

# Superoxide-mediated Formation of Tyrosine Hydroperoxides and Methionine Sulfoxide in Peptides through Radical Addition and Intramolecular Oxygen Transfer\*<sup>§</sup>

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The chemistry underlying superoxide toxicity is not fully understood. A potential mechanism for superoxide-mediated injury involves addition to tyrosyl radicals, to give peptide or protein hydroperoxides. The rate constant for the reaction of tyrosyl radicals with superoxide is higher than for dimerization, but the efficiency of superoxide addition to peptides depends on the position of the Tyr residue. We have examined the requirements for superoxide addition and structurally characterized the products for a range of tyrosyl peptides exposed to a peroxidase/O<sub>2</sub><sup>-</sup> system. These included enkephalins as examples of the numerous proteins and physiological peptides with N-terminal tyrosines. The importance of amino groups in promoting hydroperoxide formation and effect of methionine residues on the reaction were investigated. When tyrosine was N-terminal, the major products were hydroperoxides that had undergone cyclization through conjugate addition of the terminal amine. With non-N-terminal tyrosine, electron transfer from O<sub>2</sub><sup>-</sup> to the peptide radical prevailed. Peptides containing methionine revealed a novel and efficient intramolecular oxygen transfer mechanism from an initial tyrosine hydroperoxide to give a dioxygenated derivative with one oxygen on the tyrosine and the other forming methionine sulfoxide. Exogenous amines promoted hydroperoxide formation on tyrosyl peptides lacking a terminal amine, without forming an adduct. These findings, plus the high hydroperoxide yields with N-terminal tyrosine, can be explained by a mechanism in which hydrogen bonding of O<sub>2</sub><sup>-</sup> to the amine increases its oxidizing potential and alters its reactivity. If this amine effect occurred more generally, it could increase the biological reactivity of O<sub>2</sub><sup>-</sup> and have major implications.

Free radical-mediated oxidative damage occurs in numerous diseases and is thought to contribute to the aging process. The primary radical generated by the reduction of oxygen is superoxide (O<sub>2</sub><sup>-</sup>), a relatively benign radical that nevertheless must be removed by superoxide dismutases (SODs)<sup>2</sup> for an organism to

survive in an aerobic environment (1). A number of potentially damaging reactions of O<sub>2</sub><sup>-</sup> have been identified (1–4). One of these, which has received relatively little attention, is the addition of O<sub>2</sub><sup>-</sup> to other radicals to form hydroperoxides (5, 6). This reaction has been shown to occur readily with tyrosine and Tyr-containing dipeptides, resulting in the formation of tyrosine hydroperoxides (5–7). Hydroperoxides are potentially damaging reactive oxygen species. Formation on proteins can result in detrimental structural and functional changes (8). Protein hydroperoxides are also oxidants that can injure other biomolecules.

Tyrosyl radicals are generated in many physiological situations and proteins are major targets for reactive oxidants (9). In proteins exposed to free radicals, regardless of the initial site of attack, the resultant radical commonly localizes to Tyr (10–13). Tyrosyl radicals are also produced from tyrosyl peptides through the action of peroxidases such as myeloperoxidase, and are generated during the catalytic cycle of enzymes such as ribonucleotide reductase and cyclooxygenase (14). Tyrosyl radicals undergo a variety of subsequent reactions. They readily dimerize to form dityrosine, which has been well documented as a product of oxidative injury (15, 16). Another oxidative biomarker, nitrotyrosine, is also formed via tyrosyl radicals (4, 15, 17). However, one of their most favored reactions is with O<sub>2</sub><sup>-</sup> (5, 7, 18, 19). The reaction has a rate constant several times higher than that for dimerization (7, 20) and is favored over dityrosine formation in situations where both tyrosyl and O<sub>2</sub><sup>-</sup> radicals are generated (7, 20).

The reaction of O<sub>2</sub><sup>-</sup> with phenoxyl radicals results in either repair of the parent phenol (reaction 2, Fig. 1*b*) or addition to form a hydroperoxide (reaction 3). With tyrosine, most of the O<sub>2</sub><sup>-</sup> reacts by addition (7, 20). The structure of tyrosine hydroperoxide has not been determined directly but inferred from NMR studies of the corresponding monoxide derivative formed by slow decomposition (7). These were shown to be bicyclic compounds formed by conjugate addition of the amino

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<sup>2</sup> The abbreviations used are: SOD, superoxide dismutase; YM, tyrosine-methionine; YM-S=O, tyrosine-methionine sulfoxide; MY, methionine-ty-

rosine; YG, tyrosine-glycine; GY, glycine-tyrosine; YGGFM, Met-Enk, methionine-enkephalin; YGGFL, Leu-Enk, leucine-enkephalin; YPFF, Endo2, endomorphin 2; GYGGFM, Gly-Met-Enk, glycine-methionine-enkephalin; Boc-YGGFM, *tert*-butoxycarbonyl-methionine-enkephalin; RFYVVM, thrombospondin-1 (1016–1021); YSFKDMGLGR, human C5a anaphylatoxin (Tyr<sup>65</sup>,Phe<sup>67</sup>)-C5a (65–74); MEVDPIGHLY, MAGE-3 antigen (167–176); HPA, *p*-hydroxyphenylacetic acid; HOHICA, 3a-hydroxy-6-oxo-2,3,3a,6,7,7a-hexahydro-1H-indol-2-carboxylic acid; HACHD, 4-alanyl-4-hydroxy-cyclohexadienone; XO, xanthine oxidase; HRP, horseradish peroxidase; LC/MS, liquid chromatography/electrospray mass spectrometry; ESI, electrospray ionization.

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group to the phenol ring (HOHICA, designated I and named in full in Fig. 1b, proposed to arise from reactions 5 and 6).

Hydroperoxide formation has been observed with peptides but only when tyrosine is N-terminal or the reaction is promoted by amino compounds (5). The amine effect has implications for hydroperoxide formation on proteins, but the mechanism is not understood. It has also been postulated that the repair mechanism involves singlet oxygen release from an intermediate (reaction 4) rather than electron transfer (reaction 2) (18), but this has not been studied experimentally.

The objectives of this investigation were to determine the structures of the hydroperoxide and any other superoxide addition products, and to understand the mechanism of formation, using a range of synthetic and physiological tyrosyl peptides. These include the opioids Leu- and Met-Enkephalin (Leu-Enk, YGGFL; and Met-Enk, YGGFM, respectively) and Endomorphin 2 (Endo2, YPPF). The opioids have a free N-terminal Tyr that is essential for activity and are potential physiological targets for inactivation by  $O_2^-$  addition. We also investigated whether the presence of a Met residue (as in Met-Enk) influences Tyr-hydroperoxide formation on the peptide and whether  $O_2^-$  addition results in the formation of methionine sulfoxide. If so, this could be a physiological mechanism for production of methionine sulfoxide, which is one of the most prevalent products of oxidative stress (21, 22).

Peptides were exposed to a xanthine oxidase (XO) system to generate  $O_2^-$  and hydrogen peroxide ( $H_2O_2$ ) plus horseradish peroxidase (HRP) to catalyze the reaction of  $H_2O_2$  with the peptide to give the tyrosyl radical (Fig. 1a). Products were analyzed using a general hydroperoxide assay ( $Fe^{2+}$ /xylenol orange or FOX assay) and by liquid chromatography/electrospray mass spectrometry (LC/MS). We have obtained structural information on the hydroperoxides, identified a mechanism of rapid intramolecular oxidation of Met residues via a hydroperoxide intermediate, and provide an explanation for why amino groups facilitate the addition of  $O_2^-$  to the tyrosyl radical.

### EXPERIMENTAL PROCEDURES

**Reagents**—Water was purified by running through a Milli-Q system (Millipore) so that its resistivity was greater than 18 M $\Omega$ -cm. All reagents and enzymes were purchased from Sigma, unless otherwise indicated. HOCl solutions were prepared from commercial bleach (Janola) and were standardized spectrophotometrically (using  $\epsilon_{292\text{ nm}} = 350\text{ M}^{-1}\text{ cm}^{-1}$ ). The peptides: YGGFM, Boc-YGGFM, YGGFM, MEVDPIGHLY, RFYVVM, and YSFKDMGLGR were purchased from Bachem (Bubendorf) and Tyr-Met (YM) and Met-Tyr (MY) were custom synthesized by Genscript (NJ). All peptides were >98% pure. Deuterium oxide (99.9%) was obtained from Cambridge Isotope Laboratories. Anthracene-9,10-diyl diethyl sulfate (EAS) was a generous gift of Prof. Paolo Di Mascio.

The Tyr-para-hydroperoxide derivative of Gly-Tyr-Gly (Gly-Tyr-Gly-OOH) was a generous gift of Prof. Michael Davies. It was generated by the reaction of Gly-Tyr-Gly with singlet oxygen, where singlet oxygen was generated *in situ* by irradiation in the presence of Rose-Bengal (23). The concentration of Gly-Tyr-Gly-OOH was 500  $\mu\text{M}$  (as measured by the FOX assay) in the presence of 2.5 mM Gly-Tyr-Gly.

