Kinetics of Peroxynitrite Reaction with Amino Acids and Human Serum Albumin*

Beatriz Alvarez‡§, Gerardo Ferrer-Sueta‡, Bruce A. Freeman**, and Rafael Radi§‡‡

From the ‡Laboratorio de Enzimología, Unidad Asociada Enzimología, and †Departamento de Fisicoquímica Biológica, Facultad de Ciencias, †Departamento de Bioquímica, Facultad de Medicina, Universidad de la República, 11800 Montevideo, Uruguay and the **Departments of Anesthesiology and Biochemistry and the UAB Center for Free Radical Biology, the University of Alabama at Birmingham, Birmingham, Alabama 35233

An initial rate approach was used to study the reaction of peroxynitrite with human serum albumin (HSA) through stopped-flow spectrophotometry. At pH 7.4 and 37 °C, the second order rate constant for peroxynitrite reaction with HSA was 9.7 ± 1.1 × 10^8 M⁻¹ s⁻¹. The rate constants for sulfhydryl-blocked HSA and for the single sulfhydryl were 5.9 ± 0.3 and 3.8 ± 0.8 × 10^8 M⁻¹ s⁻¹, respectively. The corresponding values for bovine serum albumin were also determined. The reactivity of sulfhydryl-blocked HSA increased at acidic pH, whereas plots of the rate constant with the sulfhydryl versus pH were bell-shaped. The kinetics of peroxynitrite reaction with all free l-amino acids were determined under pseudo-first order conditions. The most reactive amino acids were cysteine, methionine, and tryptophan. Histidine, leucine, and phenylalanine (and by extension tyrosine) did not affect peroxynitrite decay rate, whereas for the remaining amino acids plots of k_{obs} versus concentration were hyperbolic. The sum of the contributions of the constituent amino acids of the protein to HSA reactivity was comparable to the experimentally determined rate constant, where cysteine and methionine (seven residues in 585) accounted for an estimated 65% of the reactivity. Nitrated aromatic amino acids occurred in HSA following peroxynitrite reaction, with nitration of sulfhydryl-blocked HSA 2-fold higher than native HSA. Carbon dioxide accelerated peroxynitrite decomposition, enhanced aromatic amino acid nitration, and partially inhibited sulfhydryl oxidation of HSA. Nitration in the presence of carbon dioxide increased when the sulfhydryl was blocked. Thus, cysteine was a preferential target of peroxynitrite both in the presence and in the absence of carbon dioxide.

Peroxynitrite anion (ONOO⁻) and its conjugated acid, peroxynitrous acid (ONOOH, pK_a = 6.8) (1), are the products of nitric oxide (NO) and superoxide (O_2⁻) radical reaction (2). Peroxynitrite is a potent and versatile oxidizing and nitrating agent, and several lines of evidence point at peroxynitrite as a key biomolecule in mediating the reactivity and toxicity of superoxide and nitric oxide (3–6). Peroxynitrite anion is a relatively stable species; its hydronated form readily isomerizes to nitrate at a rate of 4.5 s⁻¹ at 37 °C (7). Target molecules are proposed to be oxidized by peroxynitrite via two main pathways. First, ground-state peroxynitrite can oxidize the substrates in a process that is first order in peroxynitrite and first order in substrate. In the second pathway, peroxynitrous acid rearranges in a rate-limiting step into a highly reactive species, which is the ultimate oxidant. This process is first order in peroxynitrite and zero order in substrate (1, 8–10).

To better understand the mechanisms of peroxynitrite cytotoxicity and its role in pathological processes, it is important to characterize its reactivity toward different biomolecules. In this sense, proteins are key targets of oxidative stress. Exposure to oxidants such as hydrogen peroxide leads to amino acid modifications and subsequent changes in protein structure and function. These alterations have been related to protein turnover, aging, and diverse pathological processes (11–13). Peroxynitrite reaction with proteins can nitrinate as well as oxidize residues. Thus, nitrotyrosine detection has been used as a probe for peroxynitrite formation in vivo (14). The reactions of peroxynitrite with free cysteine, methionine, tyrosine, and tryptophan are characterized (1, 15–19), with no data available for other amino acids. Peroxynitrite-mediated oxidation and nitrination of amino acids proceeds through both one- and two-electron mechanisms, the former leading to the formation of protein radicals (20–22). In addition to modifying amino acids, peroxynitrite can affect prosthetic groups. Metalloproteins react with peroxynitrite at rates of 10^8 to 10^7 M⁻¹ s⁻¹ (23). These reactions are among the fastest reported for peroxynitrite and can lead to reversible redox modification of metal centers, as for cytochrome c (24) or disruption of the center followed by liberation of the metal, as for aconitase and alcohol dehydrogenase (4, 25).

