Peroxynitrite Isomerization Catalyzed by His64 Myoglobin Mutants

Susanna Herold,*,† Toshitaka Matsui,‡ and Yoshihito Watanabe‡

Laboratory of Inorganic Chemistry, Universitätsstrasse 6, ETH Zürich, CH-8092 Zürich, Switzerland
Institute for Molecular Science Myodaiji, Okazaki 444-8585, Japan

Received January 12, 2001

Peroxynitrite,[1] a strong oxidizing and nitrating agent that can be formed in vivo from the nearly diffusion-controlled reaction of nitrogen monoxide with superoxide, has attracted increasing interest over the past decade.[2,3] Because of the instability of peroxynitrite under physiological conditions,[3] the detection of 3-nitrotyrosine (NO_2-Tyr)[4] has become a biochemical marker for the presence of peroxynitrite in pathophysiological processes. The biological significance of tyrosine nitration is a subject of great interest, because extensive evidence supports the formation of NO_2-Tyr in vivo in diverse pathological conditions.[5] These observations have led to the search for a drug that can scavenge this powerful oxidizing and nitrating agent. Indeed, the ability to intercept and decompose peroxynitrite may represent a novel and critical point of therapeutic intervention in diseases associated with the overproduction of nitrogen monoxide and superoxide.

Recently, it has been shown that a series of water-soluble iron(III)porphyrin complexes catalyze the isomerization of peroxynitrite to nitrate at physiological relevant pH and temperature.[6] In particular, $[Fe^{III}(TMPS)]^{7-}$ protects cells in culture from exogenously added peroxynitrite and cytoprotection correlates well with a reduction in the NO_2-Tyr content of released cytosolic proteins.[7] Moreover, it has been reported that $[Fe^{III}(TMPS)]^{7-}$ reduces ischemia/reperfusion injury via direct scavenging and/or reduction of peroxynitrite production during reperfusion.[8] Despite these promising results, recent studies have shown that iron(III)-porphyrin complexes also catalyze the nitration as well as the oxidation of added phenolic compounds.[9] It is conceivable that nitrogen dioxide and oxoiron(IV), generated from the one-electron reduction of peroxynitrite by iron(III), may be the species responsible for nitration.[9] Interestingly, the iron(III) form of myoglobin (metMb) is the only heme-containing protein studied up to now in which the heme-center does not appear to react with peroxynitrite.[10] In the present work we show that the reactivity of metMb toward peroxynitrite is regulated by the presence of the distal histidine, which partly blocks the active site and stabilizes, via a strong hydrogen bond, the water ligand coordinated to the iron.

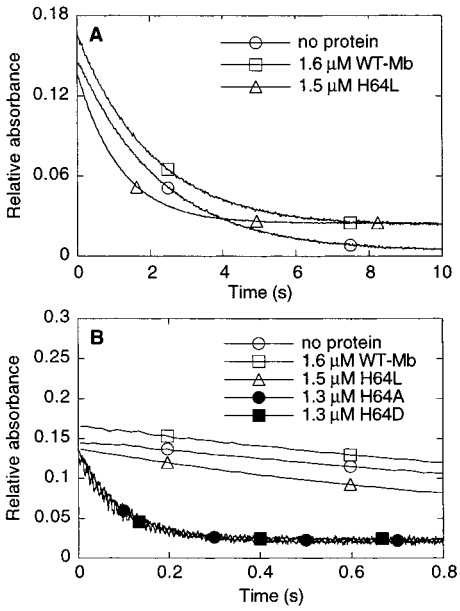

Figure 1. Time courses measured by stopped-flow spectroscopy at 302 nm for the decomposition of 100 μM peroxynitrite in 0.05 M phosphate buffer at pH 7.0, 20 °C: (A) in the absence of added proteins, with added WT-metMb, or H64L-metMb; (B) same as in part A plus added H64A-metMb, or H64D-metMb.