YM-sulfoxide (YM-S=O) was prepared by oxidation of YM by 1 M equivalent HOCl. No other components were detected by LC/MS indicating ~100% conversion.

The concentrations of stock solutions of  $H_2O_2$  were determined iodometrically. Concentrations in solutions prepared from the stock were confirmed spectrophotometrically ( $\epsilon(H_2O_2)_{240\text{ nm}} = 43.6\text{ M}^{-1}\text{ cm}^{-1}$ ). Stock solutions of XO were prepared by dilution of an ammonium sulfate suspension with 50 mM phosphate buffer, pH 7.4, and spinning through a G-25 Sephadex column to remove the ammonium sulfate. The activity of XO was measured by the cytochrome *c* assay and by quantifying  $H_2O_2$  formation using the FOX assay. Enzyme and acetaldehyde stock solutions were prepared fresh daily and stored on ice.

**Peroxidase-mediated Oxidation of Tyrosine-containing Peptides in the Presence of Superoxide**—Reaction mixtures consisted of acetaldehyde (1 mM unless stated otherwise), XO (typically 0.001 unit/ml), HRP (typically 140 nM), and 0.2 mM peptide in 50 mM phosphate buffer plus 50  $\mu\text{M}$  diethylenetriaminepentaacetic acid (except for the FOX assay experiments). This amount of XO with 1 mM acetaldehyde corresponds to an initial rate of 2.8  $\mu\text{M}/\text{min}$   $O_2^-$  and total production of 36  $\mu\text{M}$   $H_2O_2$  over a 30-min reaction period. Reactions were started by addition of XO. They were carried out at 20–25  $^\circ\text{C}$ , typically for 30 min for LC/MS, 10 min for FOX and dimer analyses, and stopped by adding 20  $\mu\text{g}/\text{ml}$  catalase to remove residual  $H_2O_2$ . When necessary the enzymes were removed from the reaction mixtures by ultracentrifugation using 10–30-kDa cutoff microconcentrators (Amicon Microcon). Samples were protected from light to avoid photochemical reactions.

**Dimer Quantification**—Dimers were measured fluorimetrically (excitation 325 nm, emission 400 nm) with a Hitachi F-4500 fluorescence spectrofluorimeter as described in Ref. 5. Results are expressed as relative fluorescence and are related to concentrations using calibration curves obtained by generating the dimer from the relevant peptide in at least 20-fold excess and known concentrations of  $H_2O_2$  in the presence of HRP. Dimer concentrations were corroborated by measuring  $A_{315}$  at pH 8.0 using  $\epsilon = 5,080\text{ M}^{-1}\text{ cm}^{-1}$ .

**Hydroperoxide Quantification**—Hydroperoxides were analyzed using a modified FOX method (5, 24) standardized against  $H_2O_2$ , under similar conditions as for dimer quantification.

**Liquid Chromatography (LC)-Electrospray Ionization (ESI)-Mass Spectrometry (MS)**—LC-ESI-MS and LC-ESI-MS/MS analyses were performed with a Thermo Finnigan LCQ Deca XP Plus ion trap mass spectrometer (San Jose, CA) coupled to a Surveyor HPLC system and PDA detector. Positive ion mode was used for all peptides and negative ion mode for *p*-hydroxyphenylacetic acid (HPA) and EAS derivatives. Data were analyzed using Finnigan Xcalibur, Thermo Finnigan Qual Browser 1.3, and High Chem Mass Frontier 3.0 programs. Fragmentation patterns were analyzed using Bioworks Browser 3.1 and peptide fragment ions were assigned and discussed based on the Roepstorff-Fohlman nomenclature. Further details on chromatography conditions and detection are given under Supplementary Methods.

**Acid Hydrolysis**—Peptides were lyophilized and vapor phase hydrolyzed with 6 M HCl containing 1% (w/v) phenol, plus 50  $\mu\text{l}$

of mercaptoacetic acid when products were analyzed for the recovery of 3,4-dihydroxyphenylalanine (25). After hydrolysis, the residual HCl was evaporated and the samples were redissolved in water and analyzed by LC-MS.

**Quantification of Met-Enk and Leu-Enk Products**—Yields of Leu-Enk hydroperoxide and Met-Enk and Gly-Met-Enk dioxides were quantified by calibrating the LC/MS peak integral for each species. Each compound was generated using the XO/HRP system and purified by LC with the same setup as for MS analysis. For Met-Enk and Gly-Met-Enk, the product peak was collected, concentrated, and a sample reinjected to check for purity. When necessary the purification step was repeated until no contaminants were detectable. The concentrations of the enkephalin dioxides in the purified solutions were established on the basis of Phe content. An aliquot of each solution was hydrolyzed and the amount of Phe determined by LC/MS using selective ion monitoring. A standard curve was created with authentic Phe, which was identified from its retention time and fragmentation pattern. Controls using internal standards of authentic Phe showed nearly 100% recovery under the applied conditions. Acid hydrolysis was >90% efficient based on Phe recovery from the parent enkephalin hydrolyzed under the same conditions. This gave the Phe content and hence the dioxide concentration in the pure sample. A known amount of pure dioxide was injected into the LC/MS to calibrate its peak integral and this calibration was used to quantify dioxide formation under experimental conditions. The instrument response was corrected by use of check standard of Leu-Enk before each set of runs.

For Leu-Enk, the hydroperoxide was not stable enough to use the same procedure. Instead, the entire Leu-Enk-OOH peak from each of two chromatographic runs was collected and the combined sample was hydrolyzed and analyzed for Phe content as above. The Phe content in half the hydrolysate was related to the mean peak integral for the original samples, using the same time interval for collection and integration. This calibration was then used to quantify the hydroperoxide peak from experimental samples that were separated at the same time as the calibration sample.

## RESULTS

### Reactions of Superoxide with Peptide Radicals

It has been observed (5, 26, 27) that radicals generated on Tyr and tyrosyl dipeptides react with  $O_2^-$  to form hydroperoxides (5, 26, 27). This occurs in competition with dimerization of the radicals, and with Tyr and the XO/HRP system as in Fig. 1*a*, more hydroperoxide than dityrosine was formed (5). We extended these observations to the enkephalins and related peptides, and found that they were all oxidized by HRP and  $H_2O_2$ . Yields of dimer formation in the XO/HRP/acetaldehyde systems were 3–4-fold higher when SOD was present, indicating that in the absence of SOD, the peptide radicals reacted with  $O_2^-$  in preference to dimerization (Fig. 2*a*). Using the FOX assay (24) to detect hydroperoxides, a positive response was obtained with Tyr-Gly (YG) but not Gly-Tyr (GY) (Fig. 2*b*), in agreement with previous observations (5) that hydroperoxide formation requires an N-terminal Tyr. Substantial  $O_2^-$ -dependent