Human serum albumin (HSA) was used as model for probing peroxynitrite reactivity toward proteins. HSA is the most abundant protein in plasma. Its physiological roles include the maintenance of colloid osmotic pressure and the transport of different ligands. In addition, HSA may have important roles as an extracellular antioxidant, by ligating free metals and scavenging reactive species and serving as a transport molecule for nitric oxide. This well characterized 66-kDa protein has names are oxoperoxynitrate (1⁻) and hydrogen oxoperoxynitrate, respectively.

The abbreviations used are: HSA, human serum albumin; BSA, bovine serum albumin; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); dtPA, diethylthreitolaminepentaoesic acid; NEM, N-ethylmaleimide; R, sulfhydryl to albumin molar ratio.

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‡‡ To whom correspondence should be sent: Dept. de Bioquímica, Facultad de Medicina, Av. General Flores 2125, 11800 Montevideo, Uruguay. Fax: 5982-9249563; E-mail: rradi@lobhm.fmed.edu.uy.

1 The term peroxynitrite is used to refer to both peroxynitrite anion (ONOO⁻) and peroxynitrous acid (ONOOH). IUPAC recommended

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18 tyrosines, 6 methionines, 1 tryptophan, 17 sulfur bridges, and only 1 free cysteine in a total of 585 amino acids, and no prosthetic groups.

In this paper, the kinetics of peroxynitrite reaction were studied for all free l-amino acids, HSA, BSA, and their single sulfhydryls. In addition, the influence of carbon dioxide/bicarbonate on the interactions between peroxynitrite and albumin was assessed. This study enables us to rationalize peroxynitrite reactivity toward proteins on kinetic terms and also contributes to understanding the fate of peroxynitrite in the intravascular space.

EXPERIMENTAL PROCEDURES

Chemicals—Human and bovine serum albumin (fraction V) were obtained from Sigma. Merccuric chloride was obtained from Merck. To prevent metal interference, 0.1 mM diethylenetriaminepentaacetic acid (dtpa) was added to the solutions. Peroxynitrite was synthesized from hydrogen peroxide and nitrous acid as described (1, 8). Contaminating hydrogen peroxide was eliminated with manganese dioxide, and peroxynitrite concentration was determined at 302 nm ($e = 1.67 \text{ M}^{-1} \text{cm}^{-1}$) (26).

Albumin Preparation—HSA was defatted with charcoal in acid solution (27). To reduce sulfhydryls oxidized during purification and storage, HSA was treated overnight with 10 mM 2-mercaptoethanol at 4°C, and excess reductant was removed by gel filtration on Sephadex G-25 using 0.01 M potassium phosphate, 0.1 mM dtpa, pH 7.4. HSA typically was 1 mM and had a sulfhydryl to albumin molar ratio (R) of 0.6–0.8, as described previously (1). The sulfhydryl of HSA was blocked by the addition of an equimolar amount of merccuric chloride (HgCl$_2$), leaving no residual sulfhydryls. Alternatively, HSA was incubated for 30 min at 20°C with a 5-fold excess of N-ethylmaleimide (NEM), followed by gel filtration. In this case, the sulfhydryl to albumin ratio was diminished to 0.015.

Biochemical Analyses—Albumin concentration was measured by absorbance at 279 nm ($e = 0.531$ (28) and $0.667$ (29), (g/liter)$^{-1}$ cm$^{-2}$) for HSA and BSA, respectively. The molecular weights were 66,486 for HSA (30) and 66,429 for BSA (31). The amino acid compositions were determined from GenBank (accession numbers A09977 for HSA and M73993 for BSA). Sulfhydryls were quantified using 5,5'-dithiobis-(2-nitrobenzoic acid) ($e_{412} = 13.6 \text{ M}^{-1} \text{cm}^{-1}$) (32). Carbon dioxide-containing solutions were prepared immediately before the experiment and used within 10 min to minimize diffusion of CO$_2$ out of the solution (33). Nitrative tyrosine residues in HSA was assessed by measuring the increase in absorbance at 430 nm after alkalinization to pH $\geq 10$ ($e_{430} = 4.4 \text{ M}^{-1} \text{cm}^{-1}$) (17).

