Chem. Res. Toxicol.*, 1993, **6**, 23.
46 P. C. Ford, D. A. Wink and D. M. Stanbury, *FEBS Lett.*, 1993, **326**, 1.
47 H. H. Awad and D. M. Stanbury, *Int. J. Chem. Kinet.*, 1993, **25**, 375.
48 M. Pires, M. J. Rossi and D. S. Ross, *Int. J. Chem. Kinet.*, 1994, **26**, 1207.
49 V. G. Kharitonov, A. R. Sundquist and V. S. Sharma, *J. Biol. Chem.*, 1994, **269**, 5881.
50 S. Goldstein and G. Czapski, *J. Am. Chem. Soc.*, 1995, **117**, 12078.
51 M. Grätzel, S. Taniguchi and A. Henglein, *Ber. Bunsen-Ges. Phys. Chem.*, 1970, **74**, 488.
52 Y. Takehara, T. Kanno, T. Yoshioka, M. Inoue and K. Utsumi, *Arch. Biochem. Biophys.*, 1995, **323**, 27.
53 K. Shibuki and D. Okada, *Nature*, 1991, **349**, 326.
54 T. Malinski, F. Bailey, Z. G. Zhang and M. Chopp, *J. Cereb. Blood Flow Metab.*, 1993, **13**, 355.
55 A. Meister and M. E. Anderson, *Annu. Rev. Biochem.*, 1983, **52**, 711.
56 A. Andersson, A. Isaksson, L. Brattström and B. Hultberg, *Clin. Chem. (Washington, D. C.)*, 1993, **39**, 1590.
57 T. Sugiura and H. Nakamura, *Int. Arch. Allergy Immunol.*, 1994, **103**, 341.
58 G. Stubauer, A. Giuffrè and P. Sarti, *J. Biol. Chem.*, 1999, **274**, 28128.
59 M. Boese, P. I. Mordvintcev, A. F. Vanin, R. Busse and A. Mülsch, *J. Biol. Chem.*, 1995, **270**, 29244.