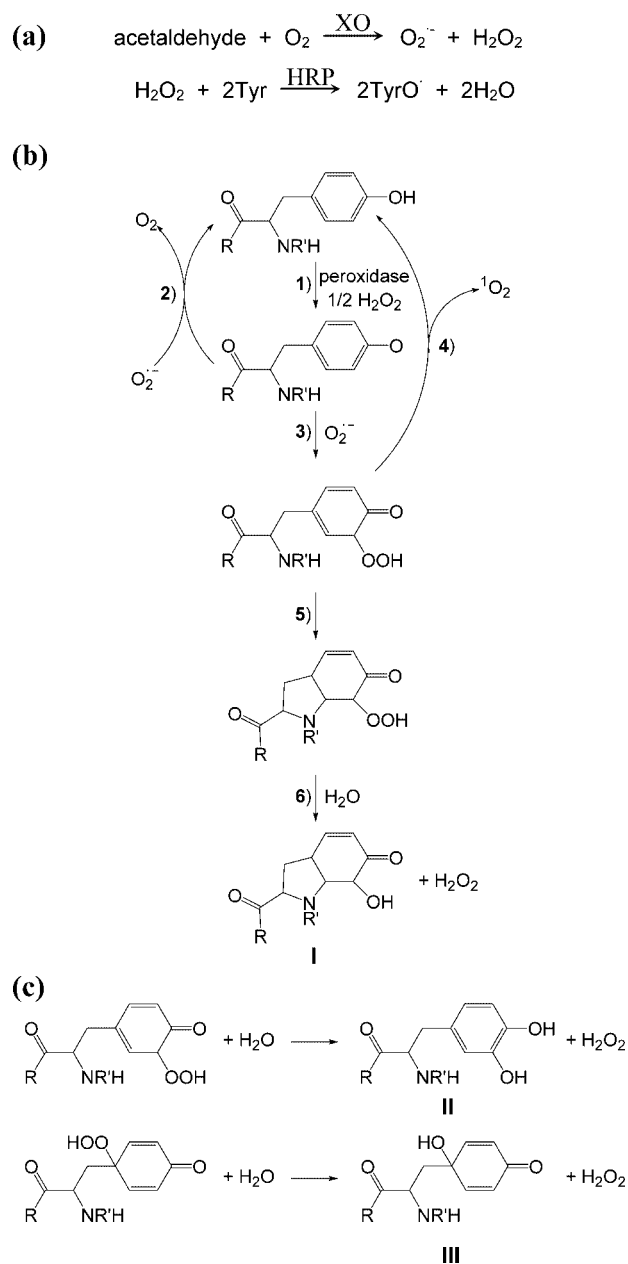


FIGURE 1. *a*, experimental system used for the generation of superoxide and tyrosyl (TyrO $^\cdot$ ) radicals. *b*, proposed mechanism for the formation and decomposition of tyrosine hydroperoxide derivatives. *R* and *R'* represent OH and H, respectively, for Tyr, or amino acid residue(s) for the peptides. Reaction 1 shows peroxidase-mediated formation of Tyr radicals, which can either dimerize (not shown) or react with  $O_2^-$  by electron transfer (reaction 2) or addition (reaction 3). Addition results in the formation of hydroperoxides (*o*- and *p*-isomers, only the *o*- isomer shown) that may exist transiently and decompose to release  $^1O_2$  (reaction 4) or form a stable species that can undergo conjugate addition of the terminal amino group are shown (when *R'* = H, reaction 5). An equivalent reaction is proposed for non-N-terminal Tyr (*R'* = amino acid residue) in which conjugation involves the amide nitrogen. Hydrolysis of the hydroperoxides that are modified by conjugate addition gives the corresponding hydroxide derivatives (*I*, 3*a*-hydroxy-6-oxo-2,3,3*a*,6,7,7*a*-hexahydro-1*H*-indol-2-carboxylic acid or HOHICA) in reaction 6. *c*, possible alternative hydrolysis products (mono-oxygenated derivatives). *II*, 3,4-dihydroxyphenylalanine derivatives from the *o*-isomer; *III*, 4-alanyl-4-hydroxy-cyclohexadienone (HACHD) derivatives from the *p*-isomer.

hydroperoxide formation was observed with Leu-Enk and Endo2, but despite having an N-terminal Tyr, Met-Enk gave no detectable hydroperoxide by FOX analysis (Fig. 2*b*).

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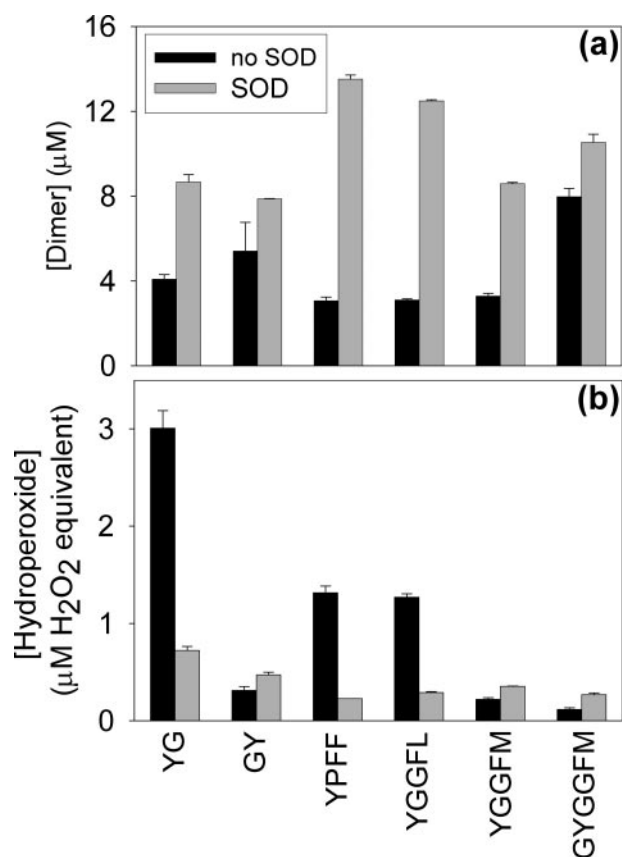


FIGURE 2. *a*, dimer; and *b*, peroxide formation from Tyr-containing peptides. Samples were incubated with HRP, acetaldehyde, and XO and the respective peptide as described under "Experimental Procedures." These peptides were shown to be good substrates for HRP, with initial rates of dimer formation measured under similar conditions of 2–16 times that of Tyr. Dimers were detected by fluorescence and hydroperoxides by the FOX assay. Zero time blanks were subtracted and *error bars* represent the range of means from two experiments. Peroxide yields are expressed as H<sub>2</sub>O<sub>2</sub> equivalents using a calibration curve that was developed with known amounts of H<sub>2</sub>O<sub>2</sub>. However, it should be noted that isotope tracer studies have demonstrated that tyrosine hydroperoxide concentrations are underestimated by the FOX assay and are approximately 6 times higher than when expressed as H<sub>2</sub>O<sub>2</sub> equivalents (5).

It should be noted that the FOX assay was calibrated with H<sub>2</sub>O<sub>2</sub> as no hydroperoxide standards are available, and this results in underestimation of hydroperoxide yields. The version of the assay used here gives a 6-fold lower response to tyrosine hydroperoxide than H<sub>2</sub>O<sub>2</sub> (5). Assuming that tyrosyl peptides behave similarly, the data in Fig. 2 indicate that the enkephalins gave more hydroperoxide than dimer. For Leu-Enk, this was confirmed by direct analysis (see below).

### Product Analysis and Structural Characterization of Hydroperoxides

**Tyr-Gly and Peptides with N-terminal Tyr Residues**—To establish the structures of the peptide hydroperoxides directly, the products of the reactions of Leu-Enk, YG, and Endo2 were analyzed by LC/MS. Each gave, in addition to the dimer, a major product with molecular mass corresponding to the native peptide + 32 (potentially the hydroperoxide) and a product at a mass of the native peptide + 16 (monoxide) (Fig. 3). In some cases two peaks of equivalent mass and fragmentation pattern were evident, presumably represent-

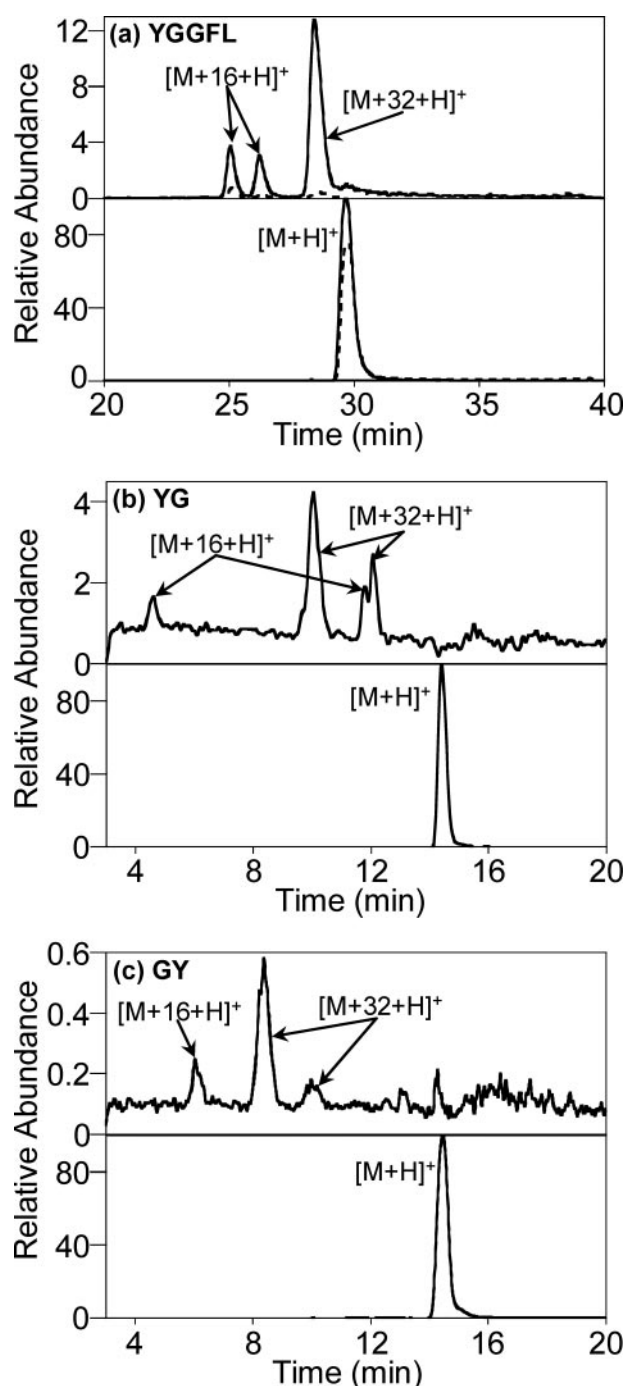


FIGURE 3. LC/MS detection of the oxygenated products from (a) Leu-Enk (YGGFL), (b) YG, and (c) GY. Samples were incubated with HRP, acetaldehyde, and XO as described under "Experimental Procedures"; chromatography conditions are described under supplementary Methods. *Bottom chromatograms* represent the native peptides, *top chromatograms* the monoxide and dioxide derivatives, all obtained by extraction from the total ion chromatograms. The peaks with *dashed lines* correspond to reactions carried out in the presence of SOD. SOD also inhibited dioxide and monoxide formation with the other peptides. Dioxide and monoxide species were also detected for Endo2 (YPFF) under similar conditions using selective ion monitoring.

ing *o*- and *p*-isomers. Formation of both species was strongly inhibited by SOD. Based on peak integrals and assuming the two species had similar MS characteristics, more dioxides than monoxides were present. The monoxides are assumed to have arisen from hydrolysis of the hydroperoxides during

the reaction and sample processing (as in Ref. 7 and shown below in Fig. 6).

