Nitric Oxide 85 (2019) 28–34

Contents lists available at ScienceDirect

Nitric Oxide

journal homepage: www.elsevier.com/locate/yniox

Acceleration of the autoxidation of nitric oxide by proteins

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A B S T R A C T

Lipoproteins and lipid membranes accelerate •NO autoxidation by increasing local concentration of •NO and O_2. Although the idea that proteins could also accelerate this reaction was presented some time ago, it was largely criticized and dismissed. Herein the effect of proteins on •NO autoxidation rates was studied following •NO disappearance with a selective electrode. It was found that human serum albumin (HSA) accelerated •NO autoxidation by a factor of 9 per g/mL of protein, much less than previously suggested. The acceleration by HSA was sensitive to pH and significantly decreased at pH lower than 4.5 coincident with the acid structure transition of HSA to a partially unfolded and rigid conformation. Other proteins with different surface hydrophobicity also accelerated •NO autoxidation and it was found to depend mostly on the protein size and dynamics. Mathematical simulations were performed to assess the physiological importance of this acceleration. It was calculated that in plasma the autoxidation of •NO is accelerated 1.38 times by HSA relative to water alone, but this becomes of little relevance when whole blood is simulated because of the rapid rate of •NO consumption by red blood cells.

1. Introduction

Nitric oxide is an important signaling molecule produced biologically by the enzymes nitric oxide synthases (NOS) that are expressed differentially in different tissues. There are two constitutive NOS isoforms (type I neuronal NOS, and type III endothelial NOS) and one inducible NOS (iNOS, type II). In the vascular system predominates the endothelial NOS (eNOS) located in endothelial cells that produces •NO as a response to different stimuli and induces vasorelaxation [1]. The biological effects of •NO depend on its reactivity towards biological targets. Despite being a free radical, •NO is not very reactive and preferentially reacts with other radical species such as superoxide anion, lipid peroxyl radicals and metals in metalloproteins [2]. The reaction with ferrous heme in soluble guanylate cyclase is one of the most important signal transduction mechanisms of •NO, leading to an increase in the production of cGMP that activates cGMP-kinases and membrane ion channels, decreases intracellular calcium levels and allows smooth muscle to relax [3]. Another important reaction is that with superoxide to yield the potent oxidant peroxynitrite that not only oxidizes cysteine and methionine residues but is also capable of nitrating tyrosines, a footprint of this biological oxidant [4].

Nitric oxide also reacts with O_2, in a complex reaction yielding oxidizing and nitrosating products ($^{\bullet}NO_2$ and N_2O_3) that in water are hydrolyzed to nitrite [5,6]:

$$2\ {}^{\bullet}NO + O_2 \rightarrow 2\ {}^{\bullet}NO_2 \quad (1)$$

$$^{\bullet}NO_2 + {}^{\bullet}N\,O \rightleftharpoons N_2O_3 \quad (2)$$

$$N_2O_3 + H_2O \rightarrow 2\ NO_2^- + 2\ H^+ \quad (3)$$

The autoxidation of •NO is kinetically an overall third-order reaction, which is second-order to •NO and first-order to O_2:

$$dNO / dt = 4k_3[^{\bullet}NO]^2[O_2] \quad (4)$$

In water, the third-order rate constant k_3 has values that range between 1.5 and $3.0 \times 10^6\ M^{-2}s^{-1}$ [5–8]. Given the low concentrations of •NO attained *in vivo*, and the dependence of the rate on the square of the concentration of •NO, this reaction is considered too slow to be biologically relevant. However, it was later shown that lipid membranes and lipoproteins, which have a well-defined hydrophobic interior, accelerate this reaction by a factor of 30–300 relative to the rate in water [6,9], and it was shown to occur because of the favored partitioning of •NO and O_2 in the hydrophobic regions [6]. The solubility of these molecules in lipid membranes has been recently related to the available free volume formed as a result of molecular mismatch and thermal motions [10,11]. The autoxidation of •NO was also observed to be accelerated by mitochondria and submitochondrial particles [12].

Some years ago it was proposed that the hydrophobic core of serum albumin could accelerate the autoxidation of •NO four orders of magnitude relative to the aqueous phase [13,14]. This report was received with skepticism and the predictions on the increase of thiol nitrosation in the presence of albumin were later refuted [15]. Proteins are not as "spongy" as lipid membranes and show average densities similar to organic crystals [16]. Nevertheless, proteins are not perfectly packed

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https://doi.org/10.1016/j.niox.2019.01.014
Received 30 November 2018; Received in revised form 19 January 2019; Accepted 25 January 2019
Available online 30 January 2019
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and hydrophobic cavities are present in their structure [16] that have been shown to accommodate hydrophobic molecules [17,18]. Furthermore, proteins undergo structural fluctuations in the nanosecond range and therefore are dynamic rather than rigid structures [19]. This was evidenced by O_2 quenching of tryptophan fluorescence in a wide range of proteins, including proteins with internal tryptophans that are not accessible to the solvent [19]. The diffusion coefficients of O_2 in the protein matrix are only 20% of that in water, indicating that despite the rigid picture given by crystal structures, proteins are very dynamic [19]. Considering the presence of hydrophobic cavities and the dynamics of proteins we thought that proteins could indeed accelerate $^{\bullet}$NO autoxidation, albeit at lower rates than reported, and this issue merited further study.