Kinetic Studies—The kinetics of peroxynitrite decomposition were studied in a stopped-flow spectrophotometer (Applied Photophysics, SF17MV) at 302 nm. Fits to kinetic traces were performed with the software provided with the instrument. Time-dependent spectra were obtained with a photodiode array (Applied Photophysics). All experiments were performed at 37°C, and the pH was measured before and after reactions.

Simulations—Computer simulations for a given reaction scheme and a set of rate constants were produced with Gepasi version 3.1 (34, 35).

Data Analyses—All experiments were repeated at least three times, and representative data are reported. Results are expressed as mean ± S.D. Graphics and mathematical fits to experimental data were performed with SlideWrite (Advanced Graphics Software) or Origin (Microcal Software).

RESULTS

Kinetics of Peroxynitrite Reaction with HSA and Its Single Sulfhydryl at pH 7.4 and 37°C—The usual approach to study the rate of peroxynitrite reaction with target molecules is through an integral rate method, under pseudo-first order conditions (36). Peroxynitrite decomposition then follows an exponential function versus time, and the rate constant is determined through the fit of the kinetic trace to this function. For peroxynitrite reaction with HSA, it was not possible to achieve pseudo-first order conditions, since the limiting maximal concentration of HSA solutions (about 1 mM) did not permit a 10-fold excess over peroxynitrite in stopped-flow experiments. The kinetic traces of peroxynitrite (0.25 mM) decomposition in the presence of HSA (r = 0.608) did not follow a single exponential function, since the logarithmic plots were not linear (Fig. 1, inset). Rather, the rate of peroxynitrite decay was initially faster as peroxynitrite reacted with both the single sulfhydryl residue present in substoichiometric amounts and with the other amino acids of the protein. When sulfhydryl-blocked HSA was used (pretreated with merccuric chloride), the decay of peroxynitrite was slower than for native HSA.

Hence, the kinetics of peroxynitrite-HSA reaction could not be studied through the integral rate method. Instead, an initial rate approach was used, using relatively similar concentrations of peroxynitrite and HSA. Since the absorbance at 302 nm (A) is proportional to peroxynitrite concentration and the change in albumin absorbance is negligible at this wavelength in the experimental conditions, the initial rate of peroxynitrite decomposition would be as shown in Equation 1.

$$- \frac{d[\text{ONOO}^-]}{dt} = -[\text{ONOO}^-] \frac{dA}{dt} / (A_s - A_i) = k_{\text{obs}}[\text{ONOO}^-]_0$$

where $dA/dt$ is the initial change in absorbance at 302 nm, and $A_i$ and $A_s$ are the initial and final values, respectively. Following the initial rate assumption that the concentration of reagents remains effectively constant during this period, the rate constant could be determined by Equation 2.

$$k_{\text{obs}} = \frac{-dA/dt}{(A_s - A_i)}$$

In order to ensure accuracy of the rate constant determinations, 200 absorbance measurements were acquired during the initial part of the reaction (~20% decrease in peroxynitrite absorbance), and 200 further points were acquired until more than 99.9% of peroxynitrite had decomposed. The initial slopes were determined for the period of linear decrease in absorbance, which corresponded to 10–15% of peroxynitrite decomposition. In the absence of HSA, the rate constant of peroxynitrite decomposition determined from the initial rate method compared well with the values determined from the integral rate method. For instance, the initial rate method yielded values of $k_{\text{obs}} = 1.11 ± 0.09 \text{ s}^{-1}$ (n = 7), with the integral method giving a $k_{\text{obs}}$ of 0.99 ± 0.03 s$^{-1}$. Differences in the values calculated from both methods were a maximum of 10%, with
the initial rate method systematically having a greater degree of dispersion than the integral one, as revealed by the increase in the standard deviation. In the presence of relatively high concentrations of sulfhydryl-blocked HSA, peroxynitrite decay better approximated an exponential function, due to the high overall concentration of target residues. In this case, the values determined through both kinetic approaches were in good agreement. To validate further the initial rate method, we confirmed that values of $k_{obs}$ determined at 320 nm were the same as those determined at 302 nm.