The yield of Leu-Enk hydroperoxide was measured under the conditions of Fig. 3a with the peak integral calibrated on the basis of the Phe content of the purified product (see "Experimental Procedures"). For three independent experiments, 10.7  $\mu\text{M}$  (S.D. 0.5) hydroperoxide and 4.5  $\mu\text{M}$  dimer (S.D. 0.4) were formed. These accounted for the majority of the total Leu-Enk loss. This result shows that the hydroperoxide was the major product in our systems.

MS/MS fragment ions were assigned for the dioxide and monoxide products. As shown for YG, the parent peptide gave the expected  $a_1$  fragment for Tyr (Fig. 4a). The monoxide (Fig. 4b) gave  $a_1$  and  $b_1$  fragment ions with the extra oxygen attached to the Tyr residue, and the dioxide (Fig. 4c) gave an  $a_1$  fragment containing both oxygens. Where there was loss of  $\text{H}_2\text{O}$  from the monoxide,  $\text{H}_2\text{O}_2$  was lost from the dioxide, implying that the two differ by an -OH or -OOH at the same site. Similar features were evident in the fragmentation patterns of the dioxide and monoxide products for Leu-Enk and Endo2 (not shown) and fits obtained using the Bioworks Browser (as for Met-Enk in Fig. S5) were consistent with the extra oxygens in these peptides being attached to the Tyr.

YG monoxide had a very similar fragmentation pattern to the monoxide formed by exposing free Tyr to the XO/HRP system (HOHICA in Fig. S1a). Both showed the same  $a_1$  fragment with an extra oxygen and the  $m/z$  134 peak (representing loss of water from the  $a_1$  fragment and thus a lack of aromaticity, see later). The  $m/z$  134 peak, plus the absence of the peak representing ammonia loss (presumably reflecting lack of a free amino group), are key features that discriminate these structures from other theoretical alternatives with the same mass (II and III in Fig. 1c; data in Fig. S1, b and c). As HOHICA (I in Fig. 1b) has been characterized as a bicyclic compound (7), we conclude that YG dioxide has a hydroperoxide on the Tyr ring that has undergone the same conjugate addition (Fig. 1b, reaction 5).

*Gly-Tyr*—Although GY gave no detectable hydroperoxide in Fig. 2b, low yields of dioxide and monoxide derivatives were detected by LC/MS (Fig. 3c). The fragmentation patterns (Fig. S2) show a hydroperoxide group, or corresponding -OH, located on the Tyr, and also suggest (as discussed below for peptides containing Met) that the Tyr ring is modified by conjugate addition of the amide nitrogen.

#### Intramolecular Oxygen Transfer in Peptides Containing Tyr and Met

The situation was different for peptides containing Met. We investigated dipeptides MY, YM, as well as Met-Enk and Gly-Met-Enk. These peptides produced dimers when exposed to the XO/HRP system, and dimerization was enhanced when  $\text{O}_2^-$  was removed with SOD (Table 1 and Fig. 2a). LC/MS analysis (Fig. 5, a–d, and Table 1) showed that a dioxygenated  $\text{M} + 32 + \text{H}^+$  species was produced from all the peptides. Formation of the dioxygenated species required  $\text{O}_2^-$  as it was suppressed by SOD. However, none of the peptides gave a positive response in the FOX assay (Table 1 and Fig. 2b), implying that the two-oxygen addition products were not hydroperoxides.

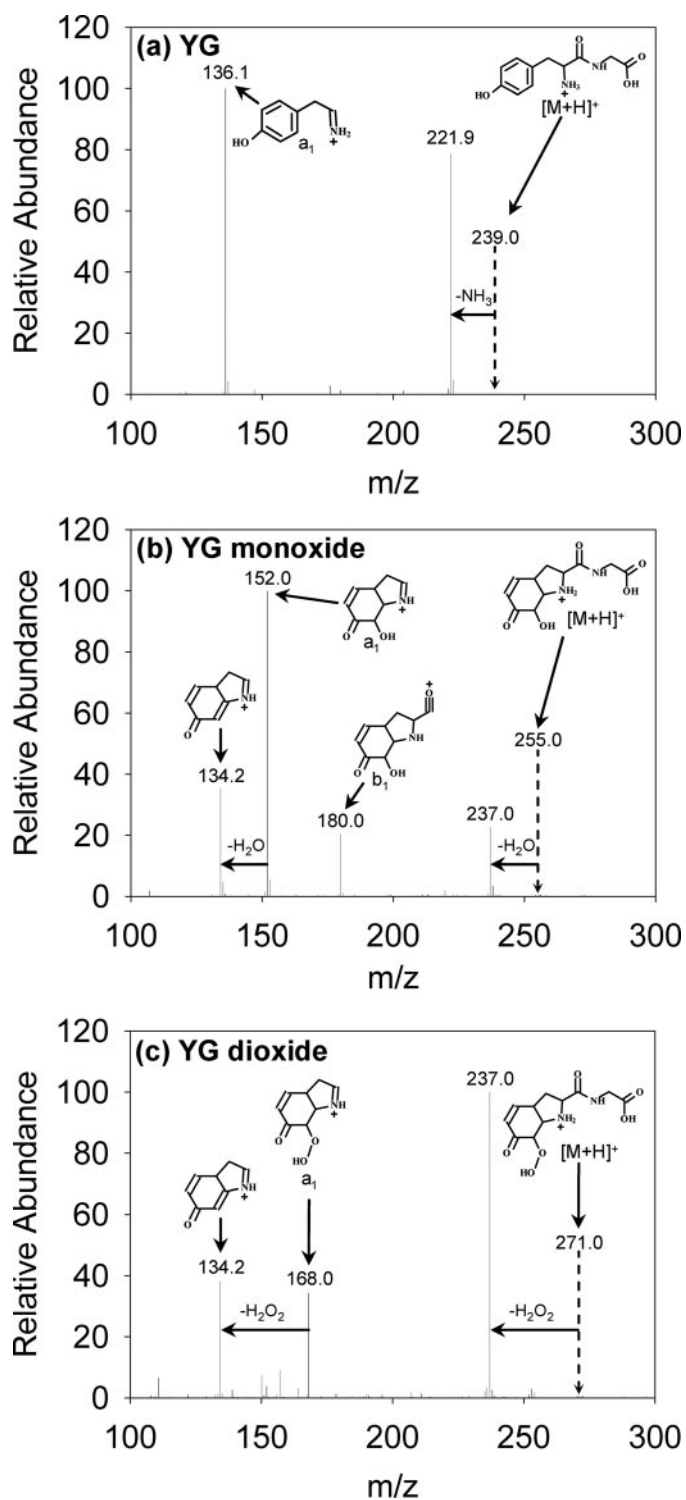


FIGURE 4. Fragmentation patterns of (a) YG, (b) YG monoxide, and (c) YG dioxide. YG was treated and analyzed as described in the legend to Fig. 3. Proposed structures and fragment assignments are discussed in the text.

Yields of dioxygenated products were quantified for Met-Enk and Gly-Met-Enk by calibrating peak integrals for each dioxide based on Phe content (see "Experimental Procedures"). Using this calibration, under the conditions of Fig. 5,  $7 \pm 2 \mu\text{M}$  Met-Enk-dioxide and  $1.8 \pm 0.4 \mu\text{M}$  Gly-Met-Enk-dioxide were formed ( $n = 4$ ). Dimer concentrations measured in the same solutions were 3.3 (S.D. 0.2) and 10.4 (S.D. 0.4)  $\mu\text{M}$ , respectively.