In this work we determined the rate of $^{\bullet}$NO reaction with O_2 using an $^{\bullet}$NO-selective electrode, which allows direct observation of any acceleration in the $^{\bullet}$NO disappearance rates produced by proteins. We focused on human serum albumin (HSA), which is the most abundant protein in human plasma, but other proteins such as ovalbumin (OVA), lysozyme (LYS) and trypsin (TRY) were also assayed for comparison. The observed acceleration factors were correlated with several molecular properties, including the volume of the cavities within the protein structure. Finally, a mathematical model was constructed including the most important reactions of $^{\bullet}$NO that could reproduce the experimental results, and allowed us to extrapolate our findings to a biological scenario like the blood vessel, to understand the fate of the different species and the relative importance of accelerated $^{\bullet}$NO autoxidation by HSA.

2. Materials and methods

2.1. Materials

Prolinonoate was from Alexis Biochemicals (San Diego, CA). All other reagents were from Sigma (St Louis, MO). Most experiments were done in 100 mM phosphate buffer, 100 μM DTPA, pH 7.4 at 25 °C. Solutions of different pH were prepared by adding HCl to this buffer and then checking the final pH. The solution at pH 3.3 was done using 100 mM citric acid with 100 μM DTPA. Human serum albumin (HSA) was delipidated as described [20] and then equilibrated in buffer by gel filtration. Other proteins were prepared directly in buffer by weight, re-equilibrated in buffer by gel filtration and the final concentration was determined by absorbance at 280 nm. This was done to ensure exactly the same buffer composition in the different experiments. This is particularly important since the solubility of both $^{\bullet}$NO and O_2 is sensitive to the concentration of dissolved salts and would affect the association to proteins. In $^{\bullet}$NO autoxidation assays, the buffer used as reference was the same buffer used to dissolve the proteins.

2.2. $^{\bullet}$NO autoxidation assays

The disappearance of $^{\bullet}$NO by autoxidation was followed using an $^{\bullet}$NO-selective electrode coupled to an Apollo 4000 analyzer (WPI Inc, Sarasota, FL). The system has a custom-made reaction glass chamber of 1300 μL and a cap that allows insertion of the $^{\bullet}$NO probe and a capillary to inject $^{\bullet}$NO into the chamber using a gastight Hamilton Syringe. The absence of headspace and the capillary ensured that gas exchange (loss of $^{\bullet}$NO) to the environment was minimal. The chamber also includes a water jacket connected to a thermostatted circulating water bath at 25 °C. The electrode was calibrated as described [6,21] and prolinonoate was prepared as before and quantitated using the calibrated electrode [6]. The $^{\bullet}$NO autoxidation assay consisted in adding ~8 μM $^{\bullet}$NO from a concentrated solution of prolinonoate to the closed chamber containing air-equilibrated buffer (or protein solution) and registering $^{\bullet}$NO decay in time. The acceleration factor A_C (Eq. (6)) was determined for each protein using four different concentrations of protein by duplicate, in at least three repeated experiments.

2.3. Determination of cavity volume in proteins

The molecular volume and surface area of proteins was determined using the "volume calculation" tool available in the 3 V web server [22], using a probe of radius 1.5 Å, equivalent to a molecule of water. The structures were downloaded from the Protein Data Bank (RCSB PDB) and were PDB ID: 1E78 for HSA [23], PDB ID: 1S81 for trypsin [24], PDB ID: 1OVA for ovalbumin [25] and PDB ID: 1LYS for lysozyme [26]. The "solvent extraction" tool was used to determine the total volume and surface of the cavities in the structure of the different proteins, as well as to extract the 3D representation of the cavities. The outer probe and the inner probe radii were set to 1.5 and 0.5 Å, respectively, in order to discard clefts and only consider cavities within the protein that are not readily accessible to the solvent. The visualization was done using Chimera [27].

2.4. Mathematical simulations

The complete system was modeled in Copasi, that is freely available [28], using irreversible or reversible mass action with the rate constants described in Table 2. The initial concentration of most components was zero, except prolinonoate that was set to 4 μM, O_2 to 220 μM and HSA to 660 μM. The only included thiyl radical reaction was with $^{\bullet}$NO, because HSA is sterically hindered to make disulfides that can lead to superoxide [15]. The complete Copasi file is provided as supplementary material.