We have studied by stopped-flow spectroscopy[11] the decomposition rate of peroxynitrite in the presence of three different sperm whale myoglobin mutants in which the distal histidine has been replaced with alanine (H64A), leucine (H64L), or aspartic acid (H64D).[12] The reaction was studied by following the absorbance changes at 302 nm, the characteristic absorbance maximum of peroxynitrite.[13] As depicted in Figure 1A, in 0.05 M phosphate buffer at pH 7.0 and 20 °C peroxynitrite decays in about 10 s. In the presence of 0.016 equiv of wild-type horse heart iron(III)myoglobin (relative to peroxynitrite), the lifetime of peroxynitrite is almost unchanged, whereas addition of 0.015 equiv of the H64L mutant slightly accelerates its decomposition rate. In contrast, in the presence of only 0.013 equiv of either H64A or H64D, peroxynitrite disappears in less than 500 ms (Figure 1B).

As shown in Figure 2, for all four proteins studied the observed peroxynitrite decay rates increase linearly with increasing protein concentration indicating a first-order dependence on the protein. The values for k_{cat} at pH 7.4 and 20 °C obtained from the linear fits (Figure 2) are as follows: $(1.4 \pm 0.1) \times 10^4$ (WT-Mb), (6.7

* To whom correspondence should be addressed. E-mail: herold@inorg.chem.ethz.ch.
† Laboratory of Inorganic Chemistry.
‡ Institute for Molecular Science.

(1) The recommended IUPAC nomenclature is oxoperoxonitrate(1−) for peroxynitrite and hydrogen oxoperoxonitrate for peroxynitrous acid. The term peroxynitrite is used in the text to refer generically to both oxoperoxonitrate-(1−) ($ONOO^-$) and its conjugate acid, hydrogen oxoperoxonitrate (ONOOH).

(2) Beckman, J. S. *Nature* **1990**, *345*, 27−28.

(3) For a recent review see: Koppenol, W. H. In *Metals in Biology*; Siegel, H., Ed.; Marcel Dekker: New York, 1999; pp 597−619.

(4) Abbreviations used: His = histidine; Mb = myoglobin; metMb = iron(III)myoglobin; NO_2-Tyr = 3-nitrotyrosine; TMPS = 5,10,15,20-tetrakis-(2,4,6-trimethyl-3,5-sulfonatophenyl)porphyrin; TMPyP = 5,10,15,20-tetrakis-(*N*-methyl-4′-pyridyl)porphyrin; WT-metMb = horse heart wild type met-myoglobin.

(5) For a recent review see: Ischiropoulos, H. *Arch. Biochem. Biophys.* **1998**, *356*, 1−11.

(6) (a) Stern, M. K.; Jensen, M. P.; Kramer, K. *J. Am. Chem. Soc.* **1996**, *118*, 8735−8736. (b) Lee, J.; Hunt, J. A.; Groves, J. T. *J. Am. Chem. Soc.* **1998**, *120*, 7493−7501.

(7) (a) Salvemini, D.; Wang, Z.-Q.; Stern, M. K.; Currie, M. G.; Misko, T. P. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 2659−2663. (b) Misko, T. P.; Highkin, M. K.; Veenhuizen, A. W.; Manning, P. T.; Stern, M. K.; Currie, M. G.; Salvemini, D. *J. Biol. Chem.* **1998**, *273*, 15646−15653.

(8) Cuzzocrea, S.; Misko, T. P.; Constantino, G.; Mazzon, E.; Micali, A.; Caputi, A. P.; Macarthur, H.; Salvemini, D. *FASEB J.* **2000**, *14*, 1061−1072.

(9) (a) Balavoine, G. G. A.; Geletii, Y. V.; Bejan, D. *NITRIC OXIDE: Biol. and Chem.* **1997**, *1*, 507−521. (b) Crow, J. P. *Arch. Biochem. Biophys.* **1999**, *371*, 41−52.

(10) (a) Martinez, G. R.; Di Mascio, P.; Bonini, M. G.; Augusto, O.; Briviba, K.; Sies, H.; Maurer, P.; Röthlisberger, U.; Herold, S.; Koppenol, W. H. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 10307−10312. (b) Exner, M.; Herold, S. *Chem. Res. Toxicol.* **2000**, *13*, 287−293. (c) Alayash, A. I.; Brockner Ryan, B. A.; Cashon, R. E. *Arch. Biochem. Biophys.* **1998**, *349*, 65−73.