TABLE 1

## Methionyl peptides examined for dioxide formation

Dimer concentrations were measured by spectrofluorimetry and hydroperoxide concentrations by the FOX assay. Peptides were separated by LC/MS using chromatographic conditions given under supplementary Methods and the  $[M + 32 + H]^+$  peaks were detected as shown in Fig. 5 for YM, MY, Met-Enk, Gly-Met-Enk, and YM-S = O.

Amino acid sequence	Molecular mass	RT <sup>a</sup> of $[M + H]^+$	RT of dioxide	Dimer		Hydroperoxide ( $H_2O_2$ eq. $\mu M$ )
				No SOD	SOD	
	<i>g/mol</i>		<i>min</i>	$\mu M$		
YM	312.4	17.9	12.7, 14.0	3.8 ± 0.1	7.0 ± 0.1	0.23 ± 0.006
MY	312.4	17.4	12.6, 14.7	5.41 ± 0.1	6.9 ± 0.2	<0.2
YGGFM	573.7	16.1	11.3, 12.5	3.3 ± 0.1	8.6 ± 0.1	<0.2
GYGFM	630.7	13.3	10.2, 11.0	8.0 ± 0.4	10.5 ± 0.4	<0.2
Boc-YGGFM	673.8	14.1	9.6	5.8 ± 0.2	11.4 ± 0.5	<0.2
RFYVVM	814.0	8.4, 10.6	7.5, 9.6	10.7 ± 0.8	17.6 ± 0.4	<0.2
YSFKDMGLGR	1244.4	10.7	9.4	1.0 ± 0.2	2.7 ± 0.2	<0.2
MEVDPIGHLY	1173.4	18.6	12.3	3.3 ± 0.3	7.8 ± 0.3	<0.2
YM-S = O	361.1	15.93	13.53, 14.07	2.55 ± 0.09	4.15 ± 0.05	0.32 ± 0.002

<sup>a</sup> RT retention time. Conditions as described under "Experimental Procedures."

The sum of the dioxide and dimers closely accounted for the loss of the corresponding native peptide. This establishes that the dioxide is the major product with Met-Enk. It also confirms that the position of the Tyr dictates the efficiency of dioxide formation as it does for the hydroperoxides and is consistent with the dioxides arising from a hydroperoxide intermediate.

Structural information was obtained by comparing the dioxide fragmentation patterns to those of the parent peptides. YM gave the expected  $a_1$  and  $y_1$  fragments and an  $M - 46 + H^+$  peak characteristic of methionyl peptides (Fig. 5e). For YM dioxide, the  $y_1$  fragment ( $m/z$  166) shows the presence of an extra oxygen, and there is a peak at  $m/z = 281$  representing loss of 64 mass units ( $-HS(=O)-CH_3$ , a characteristic loss for sulfoxides (28). This is clear evidence that one of the oxygens is present as Met sulfoxide (Fig. 5f). There is a peak at  $m/z$  134, as seen with YG monoxide and HOHICA, and no peak corresponding to ammonia loss. From this fragmentation pattern, we conclude that the other oxygen is located on the Tyr, which has undergone conjugate addition.

Fragmentation of MY-dioxide also gave a major peak at  $M - 64 + H^+$  (Fig. S3b), indicating that it is also the sulfoxide derivative. The pattern is more complex than for YM-dioxide, with no clear  $y_1$  peak. However, successive loss of two waters from both the parent ion and  $M - 64 + H^+$  peak implies a labile -OH group in addition to the carboxyl group on the Tyr. As discussed under Fig. S3, possible Tyr-OH structures with the requisite mass include a 3,4-dihydroxyphenylalanine derivative or its *p*-equivalent (II and III in Fig. 1c) and a conjugate addition product (I in Fig. 1b). Only I and III would have a labile -OH. Acid hydrolysis of the dioxides purified from MY and Gly-Met-Enk gave no detectable 3,4-dihydroxyphenylalanine or III under conditions where they should have been detected (for details, see Fig. S4). On this basis we propose that the Tyr is most likely modified by conjugate addition, possibly with the amide nitrogen.

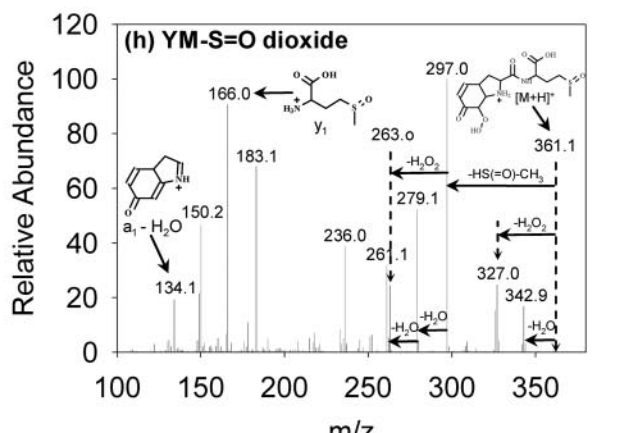
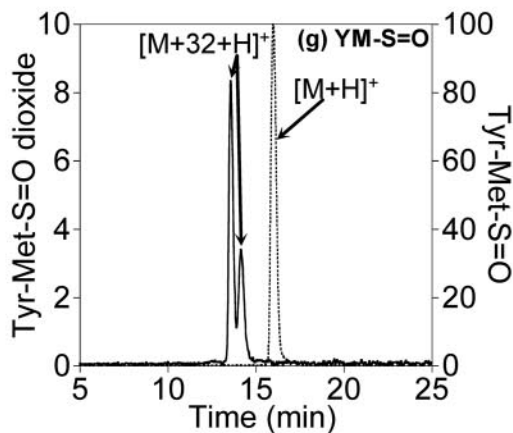
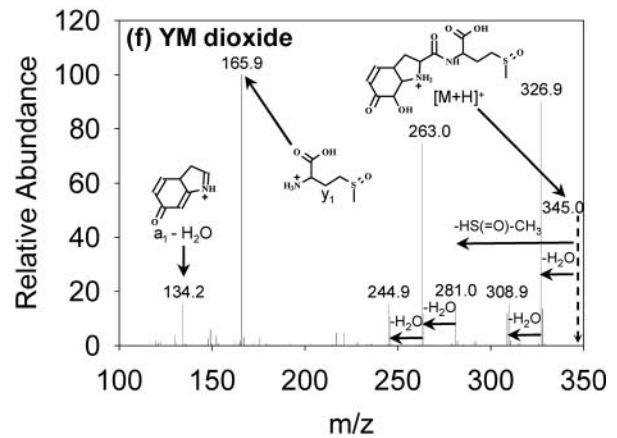
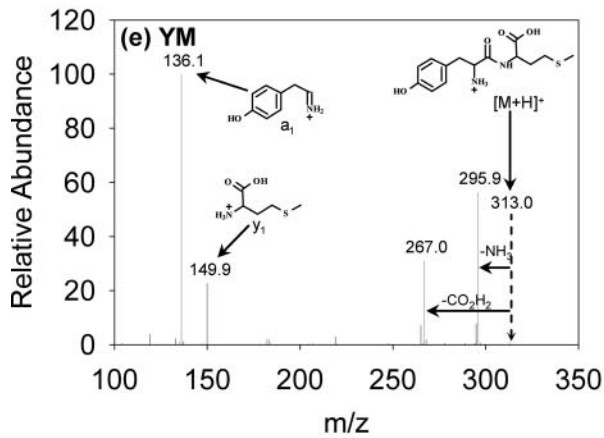
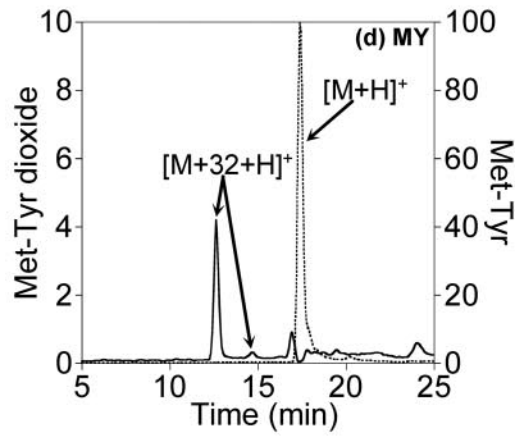
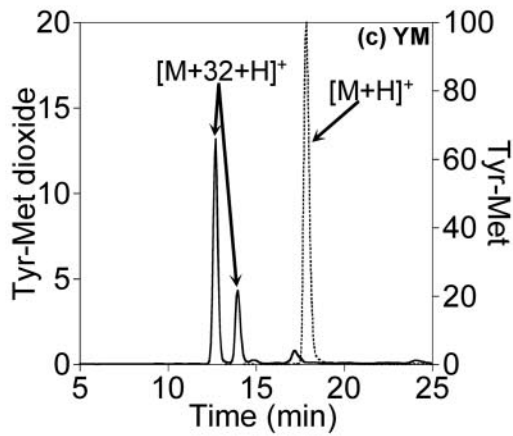
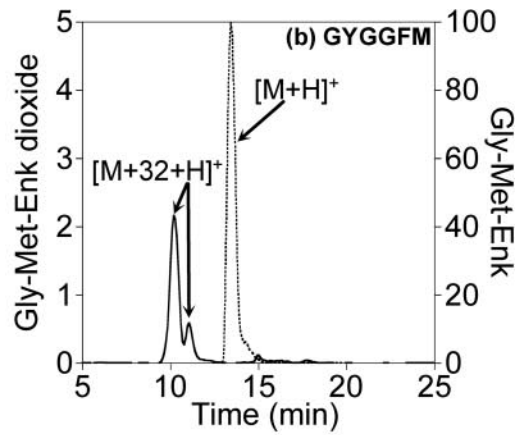
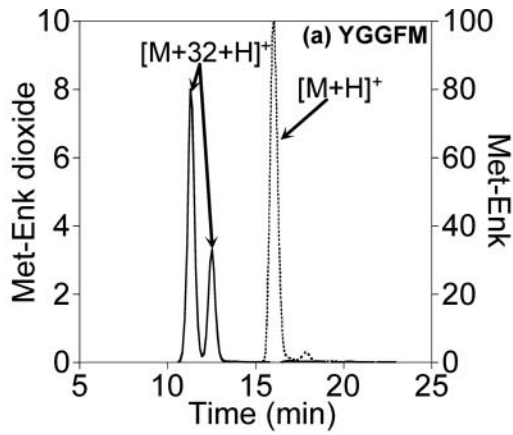
When YM was first converted to its sulfoxide derivative then exposed to the  $O_2^-/H_2O_2/HRP$  system, the major product (in addition to the dimer) had a molecular mass corresponding to  $[YM + 48 + H^+]$ . This represents the addition of two extra oxygens to YM-S=O (Fig. 5g). The formation of YM-trioxide was greatly inhibited by SOD.  $O_2^-$ -dependent formation of a hydroperoxide was detected by the FOX assay (Table 1). This, together with the obtained fragmentation pattern (Fig. 5h) indicates that YM-trioxide is the Tyr hydroperoxide derivative of YM-S=O.