3. Results

3.1. Acceleration of $^{\bullet}$NO autoxidation by HSA

The autoxidation of $^{\bullet}$NO was studied after injecting 8 μM $^{\bullet}$NO from a prolinonoate stock solution to either 100 mM phosphate, 100 μM DTPA, pH 7.4 buffer or to defatted HSA solution in the same buffer. Prolinonoate is a rapid-release $^{\bullet}$NO donor that was used to ensure a correct mixing and avoid the high local concentration of a pure $^{\bullet}$NO bolus addition that could lead to experimental artifacts. The reaction was followed using an $^{\bullet}$NO -selective electrode as before [6].

Given that the $^{\bullet}$NO autoxidation rate is second-order relative to $^{\bullet}$NO, the integration of the rate equation is Eq. (5),

$$\frac{1}{[NO]} = k_{obs}t + \frac{1}{[NO]_0} \quad (5)$$

Therefore there is a linear dependence between $1/[^{\bullet}NO]$ and time, and the slope is k_{obs} (Fig. 1B, black trace). This k_{obs} stands for $4k_3[O_2]$, where O_2 is in excess (220 μM), and k_3 is the third-order rate constant for $^{\bullet}$NO autoxidation. For these experiments k_3 in buffer was $2.8 \times 10^6\,M^{-2}s^{-1}$, in agreement with previous results [5–8]. When the experiment was done in the presence of HSA, an increase in $^{\bullet}$NO autoxidation rate was observed (Fig. 1A, red trace) that is more evident in the linear fitting to the second-order reaction (Fig. 1B, red trace). The increase in $^{\bullet}$NO autoxidation rate was directly proportional to the concentration of HSA (Fig. 1C).

To evaluate the effect of HSA on the rate of $^{\bullet}$NO autoxidation, we introduced the term "A_C", that stands for *acceleration* and indicates how much the reaction rate is affected by HSA [6]:

$$k_{obs}^{HSA}/k_{obs}^{buffer} = 1 + A_C \times C_{HSA} \quad (6)$$

Therefore, we can determine the factor A_C from the slope of a secondary plot such as in Fig. 1C, and $A_C = 9\ (g/mL)^{-1}$ was obtained for HSA at 25 °C and pH 7.4.

3.2. Is it a core or a surface effect? The acid transition of HSA

HSA undergoes an important conformational change below pH 4.3, leading to an "open" form which has a higher surface area and a lower

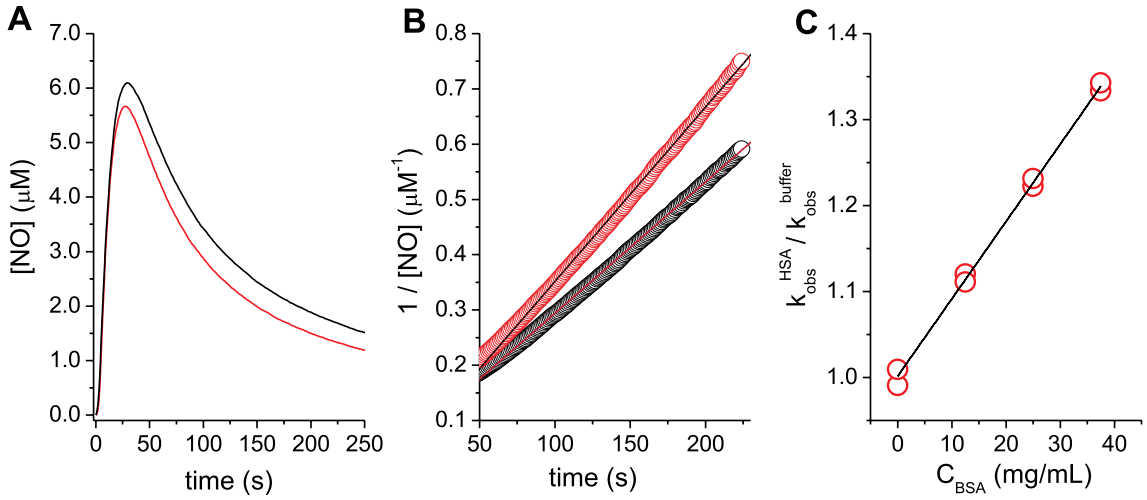

Fig. 1. Acceleration of ˙NO autoxidation by HSA. A) ˙NO autoxidation was studied by following ˙NO disappearance using an ˙NO-selective electrode in a sealed chamber with no headspace. 8 μM ˙NO was added from prolinonoate at time 0 in buffer alone (black) or in the presence of 37.5 mg/mL HSA (red). **B)** Linearization of the second-order reaction on ˙NO. The slopes are the pseudo-second-order rate constants of autoxidation (k_{obs}). The higher rate of autoxidation by HSA is observed as a greater slope. **C)** The increase in rate was proportional to protein concentration. The slope of this plot gives the acceleration factor A_C = 9 (g/mL)$^{-1}$ for HSA at 25 °C and pH 7.4.