For the following reaction schemes (Equations 3–5)

$\text{ONOOH} \rightarrow \text{NO}_3^- + \text{H}^+$  \hspace{1cm} (Eq. 3)

$\text{ONOOH} + \text{HSA} \rightarrow \text{products}$  \hspace{1cm} (Eq. 4)

$\text{ONOO}^- + \text{HSA-SH} \rightarrow \text{products}$  \hspace{1cm} (Eq. 5)

$k_1$, $k_2$, and $k_3$ are the rate constants of peroxynitrite isomerization and reaction with sulfhydryl-blocked HSA and with the sulfhydryl, respectively. Defining $k'_1$, $k'_2$, and $k'_3$ as the corresponding apparent rate constants at a fixed given pH of 7.4 and 37 °C, the observed rate constant of peroxynitrite disappearance would be as shown in Equation 6.

$$k_{obs} = k'_1 + k'_2[\text{HSA}] + k'_3[\text{HSA-SH}]$$  \hspace{1cm} (Eq. 6)

Since $R$ was defined as the sulfhydryl to albumin molar ratio, then (see Equation 7)

$$k_{obs} = k'_1 + (k'_2 + k'_3)R[\text{HSA}]$$  \hspace{1cm} (Eq. 7)

As observed in Fig. 2, $k_{obs}$ determined through the initial rate approach increased linearly with HSA concentration, in agreement with Equation 7. For native HSA, the second order rate constant of peroxynitrite reaction with HSA ($R = 0.636$) at pH 7.47 ± 0.02 and 37 °C was determined to be $8.3 \pm 0.3 \times 10^3$ M$^{-1}$ s$^{-1}$. The plot was consistently observed to intercept the y axis at a value lower than that for peroxynitrite spontaneous decomposition, as noted previously for other molecules (15, 37, 38). For sulfhydryl-blocked HSA (pretreated with mercuric chloride), the slope of the plot decreased. In this case, Equation 7 simplifies to Equation 8.

From Equation 8, $k'_2$, the rate constant of peroxynitrite reaction with sulfhydryl-blocked HSA was $5.9 \pm 0.3 \times 10^3$ M$^{-1}$ s$^{-1}$. According to Equations 7 and 8, $k'_3$, the rate constant of peroxynitrite reaction with the single sulfhydryl of HSA, can be calculated by dividing the difference in the slopes with and without mercury treatment by $R$. In this way, $k'_3$ was determined to be $3.8 \pm 0.8 \times 10^3$ M$^{-1}$ s$^{-1}$. By adding the values of the rate constants with sulfhydryl-blocked HSA and with the sulfhydryl, the total rate constant of peroxynitrite reaction with HSA, assuming $R = 1$, can be calculated to be $9.7 \pm 1.1 \times 10^3$ M$^{-1}$ s$^{-1}$.

Kinetics of Peroxynitrite Reaction with BSA—The rate constants of peroxynitrite reaction with BSA at pH = 7.43 ± 0.03 and 37 °C were determined in the same way as with HSA (data not shown), and the total rate constant with BSA (assuming $R = 1$) was determined to be $7.5 \pm 0.8 \times 10^3$ M$^{-1}$ s$^{-1}$. The rate constant with the single sulfhydryl of BSA was $k'_2 = 3.9 \pm 0.5 \times 10^3$ M$^{-1}$ s$^{-1}$. This value is in good agreement with the one determined for HSA and higher than the one reported previously for BSA of 2.6–2.8 \times 10^3 M^{-1} s^{-1} (1). The rate constant for peroxynitrite reaction with sulfhydryl-blocked BSA was $k'_2 = 3.6 \pm 0.3 \times 10^3$ M$^{-1}$ s$^{-1}$, significantly lower than that for HSA and again higher than the previously reported value of $2.5 \times 10^3$ M$^{-1}$ s$^{-1}$ (1). The differences with the previous values are likely to be due to the use of an integral rate approach.

Kinetics of Peroxynitrite Reaction with the Free Amino Acids—The kinetics of peroxynitrite decomposition in the presence of all the l-amino acids was studied under pseudo-first order conditions at pH = 7.40 ± 0.05 and 37 °C. The initial peroxynitrite concentration was 0.2–0.6 mM, and the amino acid concentration ranged from 0 to 100 mM where solubility permitted. Leu, Phe, Met, and Trp were studied at lower concentrations (up to 67, 60, 25, and 3.5 mM, respectively). Tyr and cystine could not be studied because of their low solubility. The decrease in absorbance at 302 nm was monitored for more than 8 $t_{1/2}$, and absorbance versus time traces were fitted to a single exponential function in order to obtain $k_{obs}$.