We propose that Met oxidation occurs via intramolecular transfer of one oxygen from an initial tyrosine hydroperoxide derivative. In support of this, there was little or no formation of products in which only the Tyr or Met residue of the peptide was oxidized. As only a small fraction of the parent peptide was modified, this would be expected if the reaction was intermolecular. Further evidence against intermolecular oxygen transfer is that the reaction of preformed Leu-Enk-OOH with Met or Met-Enk occurred over hours (Fig. 6).

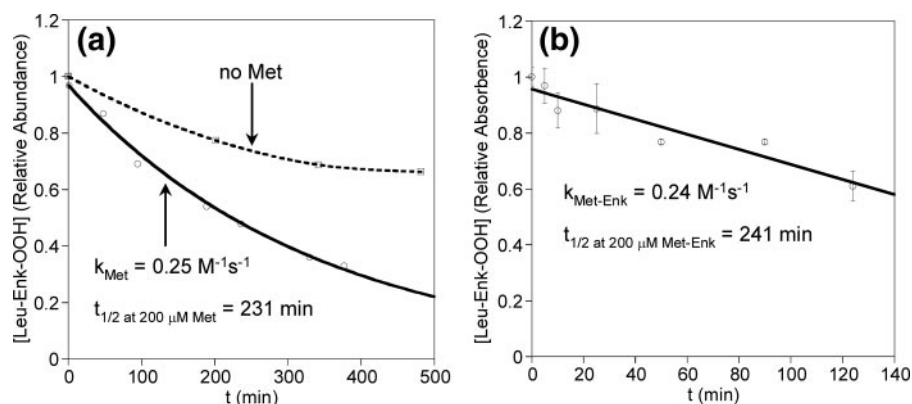
The effect of the relative positions of the Tyr and Met residues on the oxygen transfer reaction was investigated using the peptides shown in Table 1. All produced dimers when exposed to the XO/HRP system (Table 1). This reaction was enhanced in the presence of SOD, but no detectable hydroperoxides were formed. In each case, a dioxygenated product was detected by LC/MS (Table 1) with a fragmentation pattern showing loss of 64 mass units characteristic of methionine sulfoxide and other features consistent with localization of the other oxygen on the Tyr residue. The closeness of fit of the observed fragments with those simulated by the Bioworks Browser for the structure representing the best fit is illustrated for Met-Enk in Fig. S5. We propose, therefore, that in peptides containing Tyr and Met, formation of a hydroperoxide on the Tyr is rapidly followed by intramolecular oxygen transfer to the Met (shown for YM in Fig. 7) and this can operate when these residues are adjacent or up to at least 9 residues apart.

### No Evidence for Singlet Oxygen Release

The low yields of hydroperoxides or other major  $O_2^-$ -dependent products with peptides in which the Tyr was not N-terminal, combined with previous observations that less of these peptides were consumed in the reaction (5), are consistent with  $O_2^-$  reacting with the peptide radicals to repair the Tyr. Two mechanisms have been proposed: repair via electron transfer (19) or release of  $^1O_2$  from an unstable hydroperoxide intermediate (7, 18) (reactions 2 or 4 in Fig. 1b, respectively). Production of  $^1O_2$  was investigated using anthracene-9,10-diyl diethyl sulfate as a trap (29) and LC/MS to quantify the endoperoxide product. The detection limit, established with  $^1O_2$  generated from hypochlorous acid and  $H_2O_2$ , was 0.5  $\mu M$  (Fig. S6). We maximized product yields from the XO/HRP system for GY and YG and reasoned that if the YG radical reacted with  $O_2^-$  mainly by addition and GY released  $^1O_2$ , the difference in



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**FIGURE 6. Intermolecular reaction of Leu-Enk hydroperoxide (Leu-Enk-OOH) with (a) Met and (b) Met-Enk.** Decay of Leu-Enk-OOH ( $10 \mu\text{M}$  in  $50 \text{ mM}$  phosphate buffer) was measured in the presence and absence of  $200 \mu\text{M}$  methionine by LC/MS, or  $200 \mu\text{M}$  Met-Enk by the FOX assay. Diethylenetriaminepentaacetic acid ( $50 \mu\text{M}$ ) was present for *a* but not *b*. Leu-Enk-OOH was generated using the conditions described under "Experimental Procedures." After 30 min incubation,  $20 \mu\text{g/ml}$  catalase was added and the enzymes were removed by centrifugation using an Amicon 10-kDa cutoff filter. The reaction of Leu-Enk-OOH with Met-Enk was also monitored by LC/MS, where a similar second-order rate constant was obtained ( $k = 0.18 \text{ M}^{-1} \text{ s}^{-1}$ ). Loss of the hydroperoxide was accompanied by increases in the Leu-Enk monoxide and Met-Enk sulfoxide peaks at similar rates. The rate of the reaction was also investigated at lower Met-Enk concentrations. The obtained pseudo-first ordered rate constants together with the fits of the pseudo-first order kinetic traces to a single exponential curve indicate that the overall reaction is indeed second-order (first-order for both [Leu-Enk-OOH] and [Met-Enk]).

hydroperoxide yield between the two should theoretically be detected with GY as  $^1\text{O}_2$ . (This is argued in more detail in Fig. S6.) As shown in Fig. S6c,  $^1\text{O}_2$  production was below the detection limit under conditions where the difference in hydroperoxide production was at least  $15 \mu\text{M}$  (measured in  $\text{H}_2\text{O}_2$  equivalents, and for reasons given above most likely in excess of  $50 \mu\text{M}$ ). At most this would represent only a few percent of the theoretical yield. Therefore, we conclude that the release of  $^1\text{O}_2$  plays little if any role in the repair pathway for non-N-terminal Tyr.

### Enhanced Superoxide Addition to Tyrosyl Radicals by Exogenous Amines

One mechanism proposed to explain the higher yields of hydroperoxide on N-terminal Tyr is that formation is facilitated by conjugate addition of the free amine (18). This was tested by adding exogenous amines to tyrosyl peptides and identifying the products using LC/MS. Lys and ethanolamine have been shown to enhance hydroperoxide yields on GY, measured by the FOX assay (5). If this were due to conjugate addition of the amine, a higher molecular weight product would be formed. With GY, both Lys and ethanolamine gave a concentration-dependent increase in hydroperoxide yield (Fig. 8). However, the hydroperoxide had the same chromatographic mobility, mass, and fragmentation pattern as the GY-OOH formed in the absence of amine (Fig. 8a, inset). There was no evidence of a product arising from intermolecular conjugate addition. Furthermore, Lys enhanced hydroperoxide formation on HPA without forming an addi-

tion product (Fig. 8b), excluding any requirement for a peptide nitrogen. The hydroperoxide yield with YG, which was already much higher in the absence of amines, was not affected by adding Lys.