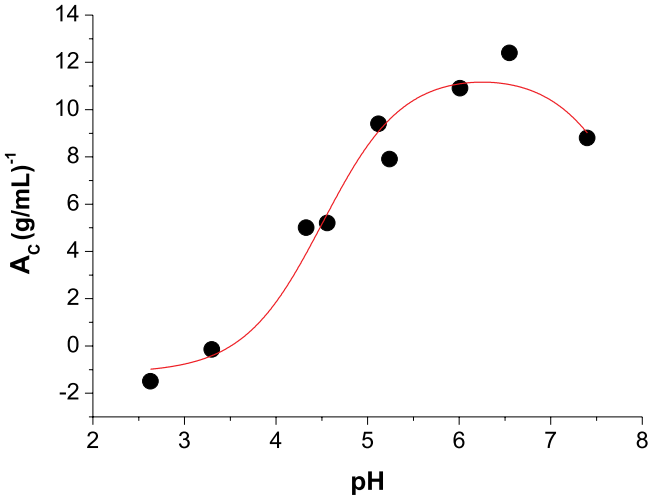

Fig. 2. Acceleration of ˙NO autoxidation by HSA at different pH. The acceleration of ˙NO autoxidation by HSA change with pH and the transitions were determined by fitting to a model with two pK_a (trace). The decrease at lower pH with a pK_a = 4.5 matched the acid transition of HSA that leads to conformational changes including an increase in surface area and decrease in volume and compressibility.

volume and compressibility [29–31]. Therefore, the importance of protein surface *vs* core effects in ˙NO acceleration could be assessed by following the ability of HSA to accelerate the autoxidation of ˙NO at different pHs. It was observed that the acceleration factor A_C slightly increased at pH 6.5 and then decreased at lower pH, with no acceleration below pH 3.5. A pK_a of 4.5 was calculated for this process that matches the pK_a for the acid transition of HSA. The basic transition occurs with pK_a = 8 [29]. Although it was not experimentally studied (because release of ˙NO from prolinonoate slows down at higher pH), it was introduced into the mathematical model and explains why acceleration is maximal at pH 6.5 (Fig. 2).

These results suggest that the acceleration of ˙NO autoxidation in the presence of HSA does not occur by adsorption to hydrophobic patches on the surface of the protein, because this surface hydrophobicity increase with the acid transition of HSA [29–31]. On the other hand, the compressibility and volume of the protein decrease when decreasing pH, in a similar manner to the observed results [30] and suggest an important role of the hydrophobic cavities and protein dynamics in accelerating ˙NO autoxidation.

3.3. Is it a core or surface effect? Comparison with other proteins

HSA has many hydrophobic surface patches that serve as binding pockets to many ligands [29]. However, the results on ˙NO autoxidation acceleration by HSA at different pHs suggested that it was not a surface effect. To further confirm this, we selected proteins that have a more polar surface than HSA, such as trypsin, lysozyme and ovalbumin, to determine whether they would be capable of accelerating ˙NO autoxidation. It was found that these proteins also accelerated ˙NO autoxidation but to different degrees (Fig. 3A and Table 1). Whereas the acceleration by ovalbumin was similar to that of HSA, the acceleration by trypsin was nearly null.

We sought for correlations between the A_C coefficients and different molecular properties like volume, density and molecular weight (Table 1). A very good correlation was found between A_C and protein adiabatic compressibility (Fig. 3B, R^2 = 0.952). This compressibility is related to fluctuations in atom packing in the protein and is an indicator of protein flexibility [32–34], underscoring the importance of protein dynamics in accelerating ˙NO autoxidation.

To correctly correlate with molecular properties, the acceleration coefficient per Molar was used ($A_M = A_C \times MW$). In this case there were generally good correlations with most properties considered. Analysis of these correlations suggest that a minimum protein volume is necessary in order to accelerate ˙NO autoxidation (Fig. 3C). Correlation with molecular volume was actually stronger than with cavity volume (R^2 = 0.88 vs 0.77, Fig. 3C and D), suggesting that protein cavities affect acceleration in a nonlinear way.

Therefore, the acceleration of ˙NO autoxidation seems to depend mainly on two factors, the size and the dynamics of the protein, and not the hydrophobic surface of the protein.

3.4. Physiological importance of ˙NO autoxidation acceleration by HSA

To understand the significance of this acceleration of ˙NO autoxidation in blood, we performed mathematical simulations of the reactions involved in ˙NO autoxidation. The reactions used for the simulation represent a minimal model and are listed in Table 2.