For the amino acid Cys, $k_{obs}$ increased linearly with concentration as before (1). For Met, $k_{obs}$ showed a hyperbolic plus linear dependence on concentration as previously at 25 °C (16). This is the case for Trp as well (19), although in this work the low concentration range used in order to prevent interference from nitrotrotyphan absorbance did not allow us to observe the linear dependence. Ala, Arg, Asn, Asp, Gln, Glu, Gly, Ile, Lys, Pro, Ser, Thr, and Val displayed hyperbolic behavior, as exemplified for Pro in Fig. 3. His, Leu, and Phe did not affect peroxynitrite decay rate to any detectable extent in the conditions studied. This is expected for Tyr as well according to the results obtained for Phe (this work), phenol, and salicylate (18).

From the plots of $k_{obs}$ versus amino acid concentration, three parameters, $a_1$, $a_2$, and $a_3$, were adjusted by nonlinear regression to the function shown in Equation 9.

$$k_{obs} = k'_1 + \frac{a_1[a]}{a_2 + [a]} + a_3[a]$$  \hspace{1cm} (Eq. 9)

where [a] is the amino acid concentration, $a_1$ represents the difference between $k_{obs}$ at infinite ($k'_3$) and zero ($k'_1$) amino acid concentrations, $a_2$ represents the concentration of amino acid, where $k_{obs}$ is halfway between $k'_1$ and $k'_3$, and $a_3$ represents the slope of the linear portion of the plot, when present. The parameters ($a_1$, $a_2$, and $a_3$) obtained for the different amino acids are shown in Table I.

pH dependence of the Reaction between Peroxynitrite and HSA—The pH profile of the apparent rate constant of peroxynitrite decay in the presence of HSA is shown in Fig. 4.
FIG. 3. Pseudo-first order rate constant for peroxynitrite decay in the presence of proline as a representative amino acid. Peroxynitrite (0.3 mM) was mixed with increasing concentrations of proline in phosphate buffer, pH = 7.42 ± 0.01, 0.1 mM dtpa. Experimental data (mean ± S.D., n = 9) were fitted to a rectangular hyperbola.

TABLE 1
Kinetic parameters of the reaction of the free amino acids with peroxynitrite

| Amino acid | \(a_1\) | \(a_2\) | \(a_3\) |
|------------|--------|--------|--------|
| Cys        | 0      | 0      | 3.8 ± 0.8 |
| Met        | 1.29 ± 0.07 | 0.81 ± 0.11 | 0.364 ± 0.007 |
| Trp        | 0.23 ± 0.02 | 0.37 ± 0.11 | 0.037 ± 0.022 |
| Ser        | 1.03 ± 0.05 | 27.6 ± 4.1 | 0 |
| Arg        | 0.89 ± 0.04 | 24.9 ± 4.1 | 0 |
| Val        | 0.37 ± 0.01 | 10.4 ± 1.0 | 0 |
| Pro        | 0.39 ± 0.02 | 20 ± 2.6 | 0 |
| Glu        | 0.48 ± 0.03 | 26 ± 5.8 | 0 |
| Thr        | 0.77 ± 0.05 | 50 ± 15 | 0 |
| Asn        | 0.80 ± 0.11 | 66 ± 22 | 0 |
| Ala        | 0.084 ± 0.012 | 6.2 ± 4.7 | 0 |
| Glu        | 0.25 ± 0.01 | 24.6 ± 3.7 | 0 |
| Gly        | 0.65 ± 0.11 | 105 ± 35 | 0 |
| Asp        | 0.87 ± 0.05 | 175 ± 22 | 0 |
| His        | 0.21 ± 0.01 | 9.4 ± 2.1 | 0 |
| Leu        | 0 | 0 | 0 |
| Tyr        | 0 | 0 | 0 |

For sulfhydryl-blocked HSA (see Equation 10), then

\[
\begin{align*}
\frac{k_{\text{obs}}}{R[HSA]} & = \frac{k_1[H^+]}{K_{\text{obs}} + [H^+]} + \frac{k_2[H^+]_i[HSA]}{K_{\text{obs}} + [H^+]_i[HSA]} \\
& = \frac{k_1[H^+]}{K_{\text{obs}} + [H^+]} + \frac{k_2[H^+]_i[HSA]}{K_{\text{obs}} + [H^+]_i[HSA]} \\
& = \frac{k_{\text{obs}}}{R[HSA]}. \\
\end{align*}
\]

(Eq. 10)

The first term represents the spontaneous decomposition of peroxynitrous acid as first proposed by Keith and Powell (39). \(k_1\) and \(pK_{\text{obs}}\) at 37 °C were determined in a separate experiment to be 4.6 ± 0.1 s\(^{-1}\) and 6.8 ± 0.1, respectively, in agreement with previous reports (1, 7). The second term in Equation 10 represents the reaction with HSA, assuming peroxynitrous acid is the reacting species. The experimental data were fitted to Equation 10. The value of \(k_2\) determined from the fit was 14.4 ± 0.5 × 10\(^3\) M\(^{-1}\) s\(^{-1}\) and \(pK_{\text{obs}}\), the apparent \(pK_a\) of peroxynitrous acid ionization in the presence of HSA, was 7.45 ± 0.05.