These observations suggest an alternative mechanism by which the proximity of amino groups promotes  $\text{O}_2^-$  addition to the tyrosyl radical. It is unlikely to be a salt effect or general acid catalysis as enhancement was observed in  $50 \text{ mM}$  phosphate buffer and was not seen when Lys was added to YG. A possible explanation is that protonated amine groups increase the electrophilicity of  $\text{O}_2^-$  via H-bonding, and this favors the addition reaction. To test this mechanism, the reaction was carried out in  $\text{D}_2\text{O}$ , in which proton exchange results in deuteration of the Lys amino group.

Deuterated Lys, which forms much weaker hydrogen bonds, gave correspondingly less enhancement of hydroperoxide formation (Fig. 8a).

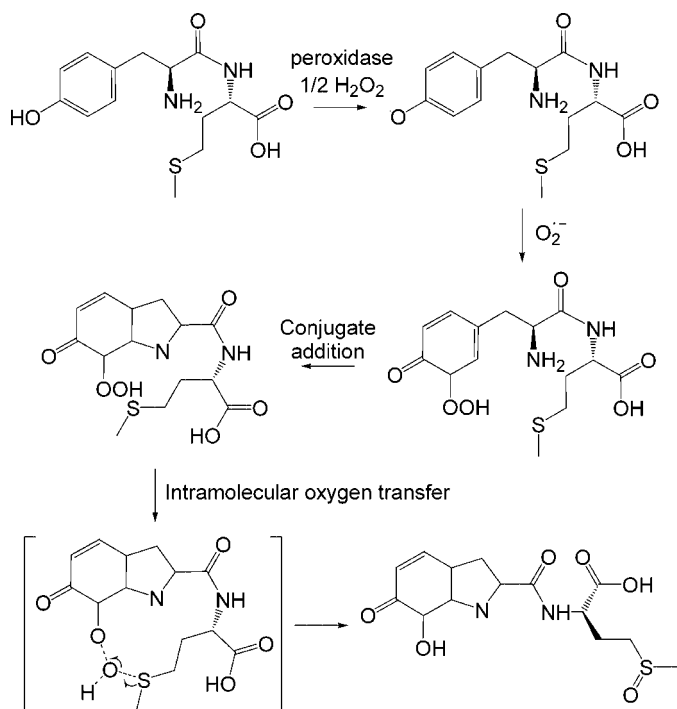
## DISCUSSION

This study has focused on  $\text{O}_2^-$  addition to tyrosyl radicals and the relevance of this mechanism to oxidative modification of peptides and proteins. We have characterized the products with physiological peptides and present evidence that the preferential formation of hydroperoxides on the N-terminal Tyr residues is due to adjacent amino groups interacting with  $\text{O}_2^-$  to promote the addition reaction. We have also identified a novel mechanism of rapid intramolecular transfer of an oxygen from an initial tyrosine hydroperoxide to a Met residue to form the sulfoxide. The biological relevance of  $\text{O}_2^-$  addition products was demonstrated by showing that Leu-Enk and Endomorphin 2 were converted to hydroperoxides and Met-Enk to a dioxide when exposed to a peroxidase/ $\text{O}_2^-$  system. With the HRP/XO system we used, more hydroperoxide or dioxide was formed than dimer. In theory this system generates a maximum of  $\sim 0.5 \text{ O}_2^-$  radicals per Tyr radical,<sup>3</sup> so in situations where  $\text{O}_2^-$  production is high, such as the surroundings of neutrophils, the

<sup>3</sup> The reduction of oxygen by xanthine oxidase is  $\sim 30\%$  by a 1-electron route to  $\text{O}_2^-$ , with the remainder reduced directly to  $\text{H}_2\text{O}_2$ . If all the  $\text{H}_2\text{O}_2$  reacted with Tyr to generate Tyr radicals and the  $\text{O}_2^-$  did not dismutate to give more  $\text{H}_2\text{O}_2$ , then there would be approximately 70 Tyr radicals generated per  $30 \text{ O}_2^-$ .

**FIGURE 5. LC/MS detection of the dioxide products from (a) Met-Enk, (b) Gly-Met-Enk, (c) YM, (d) MY, and (g) YM-S=O and fragmentation patterns of (e) YM, (f) YM dioxide, and (h) YM-S=O dioxide.** Chromatograms represent the native peptides (dashed lines) and dioxides (solid lines) obtained by selective ion monitoring (a and c) or by extraction from the total ion chromatograms (b, d, and g). Amounts of products (left ordinate axis) are shown as relative abundance compared with the parent peptide set as 100%. Reaction conditions are described under "Experimental Procedures" and chromatographic conditions under supplementary Methods. Dioxide yields were inhibited  $>90\%$  by  $10 \mu\text{g/ml}$  SOD. Some peptide-monoxide derivatives were also detected, but except for YM where a small amount of Tyr-OH was detected, it was due to sulfoxide impurity in the authentic sample. A small amount of YM-S=O monoxide (i.e. YM dioxide) was also detected in the YM-S=O system that is presumably due to the hydrolysis of the YM-S=O hydroperoxide (in analogy with the YG and Leu-Enk hydroperoxides, see Fig. 3).



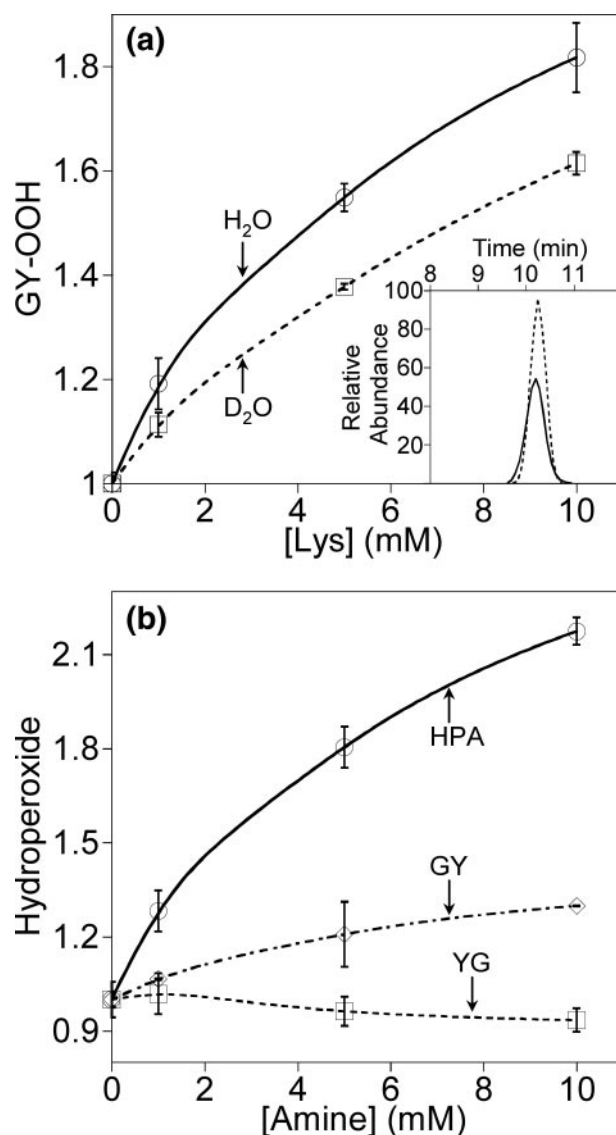


**FIGURE 7. Proposed mechanism for the formation of Tyr-Met-dioxide.** The Tyr residue is oxidized in a peroxidase-mediated one-electron oxidation reaction by  $\text{H}_2\text{O}_2$  to give the phenoxyl radical, which adds to superoxide to form the hydroperoxide intermediate. The hydroperoxide undergoes conjugate addition of the amine nitrogen to the Tyr ring and the Met residue is oxidized to its sulfoxide via intramolecular oxygen transfer from the Tyr hydroperoxide. The reaction is shown for the *ortho* hydroxyl isomer; an equivalent reaction can be written for the *para* form.

hydroperoxide would be even more favored. Therefore, superoxide addition to tyrosyl radicals should occur physiologically and could result in inactivation of enkephalins at sites of inflammation. Indeed, stimulated neutrophils use myeloperoxidase and  $\text{O}_2^-$  to oxidize enkephalins by this mechanism.<sup>4</sup>

**Structural Characterization of Hydroperoxides as Conjugate Addition Products**—The structures of hydroperoxides on N-terminal Tyr residues and the equivalent monoxide on Met-containing peptides were confirmed as bicyclic conjugate addition products. Those detected in much lower yields when Tyr was not N-terminal also appeared to be conjugates, possibly with the amide nitrogen although definite characterization is still required. This is different from the unconjugated hydroperoxides generated by  $^1\text{O}_2$  on peptides such as GYG (23), but equivalent conjugate addition of an amide nitrogen has been observed for photooxygenation of *N*-acetyltyramine (30).