The model represented the experimental results very well, after adjusting some parameters (R^2 = 0.9995, Fig. 4A). In particular, the

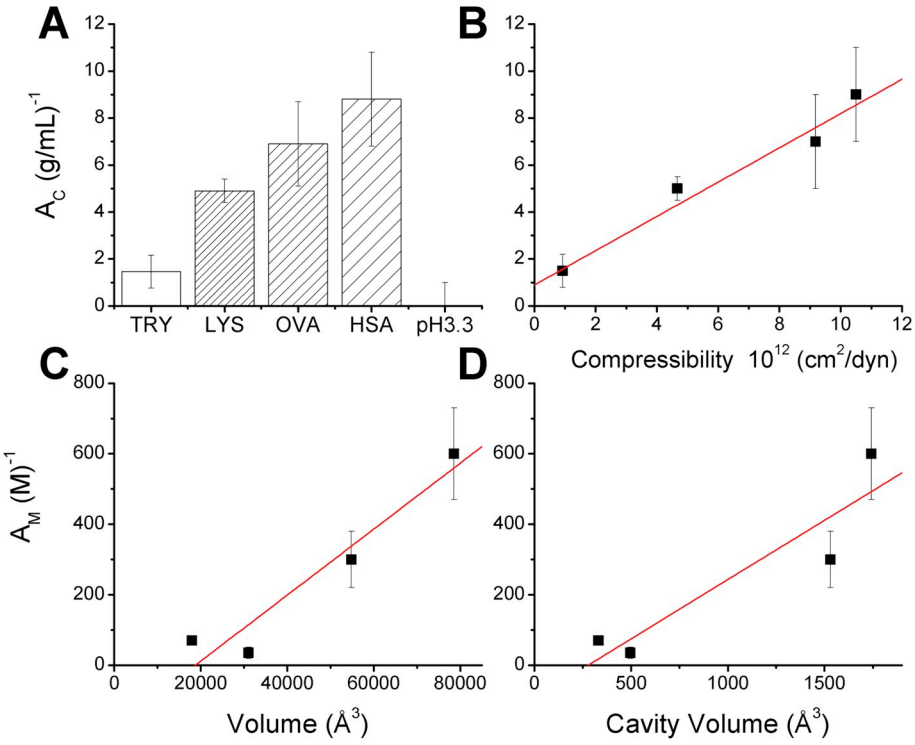

Fig. 3. A) Acceleration of ˙NO autoxidation by different proteins: trypsin (TRY), lysozyme (LYS), ovalbumin (OVA) and human seroalbumin (HSA). It can be observed that proteins that have a more hydrophilic surface than HSA are also able to accelerate the autoxidation of ˙NO. **B)** A good correlation between Ac and protein compressibility was found (R^2 = 0.952), indicating that protein dynamics is an important factor regulating acceleration. **C)** The correlation between A_M (Ac x MW) and molecular volume was not so good (R^2 = 0.88) and suggests a minimum volume is required to allow acceleration of ˙NO autoxidation. **D)** The correlation between A_M and cavity volume was poor (R^2 = 0.77), suggesting that this factor affects acceleration in a nonlinear way.

rate of injection of prolinonoate (Reaction 1 in Table 2), the rate of ˙NO release from prolinonoate (Reaction 2 in Table 2), and the exact amount of prolinonoate used (4.35 μM) were obtained by fitting the model to the experimental results of ˙NO disappearance in buffer. The rate of ˙NO autoxidation obtained by fitting the model was practically the same to the one obtained in Fig. 1 by linearization (2.7 and 2.8 × 10^6 $M^{-2}s^{-1}$, respectively). These parameters obtained by model optimization were then included to simulate the acceleration by HSA, using the experimentally determined acceleration at 37.5 mg/mL HSA (Fig. 1C), and a very good fit was obtained (R^2 = 0.9983, Fig. 4A).

Next, to simulate the acceleration of ˙NO autoxidation in plasma by HSA, we considered the concentration of HSA in plasma (42 mg/L [29]) and the acceleration factor A_C determined herein (Table 1). An overall acceleration of 1.38 times by HSA in whole plasma was calculated. The addition to the model of reactions between $^{\cdot}NO_2$, N_2O_3 and HSA (reactions 6–8 in Table 2) had no effect on the ˙NO disappearance rate, as expected for a process limited by ˙NO autoxidation. In the absence of HSA, transient concentrations of $^{\cdot}NO_2$ and N_2O_3 reached picoMolar levels, and decreased one order of magnitude after adding the reactions with HSA (Fig. 4B).

Although we did not measure it, we simulated the nitrosation of the thiol in HSA by both radical ($^{\cdot}NO_2$) and N_2O_3 pathways (reactions 6–8 in Table 2, Fig. 4C). In the absence of acceleration by protein, radical nitrosation yielded 10.3 times more nitrosothiol than N_2O_3-mediated nitrosation. After including acceleration by HSA, a slight increase in the rate of nitrosation was obtained, and the radical pathway yielded 10.6 times more nitrosated HSA than the N_2O_3 pathway, but the overall yield remains almost the same: 45 vs 46% relative to added ˙NO. Notice that the efficiency of nitrosation is high because we only considered this minimal model that is nonetheless useful to illustrate several points. For one, this accelerated nitrosation is expected to be of little consequence and difficult to assess experimentally, because there is only a small increase in the *rate (and not yield) of nitrosation*, that would be missed by experimental error.