For native HSA, assuming peroxynitrite anion is reacting with hydronated sulfhydryl (see Equation 11), then

\[
k_{\text{obs}} = \frac{k_1[H^+]}{K_{\text{obs}} + [H^+]} + \frac{k_2[H^+]_i[HSA]}{K_{\text{obs}} + [H^+]_i[HSA]} + \frac{k_3K_{\text{obs}}[H^+]_i[HSA]}{[K_{\text{obs}} + [H^+]_i[HSA]]^2[HSA]} \\
\]

(Eq. 11)

In order to fit the experimental data to Equation 11, the value of \(k_3\) and \(pK_{\text{obs}}\) used were those just determined for sulfhydryl-blocked HSA. Thus, \(k_3\) was 5.0 ± 0.9 × 10\(^3\) M\(^{-1}\) s\(^{-1}\) and the dissociation constant of the sulfhydryl was \(pK_{\text{obs}}\) = 8.6 ± 0.4. To confirm these values, \(k_{\text{obs}}\) for sulfhydryl-blocked HSA were subtracted from \(k_{\text{obs}}\) for native HSA at each pH and divided by the sulfhydryl concentration in order to fit to the following function shown in Equation 12.

\[
k_{\text{obs}} = \frac{k_{\text{obs}} - k_{\text{obs(blocked)}}}{[R[HSA]]} = \frac{k_1K_{\text{obs}}[H^+]_i}{[K_{\text{obs}} + [H^+]_i[HSA]]^2[HSA]} \\
\]

(Eq. 12)

Despite the relatively high degree of dispersion arising from data processing, the plot of \(k_{\text{obs}}\) as a function of pH fitted this bell-shaped function, with a maximum around pH 7.55 (Fig. 4, inset). The values of \(k_2\) and \(pK_{\text{obs}}\) were calculated to be 5.5 ± 0.5 × 10\(^3\) M\(^{-1}\) s\(^{-1}\) and 8.3 ± 0.2, respectively, and are in good agreement with those determined from the fit to Equation 11. With the values of \(k_3\) and \(pK_{\text{obs}}\) determined from Fig. 4, it can be calculated from Equation 12 that \(k_{\text{obs}}\) at pH = 7.4 would be 3.7 × 10\(^3\) M\(^{-1}\) s\(^{-1}\), in accordance with the value of 3.8 ± 0.8 × 10\(^3\) M\(^{-1}\) s\(^{-1}\) obtained experimentally at pH 7.4 in Fig. 2.

From the data reported in Fig. 4 it cannot be elucidated which the reacting species are, since the pH dependence of the kinetics of peroxynitrite anion reacting with hydronated sulfhydryl are homomorphic with those of peroxynitrous acid reacting with the thiolate. Although Equations 11 and 12 correspond to the former, it can be calculated that for peroxynitrous acid reacting with the thiolate, the pH-independent rate constant would be \(k_3 = 335 ± 39 × 10^3\) M\(^{-1}\) s\(^{-1}\).
Role of the Sulfhydryl in Protein Nitration in the Presence and Absence of Carbon Dioxide—Since the kinetic constant of peroxynitrite reaction with the single sulfhydryl can account for about 39% of the reactivity of HSA, it could be expected that the sulfhydryl would inhibit nitration of aromatic residues. HSA (0.270 mM, R = 0.526) was reacted with peroxynitrite (1 mM) in the stopped-flow spectrophotometer, and time-dependent spectra were recorded throughout the reaction period (Fig. 5). Exposure of native HSA to peroxynitrite led to a minimal change in absorbance in the 400–450 nm range. As expected, the time required for 50% decrease in peroxynitrite absorbance (t_{1/2}) diminished to ~46% in the presence of HSA compared with that of peroxynitrite decomposition alone (0.76 ± 0.03 s, n = 4). With sulfhydryl-blocked HSA, the t_{1/2} decreased instead to ~55%, whereas the absorbance at 430 nm increased with a comparable t_{1/2}, suggesting aromatic residues were being nitrated. To estimate the increase in nitration better, absorbance at 430 nm was measured after alkalinization to pH ≈ 10 in a parallel experiment (Table II). Blockage of the sulfhydryl increased nitration 2-fold. This increase could not be attributed to mercury ions catalyzing the reaction, since controls showed no increase in HSA nitration with mercury treatment as opposed to NEM treatment (data not shown).