(11) For details on the stopped-flow apparatus and ion chomatographer used see: Herold, S. *Arch. Biochem. Biophys.* **1999**, *372*, 393−398.

(12) The H64A, H64L, and H64D sperm whale myoglobin mutants were prepared according to: Matsui, T.; Ozaki, S.; Watanabe, Y. *J. Am. Chem. Soc.* **1999**, *121*, 9952−9957.

(13) Peroxynitrite was prepared according to: Koppenol, W. H.; Kissner, R.; Beckman, J. S. *Methods Enzymol.* **1996**, *269*, 296−302.

10.1021/ja010111d CCC: $20.00
Published on Web 04/06/2001

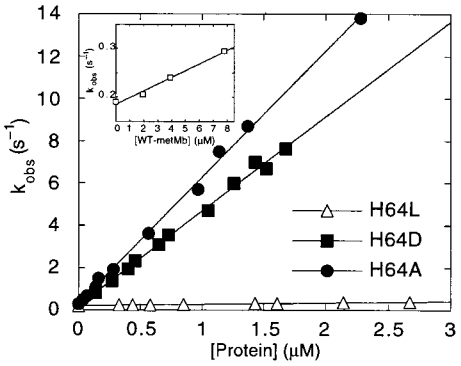

Figure 2. Plots of k_{obs} versus protein concentration for the protein-catalyzed decomposition of peroxynitrite in 0.05 M phosphate buffer at pH 7.4 and 20 °C. The second-order rate constants resulting from the linear fits depicted are given in the text.

Table 1. Percentage of Yield of Nitrate and Nitrite from the Decomposition of 159 μM Peroxynitrite in 0.05 M Phosphate Buffer at pH 7.4 (20 °C) in the Absence and the Presence of Added H64A

product	no protein	0.2 μM H64A	1.6 μM H64A
NO_3^-	88	93	99
NO_2^-	13	7	<1

$\pm$ 0.2) $\times$ 10^4 (H64L), (4.5 $\pm$ 0.1) $\times$ 10^6 (H64D), and (6.0 $\pm$ 0.1) $\times$ 10^6 M^{-1} s^{-1} (H64A). The rates for H64A and H64D are among the largest values measured for reactions with peroxynitrite[3] and are comparable to those of the most efficient iron(III)-porphyrin catalysts (2.2 $\times$ 10^6 M^{-1} s^{-1} for $[Fe^{III}(TMPyP)]^{5+}$ at pH 7.0 and 37 °C).[6a]

The value of k_{cat} obtained for the reaction of peroxynitrite with H64A was found to increase with decreasing pH. At pH 6.9 we obtained (1.3 $\pm$ 0.1) $\times$ 10^7 M^{-1} s^{-1}, whereas at pH 7.8 k_{cat} = (2.0 $\pm$ 0.2) $\times$ 10^6 M^{-1} s^{-1}. These data suggest that peroxynitrous acid (HOONO) might be the species that reacts with the protein in our system. Alternatively, the ionization of one or more groups in the protein could also be responsible for the observed pH dependence.

As summarized in Table 1, analysis by ion chromatography[11] of the nitrogen-containing products revealed that in the presence of 0.01 equiv of H64A peroxynitrite isomerizes completely to nitrate. Decay of peroxynitrite under the same conditions but in the absence of the protein yields about 13% nitrite. This result indicates that the myoglobin mutants studied are efficient peroxynitrite isomerases.

To evaluate whether the studied Mb mutants can also protect against nitration of tyrosine, we determined the yield of NO_2-Tyr formed by the reaction of peroxynitrite with added free tyrosine in the presence of H64A, H64L, or H64D. In contrast to the iron(III)porphyrin complexes,[9] the myoglobin mutants efficiently prevent nitration of added tyrosine. As shown in Table 2, HPLC analyses[14] revealed that addition of 0.01 equiv of H64A or H64D (relative to tyrosine) lowered the yield of NO_2-Tyr to less than 10% of the amount found in the absence of the proteins. In the presence of 0.05 equiv of H64A the nitration was prevented almost completely. The efficiency of the protection reaction correlates with the value of k_{cat}. In the presence of 0.01 equiv of WT-metMb the relative yield of NO_2-Tyr was still 87%.