Next we considered the whole blood scenario. In this case, ˙NO will react preferentially with oxyhemoglobin in red blood cells to form nitrate [39,40]. To include this reaction in the simulation we had to consider the limitation imposed by the localization of hemoglobin inside red blood cells, that actually lowers the rate of ˙NO decomposition

Table 2
Reactions used in the mathematical simulation.

#	Reaction	Rate constant	Ref.
1	Stock → prolinonoate	0.31 s^{-1}	This work
2	prolinonoate → 2 ˙NO	0.077 s^{-1}	This work
3	2 ˙NO + O_2 → 2 $^{\cdot}NO_2$	2.8 × 10^6 $M^{-2}s^{-1}$	[5,6,8]
3 Ac	2 ˙NO + O_2 → 2 $^{\cdot}NO_2$	3.86 × 10^6 $M^{-2}s^{-1}$	This work
4	$^{\cdot}NO_2$ + ˙NO ⟷ N_2O_3	1.1 × 10^9 $M^{-1}s^{-1}$ 8.1 × 10^4 s^{-1}	[5]
5	N_2O_3 → 2 HNO_2	9930 s^{-1}	[5,15]
6	HSA + N_2O_3 → HSA-SNO + HNO_2	6.6 × 10^7 $M^{-1}s^{-1}$	[35]
7	HSA + $^{\cdot}NO_2$ → HSA-S˙ + HNO_2	2.2 × 10^7 $M^{-1}s^{-1}$	[36]
8	HSA-S˙ + ˙NO → HSA-SNO	3 × 10^9 $M^{-1}s^{-1}$	[37]
9	˙NO → NO_3^- (RBC)	498 s^{-1}	[38]

Table 1
Acceleration factors of ˙NO autoxidation and physical properties of the proteins.

	MW (kDa)	A_C [a] (g/mL)$^{-1}$	A_M (M)$^{-1}$	Volume (Å^3)	Cavity volume (Å^3)	$\bar{\nu}$ (mL/g)	Compressibility 10^{12} (cm^2/dyn)
HSA	66.5	9 ± 2	600 ± 130	78545	1742	0.733	10.5
HSA/pH 3.3	66.5	0 ± 1	0				
OVA	42.8	7 ± 2	300 ± 80	54851	1530	0.746	9.18
TRY	23.5	1.5 ± 0.7	35 ± 16	31048	496	0.719	0.92
LYS	14.3	4.9 ± 0.5	70 ± 7	17951	331	0.712	4.67

[a] The reported A_C is the average value of three independent experiments plus minus the standard deviation.