Carbon dioxide reacts with peroxynitrite at a rate of $5.8 \times 10^4 \text{m}^{-1} \text{s}^{-1}$ at 37 °C (40, 41). The adduct formed (ONOOCO$_2$) can react with target molecules or recycle to carbon dioxide, thus redirecting peroxynitrite reactivity (41–44). In the presence of 10 mM added sodium bicarbonate, where the concentration of carbon dioxide in equilibrium with it would be 0.48 mM (apparent pK$_a$ = 6.1), peroxynitrite decomposition was accelerated, and the t$_{1/2}$ was 0.028 ± 0.002 s. Carbon dioxide has been reported to inhibit sulfhydryl oxidation and enhance nitration of aromatics (19, 41, 45, 46). Indeed, when 10 mM sodium bicarbonate was added, sulfhydryl oxidation was inhibited by 50% whereas nitration increased 4-fold, with the t$_{1/2}$ of nitration ~0.017 s (Fig. 5 and Table II). In the presence of carbon dioxide, aromatic nitration of sulfhydryl-blocked HSA also increased as compared with native HSA, with nitration 25% higher when the sulfhydryl was blocked (Fig. 5 and Table II).

**DISCUSSION**

In this paper, we report the kinetics of peroxynitrite reaction with amino acids and human serum albumin.

**TABLE II**

| Condition | Mole SH/mol HSA | Mole nitro-Tyr/mol HSA |
|-----------|-----------------|-----------------------|
| Without added NaHCO$_3$ |              |                       |
| Native HSA | 0.089 ± 0.037          | 0.085 ± 0.007          |
| Sulfhydryl-blocked HSA | 0° | 0.159 ± 0.007          |
| With 10 mM added NaHCO$_3$ |              |                       |
| Native HSA | 0.363 ± 0.015          | 0.356 ± 0.007          |
| Sulfhydryl-blocked HSA | 0° | 0.437 ± 0.015          |

* Treatment with mercuric chloride left no residual sulfhydryls.

For HSA, the second order rate constant for reaction with peroxynitrite at pH 7.4 and 37 °C was 9.7 ± 1.1 × 10^8 m$^{-1}$ s$^{-1}$ (assuming R = 1). With the single sulfhydryl the rate constant was 3.8 ± 0.8 × 10^8 m$^{-1}$ s$^{-1}$, which can be compared with that found for BSA and those reported for cysteine (4.5 × 10^8 m$^{-1}$ s$^{-1}$) (1, 15) and glutathione (1.35 × 10^8 m$^{-1}$ s$^{-1}$) (7). The plot of the rate constant with the sulfhydryl versus pH was bell-shaped (Fig. 4). The ionization constant of the sulfhydryl (pK$_a$ = 8.6 ± 0.4) can be compared with that of cysteine (8.36) (47), glutathione (8.75) (48), and BSA (8.0–8.5) (1, 48). The reactivity of sulfhydryl-blocked HSA with peroxynitrite increased as pH decreased, suggesting peroxynitrous acid as the main reactive species (Fig. 4). However, if peroxynitrous acid was the only reacting species toward a single target, the expected pK$_a$ for the reaction would be 6.8, as for tryptophan (19). The increase in the pK$_a$ for peroxynitrite reaction with sulfhydryl-blocked HSA to 7.45 ± 0.05 could be due to the fact that either unhydrated HSA residues were reacting with peroxynitrous acid or that peroxynitrite anion was also reacting. In addition, the pH dependence of the reactivity of HSA toward peroxynitrite may be affected by conformational changes of albumin (49), but spin trapping of thyl radicals formed during BSA reaction with peroxynitrite suggest that the environment of the cysteine is not critically changed at pH lower than 9 (20).

The parallel study of peroxynitrite reaction kinetics with free...
amino acids showed that the most reactive amino acids were Cys, Met, and Trp. The other amino acids were 1–3 orders of magnitude less reactive, as calculated from Equation 9 and Table I. The amino acids that reacted slowly with peroxynitrite could possibly still have been modified, by reaction with highly reactive species formed from peroxynitrite in rate-limiting steps. Reaction of these species with tyrosine are likely to lead to tyrosine nitration (17, 18).