(14) For details on the HPLC analysis see: Herold, S.; Exner, M.; Nauser, T. *Biochemistry* **2001**, *40*, 3385−3395.

Table 2. Relative Yield[a] of NO_2-Tyr Formed from the Reaction of 200 μM Peroxynitrite with 100 μM Tyrosine (in 0.05 M phosphate buffer at pH 7.4 and 20 °C) in the Presence of Different Concentrations of Proteins and (last lane) 1.2 mM CO_2

[protein] (μM)	WT-Mb	H64L	H64D	H64A
0	100	100	100	100
1	86	67	7	7
5	73	30	4	3
5 (1.2 mM CO_2)	85	54	7	4

[a] Relative yield = (yield with added protein/yield with no protein) $\times$ 100%.

Preliminary results show that H64A is an efficient scavenger also in the presence of 1.2 mM CO_2 (k_{cat} = (9.6 $\pm$ 0.2) $\times$ 10^6 M^{-1} s^{-1}, at pH 7.3 and 20 °C). As shown in Table 2, 0.05 equiv of H64A prevent the nitration of tyrosine almost completely also in the presence of 1.2 mM CO_2.

In contrast to the reaction of peroxynitrite with iron porphyrin complexes, which results in the rapid formation of the oxoiron(IV) form, no change was observed in the UV−vis spectrum of the protein solutions mixed with peroxynitrite (data not shown). This observation indicates that either isomerization proceeds via a different mechanism which does not involve the oxoiron(IV) species or that the reduction of this intermediate is very rapid and its formation is the rate-determining step. We have recently shown that a peroxynitrito-metMb complex is formed as an intermediate of the nitrogen monoxide-mediated oxidation of oxyMb and can partly be stabilized under alkaline conditions.[15] Attempts were made to identify a similar intermediate in the course of the reaction of peroxynitrite with the Mb mutants, but also under alkaline conditions (pH 9.5) no change was observed in the UV−vis spectrum.

The significant difference in the reactivity of peroxynitrite toward WT-metMb and the His64 mutants studied is likely to arise from the relief of steric hindrance imposed by the imidazole side chain and the rupture of the hydrogen bond to the coordinated water ligand. Indeed, it has been shown that the rate-determining step for azide binding to metMb is the dissociation of coordinated water and that this reaction proceeds at a significantly larger rate with the H64A mutant.[16] The two most efficient peroxynitrite isomerases, the mutants H64A and H64D, have a water ligand bound to the iron whereas H64L is 5-coordinate. Therefore, our results suggest that once the H-bond which stabilizes the coordinated water is ruptured the reactivity toward peroxynitrite is regulated mostly by steric factors.

In conclusion, in this work we have shown that two His64 metMb mutants are extremely active catalysts for the isomerization of peroxynitrite to nitrate as well as efficient protecting agents against peroxynitrite-mediated nitration of free tyrosine under physiological conditions. Therefore, these proteins might find an application as scavengers against peroxynitrite-mediated damage. More studies are needed, and are in course in our laboratory, to elucidate the mechanism of action of these catalysts, in particular to explain the differences in reactivity between the Mb mutants and the iron(III)porphyrin complexes.

Acknowledgment. We thank Michael Exner for his help with the HPLC measurements. These studies were supported by the ETH Zürich.

JA010111D

(15) Herold, S. *FEBS Lett.* **1999**, *443*, 81−84.

(16) Brancaccio, A.; Cutruzzola, F.; Travaglini-Allocatelli, C.; Brunori, M.; Smerdon, S. J.; Wilkinson, A. J.; Dou, Y.; Keenan, D.; Ikeda-Saito, M.; Brantley, R. E., Jr.; Olson, J. S. *J. Biol. Chem.* **1994**, *269*, 13843−13853.