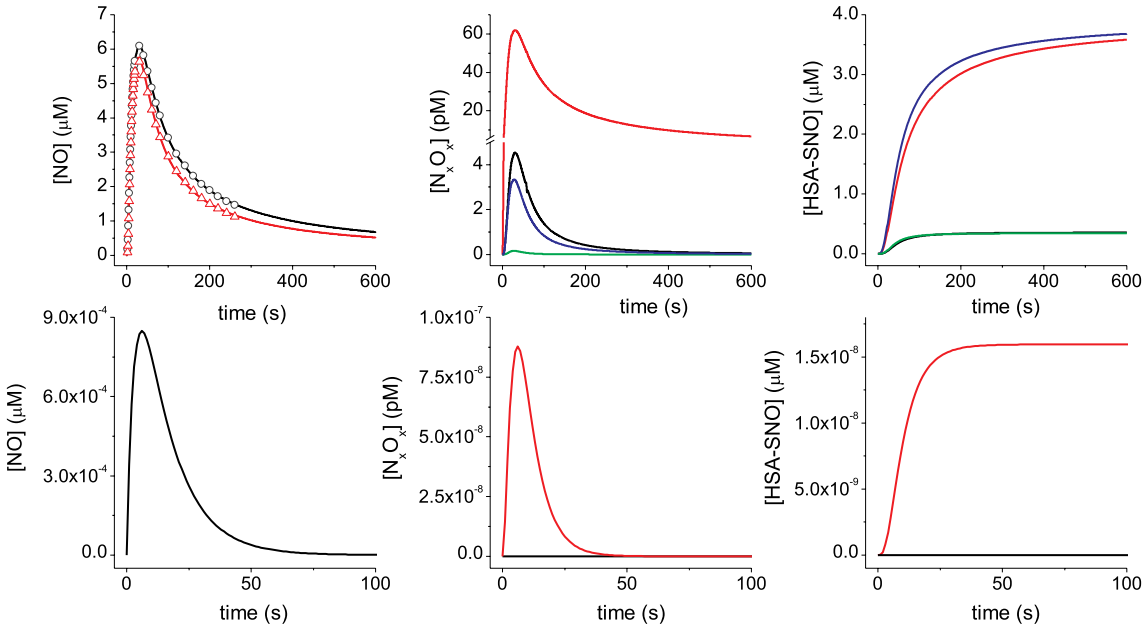

Fig. 4. Mathematical modeling of ˙NO autoxidation. A) The experimental results for ˙NO autoxidation in buffer (black circles) and in the presence of HSA (red triangles) in Fig. 1A were correctly simulated by the mathematical model (lines). **B)** The transient concentrations of nitrogen dioxide (red) and dinitrogen trioxide (black) were determined to be in the pM range, and to decrease significantly by adding reactions with HSA (blue and green, for $^{\cdot}NO_2$ and N_2O_3 respectively). **C)** The theoretical nitrosation of HSA was found to occur mostly by the radical pathway (red, reactions 7–8 in Table 2), vs N_2O_3 pathway (black, coincident with green, reaction 6 in Table 2). Introducing the acceleration by HSA led to a higher rate of nitrosation, but overall similar yields in nitrosated products (blue and green for radical and N_2O_3-mediated nitrosation, respectively). **D)** To simulate the same experiment in whole blood, we introduced the reaction with red blood cells (reaction 9 in Table 2) to the accelerated model and found that red blood cells consume virtually all of the ˙NO very rapidly. **E)** Red blood cells also cause a dramatic decrease in the formation of nitrogen oxides (references as in B). **F)** The effect of red blood cells on nitrosation is also dramatic and decreases HSA nitrosation 8 orders of magnitude (references as in C).

relative to free hemoglobin [38]. We used the rate constants determined by Liu et al. extrapolated to whole blood [38]. We still used prolinonoate as a short burst of ˙NO, that may be equated to a rapid and short release of ˙NO by cells. In this case, erythrocytes consume virtually all of the ˙NO that would be produced by prolinonoate, lowering its concentration almost 4 orders of magnitude to sub-nanoMolar (Fig. 4D). The concentration of the intermediates $^{\cdot}NO_2$ and N_2O_3 is lowered 8 orders of magnitude from picoMolar level (Fig. 4E). The concentration of nitrosated HSA is also lowered 8 orders of magnitude from microMolar level (Fig. 4F). Therefore, although the autoxidation of ˙NO is accelerated by proteins, this effect will be of little consequence in ˙NO physiology, especially in the vascular space. In the extravascular space, in the absence of red blood cells, the acceleration of ˙NO autoxidation by proteins may contribute only a very small amount of nitrosating species, as suggested by the minor effect of protein acceleration on nitrosation in Fig. 4C.

4. Discussion

This work fills a gap between the works of Nudler et al. and Keszler et al. regarding the interaction of ˙NO with proteins [13–15]. The first publication suggested that the hydrophobic core of proteins could accumulate 120 times more ˙NO than an equivalent volume of water, thus leading to micellar catalysis that was calculated to accelerate ˙NO autoxidation 1.4×10^4 fold, which in turn translated into an enhanced nitrosation of protein and low molecular thiols [13]. This claim was later refuted by Keszler et al. who analyzed the system in depth by different approaches and found no increase in the nitrosation yields of either protein or low molecular weight thiols [15]. However, no study of the kinetics of ˙NO disappearance in the presence of proteins was conducted in this work [15]. Yet, the idea that hydrophobic core of proteins could accelerate ˙NO autoxidation sounded reasonable, so we performed experiments to specifically address this issue. Our results indicate that proteins can accelerate ˙NO autoxidation but to a much lesser degree than first proposed.

Most of the volume of the proteins is occupied by the atoms of amino acids that present an atom density comparable to that of organic crystals. However, the packing of amino acids is not perfect and cavities are formed that are not directly accessible to the solvent neither occupied by any other atoms (see Figs. S1–S4). Furthermore, proteins are not completely rigid structures and are known to undergo rapid structural fluctuations (the so called “protein breathing”) [19]. As an example, it can be observed in Fig. S1 how HSA can accommodate hydrophobic ligands such as myristic acid in previously inexistent cavities. Considering this protein dynamics, it could be envisioned that proteins could indeed favor the solubility of ˙NO and O_2, increase their local concentration and accelerate the ˙NO autoxidation reaction.

Our results show that HSA and other proteins are able to accelerate ˙NO autoxidation in a manner that depends on the size and dynamics of the protein. HSA was the largest protein assessed, also the most dynamic protein according to measurements of compressibility, and the one that led to the largest acceleration of ˙NO autoxidation. Structural alterations of HSA induced by decreasing pH include a partial unfolding, an increase in surface area and a decrease in protein compressibility [29,30], and were found to decrease the acceleration of ˙NO autoxidation (Fig. 2). Besides HSA, other proteins were found to accelerate ˙NO autoxidation in a size-dependent manner (Fig. 3). These proteins also differed in their compressibility and a good correlation was found with the acceleration factor (Fig. 3B), indicating an important role of the protein structural dynamics in facilitating ˙NO and O_2 solubilization and reaction.