We estimated the contribution of the individual amino acids to the increase in peroxynitrite decomposition rate by HSA or BSA (Table III). The rate constants predicted for HSA and BSA from the sum of the individual contributions compared well with the experimental values. In fact, they were 30–40% higher, since it is likely that primary, secondary, and tertiary structure of proteins will affect reactivity, for instance by diminishing the accessibility of reactive amino acids. These observations indicate that it is possible to make rough predictions about peroxynitrite reactivity toward a certain protein from its amino acidic composition. In addition to the presence of prothleteic groups, these data reveal that the most relevant factor in protein reactivity would be usually the amount of Cys and Met. As for most proteins Trp is not a common amino acid, its contribution to overall reactivity will be often minor. In HSA, the sulfur-containing amino acids (seven residues in a total of 585) accounted for 65% of the reactivity of HSA toward peroxynitrite (Table III). Although the other amino acids were much less reactive, their high content explains net contributions to HSA reactivity.

The critical role of the single free cysteine was evident in the fact that nitration increased in sulfhydryl-blocked HSA. This increase may in part be due to simple kinetic competition of the sulfhydryl for reaction with peroxynitrite. Computer simulation showed, however, that kinetic competition was not sufficient to explain the increase in nitration, suggesting that additional mechanisms may be operative, such as the sulfhydryl acting as a radical sink (50).

In the presence of carbon dioxide nitration was enhanced due to ONOO⁻CO₂⁻ or its secondary intermediates being good nitrat- ing species (Fig. 5). As tyrosine nitration by peroxynitrite in HSA is a rather low yield process (Table II), the presence of CO₂ in biological systems provides a way to promote nitration processes that reflect peroxynitrite formation and reactions. Remarkably, under conditions that most peroxynitrite (90%) will be forming the adduct with carbon dioxide, nitration was further enhanced in sulfhydryl-blocked HSA (Fig. 5C), suggesting that the adduct or its secondary products are reacting with the sulfhydryl, as proposed previously (41, 46), with this scavenging reaction preventing aromatic nitration.

Herein, we have established a link between peroxynitrite reactivity with HSA and its constituent amino acids. In addition, since HSA is the principally oxidizable protein of plasma, and peroxynitrite is produced by vascular cells at increased rates during inflammation (51), it is likely that this work will also provide insights into the molecular mechanisms of vascular injury.

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Note Added in Proof—Recent observations in our laboratory with lower peroxynitrite concentrations than reported herein revealed that

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**Table III**

*Contribution of the individual amino acids to peroxynitrite reaction with HSA and BSA*

| Amino acid | Residues | s⁻¹ | Residues | s⁻¹ |
|-----------|----------|-----|----------|-----|
| Cys       | 1        | 1.52| 1        | 1.52|
| Met       | 6        | 1.84| 4        | 1.44|
| Trp       | 1        | 0.134| 2       | 0.187|
| Ser       | 24       | 0.266| 28      | 0.297|
| Arg       | 24       | 0.248| 23      | 0.241|
| Val       | 41       | 0.226| 36      | 0.215|
| Pro       | 24       | 0.126| 28      | 0.14 |
| Glu       | 62       | 0.234| 59      | 0.228|
| Thr       | 28       | 0.141| 33      | 0.161|
| Asn       | 17       | 0.075| 14      | 0.063|
| Lys       | 59       | 0.067| 59      | 0.063|
| Ala       | 62       | 0.124| 47      | 0.121|
| Gln       | 20       | 0.046| 20      | 0.046|
| Gly       | 12       | 0.024| 16      | 0.031|
| Asp       | 36       | 0.051| 40      | 0.056|
| Ile       | 8        | 0.053| 14      | 0.078|
| Phe       | 31       | 0      | 27      | 0     |
| His       | 16       | 0      | 17      | 0     |
| Leu       | 61       | 0      | 61      | 0     |
| Cys-Cys   | 34       | 0      | 34      | 0     |
| Tyr       | 18       | 0      | 20      | 0     |
| Total (s⁻¹) | 585      | 5.174| 583     | 4.886|
| Without Cys (s⁻¹) | 584   | 3.654| 583     | 3.366|

\[ k_2 \text{ (M}^{-1}\text{s}^{-1}) \]

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Assumed not to alter peroxynitrite decomposition rates.
the parameter a₁ in Table I was often decreased. This affirms that Cys, having an a₁ = 0, will be an even more significant component of protein reactivity with peroxynitrite.

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