**Mechanism of Superoxide Addition Versus Radical Repair**—Our data, along with earlier findings (5), indicate that peptides with non-N-terminal Tyr undergo more efficient radical repair. Others have observed for a range of phenoxyl radicals that the contribution of repair relative to  $\text{O}_2^-$  addition decreases progressively with increasing reduction potential of the phenol (19). This was explained by the repair reaction occurring by electron transfer (which, in accordance with the Marcus theory, is favored when the difference in reduction potential between



**FIGURE 8. Increases in superoxide-dependent hydroperoxide formation in the presence of amines as detected by LC/MS.** *a*, formation of GY-OOH in the presence of Lys in normal (○) or deuterated (□) water. Data points represent the peak areas of the GY-OOH relative to control with no amine (mean  $\pm$  S.D. of triplicates). All experiments were performed at least twice. *Inset*, chromatograms represent the relative abundance of GY-OOH at 0 mM (solid line) and 10 mM (dashed line) Lys and were recorded by selective ion monitoring at  $m/z = 271$ . *b*, relative peak areas of the dioxide derivatives of HPA (○) and YG (□) at different Lys concentrations and GY (◇) at different ethanolamine concentrations. Reaction conditions were as described under "Experimental Procedures" except: [acetaldehyde] = 2.5 mM, [peptide] = 1 mM, and [Lys] or [ethanolamine] = 0–10 mM. Before quantification the enzymes were removed using 10-kDa microconcentrators. As controls, Lys had no effect on the rates of production of  $\text{O}_2^-$  or  $\text{H}_2\text{O}_2$  by XO as measured with the cytochrome *c* and FOX assays, respectively. Lys and ethanolamine also did not affect the rate of the HRP-catalyzed oxidation of GY by  $\text{H}_2\text{O}_2$  or the yields of dimer in the actual samples. In deuterated solvents pD was measured using a glass electrode by adding 0.4 pH values to the pH meter reading. The activity of XO was measured in  $\text{D}_2\text{O}$  when the reactions were carried out in deuterated media.

the two couples is high). On this basis, Tyr and Tyr-peptides, which all have a reduction potential ( $\text{PhO}^\bullet/\text{PhO}^-$ ) of  $\sim 0.64$  V, should react predominantly by electron transfer. The small amount of  $\text{O}_2^-$  addition to non-N-terminal Tyr peptides fits well with this relationship, but the efficient addition to N-terminal Tyr is anomalous. To explain this, von Sonntag and co-workers (18) proposed that rather than competition between addition

<sup>4</sup> P. Nagy, A. J. Kettle, and C. C. Winterbourn, unpublished results.

## Radical Targets for Superoxide

and electron transfer, the phenoxyl and  $O_2^-$  radicals combine to form a transient intermediate that either breaks down to release oxygen or converts to a stable hydroperoxide (Fig. 1b, reactions 4 and 5, respectively). The driving force for stabilization of the hydroperoxide was proposed to be conjugate addition of the terminal amine. Our data do not support this mechanism on two counts. We saw no formation of  $^1O_2$ , which should have been released in reaction 4 to obey the spin conservation rule. Also, if conjugate addition were the stabilizing factor, enhancement of hydroperoxide formation by exogenous amines should be associated with conjugation of the amine to the tyrosyl ring. This was not seen with GY or HPA even though the amines increased hydroperoxide yields.

We propose an alternative mechanism that accounts for the Tyr anomaly. In this mechanism the two radicals react either by electron transfer or radical addition as proposed (19), but hydrogen bonding to an amine group alters the reactivity of  $O_2^-$  so as to favor the addition pathway. This mechanism is consistent with our findings that Lys promoted less hydroperoxide formation when it was deuterated. It is also supported by experimental evidence of hydrogen bonding of  $O_2^-$  to protonated amines (31, 32), and calculations showing that hydrogen bonding to N-H groups alters its spin and charge densities (31, 33). The effect of hydrogen bonding is to increase the electrophilicity and reduction potential of the  $O_2/O_2^-$  couple. The impact on the  $O_2^-$ /phenoxyl radical reaction would be to decrease the potential difference between the two redox couples, and as reasoned above (19), favor the radical addition pathway. Thus, promoting  $O_2^-$  addition through hydrogen bonding to the terminal amino group would explain the Tyr anomaly. The greater hydroperoxide formation with N-terminal Tyr than with exogenous amines implies that hydrogen bonding provides more effective promotion of the addition pathway when the reaction is intramolecular. This could be explained by a lowering of transitional entropy (34).

The amine effect could represent rate enhancement of the radical-radical reaction or a change in preference for addition over electron transfer. As the rates are almost diffusion controlled and rate constants show only minor differences between phenols that add or transfer electrons (19), the latter explanation is more likely. It is further supported by our pulse radiolysis measurements of similar rate constants for the reaction of  $O_2^-$  with free Tyr and Tyr peptides ( $1.5 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ ) regardless of whether or not they form hydroperoxides.<sup>5</sup> Although our results fit with this mechanism, we cannot exclude the possibility that there is initial formation of an associative intermediate between superoxide and tyrosyl radicals and then interaction of this intermediate with an amine to favor its breakdown by addition rather than electron transfer.

Reaction with  $O_2^-$  should be a favored reaction for tyrosyl radicals generated on proteins and a likely route for formation of protein hydroperoxides. Interaction with amino groups should have a major influence on where addition as against repair of the Tyr residue occurs, with peroxides formed not

only when Tyr is N-terminal but also when it is favorably aligned to amino groups that could promote the reaction.

*Intramolecular Oxygen Transfer to Methionine*—The finding that peptides containing Met did not form stable hydroperoxides was unexpected, but led us to identify an intramolecular oxygen transfer mechanism for methionine sulfoxide formation. We propose that the reaction proceeds through an initial hydroperoxide. The formation of a stable hydroperoxide on preformed YM-S=O, when the oxygen transfer route is blocked, plus the 5-fold higher yield of intramolecular oxygen transfer product with Met-Enk than Gly-Met-Enk provide support for this mechanism. Also, oxidation of free methionine by Leu-Enk hydroperoxide was much too slow for intermolecular transfer to account for the reaction. Electron transfer between Met and Tyr residues has been observed in situations where Met residues are oxidized by a 1-electron mechanism (10, 12, 13). The electron transfer is in the opposite direction to oxygen transfer, regenerating Met from its radical and forming a Tyr radical. Our findings are not explained by this process and, as far as we are aware, represent a previously unrecognized mechanism of oxygen transfer. It has some similarity to the oxidation of Met residues in apoA by lipid hydroperoxides, which is facilitated by the presence of both reactants in high density lipoprotein (35, 36). We observed oxygen transfer in peptides with little discrimination for the relative positions of the Tyr and Met. The chances of it occurring in proteins must therefore be high. Proximity to Tyr in the tertiary structure might give preference to modification of particular Met residues.

There has been one other study of the reaction of  $O_2^-$  with the tyrosyl radical of Met-Enk (20). Using radiolytic methods, these authors measured a reaction rate with  $O_2^-$  similar to that reported for other phenoxyl radicals. They detected a dioxygenated product but did not show MS analysis and assumed it to be a hydroperoxide. They concluded that no oxygenated products were formed from N-terminal blocked Boc-Met-Enk, although the main peak in the mass spectrum of their reaction mixture ( $m/z$  706.2, which they did not comment on) has the mass of a dioxygenated species.

*Physiological Significance*—Superoxide and tyrosyl radicals are among the most prevalent radicals generated biologically during oxidative stress. Reaction between the two is highly favored. We have shown with tyrosine and the enkephalins that this results in the formation of additional products in greater yields than dityrosine derivatives, the tyrosine oxidation products that generally receive most attention in relation to oxidative injury. We have characterized the tyrosine hydroperoxides formed and proposed a mechanism involving hydrogen bonding to amino groups that explains why formation is favored on N-terminal Tyr. The repair reaction that predominates when these conditions are not met does not release  $^1O_2$ .  $O_2^-$  addition to tyrosyl peptides that also contain Met and subsequent intramolecular oxygen transfer may represent an unrecognized mechanism of methionine sulfoxide formation in proteins under conditions of oxidative stress. Interaction of  $O_2^-$  with neighboring amines could be important for determining structural selectivity of  $O_2^-$  addition to Tyr residues on proteins. Moreover, the proposed mechanism, whereby the electrophili-

<sup>5</sup> P. Nagy and T. Nauser, unpublished data.

licity of  $O_2^-$  is increased through hydrogen bonding, has wide biological implications. This would bring the reactivity of  $O_2^-$  closer to that of the more oxidizing hydroperoxyl radical and should increase its ability to react with more biological substrates. The relevance of this mechanism to  $O_2^-$  biology warrants further investigation.

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