If we consider that this acceleration occurs by a similar mechanism to that in lipid membranes [6,9,41], then these results suggest that ˙NO and O_2 are indeed more soluble in the hydrophobic core of proteins than in buffer. However, the measurement of O_2 solubility in horse plasma actually shows that proteins do not increase O_2 solubility

relative to an isotonic solution [42]. This can be reconciled with our interpretation considering that O_2 is on average excluded from a large part of the volume occupied by the atoms of the protein but favorably located inside protein cavities that account approximately 2% of the total protein volume (Table 1). Taking into account the observed acceleration of ˙NO autoxidation by proteins and its relationship with protein size, cavities and dynamics, it appears that the reaction is not accelerated by a single cavity, but that single steps may occur in different regions of the protein. In addition, we have to consider that ˙NO autoxidation, even though is kinetically a termolecular reaction (Eqs. (1) and (4)), it actually involves two separate bimolecular steps and one homolysis [5,43]:

$$^{\cdot}NO + O_2 \rightleftharpoons ONOO^{\cdot} \quad (7)$$

$$ONOO^{\cdot} + {}^{\cdot}NO \rightarrow ONOONO \quad (8)$$

$$ONOONO \rightarrow 2\ {}^{\cdot}NO_2 \quad (9)$$

Considering the high diffusivity of ˙NO and O_2 [19,44], it is likely that the acceleration of ˙NO autoxidation by proteins can occur by the following mechanism. First, ˙NO and O_2 locate transiently in different hydrophobic cavities within the protein (energetically more favorably than in water). While diffusing to another hydrophobic cavity, both ˙NO and O_2 collide with each other in a channel that connects both cavities formed by the motion of the peptide chains, leading to the transient ONOO˙. Next, a second molecule of ˙NO that diffused to a nearby hydrophobic cavity reacts with ONOO˙ and leads to ONOONO, that splits in two $^{\cdot}NO_2$ that diffuse to the aqueous phase. This theory explains why the acceleration of ˙NO autoxidation depended greatly on protein dynamics (Fig. 3B) and size, especially why there is a minimum size of protein that favors the acceleration (Fig. 3C). Furthermore, it also explains why there is such an important decrease in A_C when HSA is partially unfolded at acid pH (Fig. 2). Although the volume of HSA stays practically the same, there are fewer hydrophobic cavities where O_2 and ˙NO can locate, and at the same time, these cavities are less connected to each other because of the higher rigidity of the protein.

To extrapolate our results to other biochemically relevant reactions such as nitrosation and to more biological scenarios such as the vascular space, we used mathematical simulations. With respect to nitrosation, the simulations were in agreement with results reported by Keszler et al. in that no significant increase in nitrosation yields was predicted, but only a minor increase in the rate of nitrosation, that would be very difficult to assess experimentally [15]. Although it has been discussed before, it is important to point out that the site of formation of nitrosating species will not necessarily favor the nitrosation of neighboring thiols, because the *rates* of nitrosation reactions (either radical or N_2O_3-mediated, reactions 6–8 in Table 2) are not limited by diffusion [41]. This means that several collisions are needed before nitrosation occurs. Considering that diffusion is at least 100 times more rapid than reaction, it is more probable that the nitrosating species will diffuse and react far from the site of production. In this context, the presence of a highly reactive thiol may be more important for an efficient nitrosation rather than proximity to a source of nitrosating species. For instance, although ˙NO autoxidation is accelerated in lipid membranes [6,9], Zhang et al. showed that cysteine residues inserted at different depths in the membrane were less nitrosated than glutathione in the aqueous phase [45]. Although it may seem unexpected, this result is explained because it is the thiolate rather than the thiol that reacts rapidly with nitrosating species, and the ionization is energetically unfavorable in the low polarity milieu of the membrane [45].

In the vascular space, the simulations show that most of the ˙NO will be rapidly consumed by red blood cells (Fig. 4D) and that the acceleration by plasma HSA is not quantitatively significant as an alternative route to ˙NO decomposition. In the extravascular space in the absence of red blood cells, the acceleration of ˙NO autoxidation by HSA may theoretically be responsible for up to 27% of the products of autoxidation such as nitrite. However, plasma has other proteins that can react with ˙NO through different mechanisms, such as ceruloplasmin, that has an important ˙NO oxidase activity [46] and may effectively compete with HSA for ˙NO, narrowing even more the possible physiological effects of the acceleration of ˙NO autoxidation by HSA.

Even though the relative importance of this protein-driven acceleration is not clear at present, the quantitative information provided here may be included in the future in more complex and realistic kinetic models of ˙NO in the vasculature, and eventually understand its role in ˙NO biology.

Acknowledgements

We are grateful to Dr Ernesto Cuevasanta and Dr Beatriz Alvarez for excellent suggestions and critical discussions.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.niox.2019.01.014.

Funding

This work was partially supported by grants C38-432 from Comisión Sectorial de Investigación Científica to AD, FCE_1_2017_1_136043 Fondo Clemente Estable from Agencia Nacional de Investigación e Innovación to MNM. MNM and AD also acknowledge Sistema Nacional de Investigadores (SNI) and Programa de Desarrollo de las Ciencias Básicas (PEDECIBA).

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