Peptides containing histidine at position 2 were observed to undergo spontaneous N-terminal oxidative deamination in aqueous solution in the presence of Ni(II), sulfite, and ambient oxygen. The reaction resulted in the formation of a free carbonyl on the N-terminal α-carbon (α-ketoamide) and was catalytic with respect to nickel. This oxidative deamination was confirmed by 13C NMR, 1H NMR, mass spectrometry, and chemical tests. No evidence of modification of histidine was found. It was demonstrated that the nickel-dependent N-terminal oxidative deamination also occurred in His-2 peptides using potassium peroxymonosulfate (oxone) as an oxidant. When oxone was used, oxygen was not required for the deamination to proceed. The results suggest that both nickel-catalyzed reactions (sulfite and oxone) produce an imine intermediate that spontaneously hydrolyzes to form the free carbonyl. These findings may provide a physiologically relevant model for oxidative carbonyl formation in vivo, as well as a useful method for producing a site-specific carbonyl on peptides and proteins.

Excessive exposures to both nickel and sulfite are known to produce serious toxic and carcinogenic effects in animals and humans (1–4). The generation of potentially harmful radicals from sulfite oxidation by transition metal catalysis has been well established (5). Nickel salts are physiologically redox-inactive, and therefore nickel toxicity in vivo is inferred to involve activation by coordination with peptides and proteins. A variety of catalytic oxidative properties of Ni(II)/peptide complexes have been reported in the literature. These include decarboxylation of the Ni(II)-Gly-Gly-His complex in the presence of molecular oxygen (6), the ability of Ni(II)-histidyl peptide complexes to function as Fenton reaction catalysts (7, 8), and the ability of the Ni(II)-Gly-Gly-γ-His complex to catalyze the oxidation and cleavage of DNA (9, 10) and to promote the decarboxylation of the Ni(II)-glycyl-glycyl-histidine (11, 12). Muller et al. (13) and Liang et al. (14) have recently reported that autoxidation of sulfite catalyzed by the Ni(II)-complexed Lys-Gly-His-amide tripeptide can oxidatively damage DNA. They postulate in situ formation of monoperoxysulfate, a strong oxidant, as an active intermediate in the damaging effect.

We have recently reported on the identification of a unique Ni(II) binding site on hemoglobin in the presence of monoperoxysulfate, produces N-terminal oxidative deamination, as well as intramolecular cross-linking, both specific to the β-globins (15). In the present study, we have employed small model peptides in order to verify the structural assignment of oxidative deamination, as well as to identify the minimal sequence requirements for reaction susceptibility. Additionally, we have tested a system that substitutes sulfite (SO3 2−) and oxygen (O2) for potassium peroxymonosulfate (oxone).1 Our findings show clearly that histidine at position 2 is a fundamental requirement for Ni(II)-catalyzed oxidative N-terminal deamination and that sulfite and ambient oxygen can readily substitute for the potent peroxy oxidant oxone. Furthermore, by contrasting some of the differences between products produced by the sulfite/oxone and the oxone reactions with Ni(II) peptides, we can exclude the formation of diffusible monoperoxysulfate (HSO5 −) as a mediator in SO3 2−/O2-promoted deamination.

EXPERIMENTAL PROCEDURES

Materials—Sodium sulfite was purchased from Fluka Biochemika. Ammonium sulfite, potassium peroxymonosulfate (oxone), nickel chloride, butylated hydroxyanisole, tert-butyl alcohol, EDTA, mannitol, ethanol, sodium cyanoborohydride, sodium phosphate (as mono- and dibasic salts), and thiourea were from Sigma. The peptides bursin (KHG-amide), carnosine (β-alanyl-histidine), and homocarnosine (γ-amino butyl-histidine) were purchased from Sigma. KGH-amide was synthesized by SynPept Corp. All other peptides were purchased from Bachem Bioscience Inc. and used without repurification.

Sulfite Reaction Standard Conditions—Unless otherwise noted, reaction mixtures contained 30 mM peptide and 5 mM nickel chloride, to which sodium sulfite was added from a 10× stock to a final concentration of 60 mM, all in 0.1 M sodium phosphate, pH 8.2. 200-µl reactions were maintained at room temperature for 24 h under ambient air in 16 × 100-mm glass test tubes protected from light. Reactions were quenched by addition of 1: 10 (v/v) of 0.5 M EDTA, pH 8, and then diluted 1:10 into 2% formic acid in water and placed at 4 °C until analysis by reversed phase chromatography.

Oxone Reaction Standard Conditions—Initial reaction mixtures contained 30 mM peptide and 30 mM nickel chloride in 0.1 M sodium phosphate, pH 8.2, to which oxone, also in 0.1 M sodium phosphate, pH 8.2, was added as a 10× stock to a final concentration of 30 mM. After approximately 5 min at room temperature, the oxone reactions were quenched by the addition of at 1:10 (v/v) of 0.5 M EDTA, pH 8, and then diluted 1:10 into 2% formic acid in water and placed at 4 °C until analysis by reversed phase chromatography.

With time, optimized conditions for the oxone reactions were established. Where noted as optimized oxone conditions, the peptides, NiCl2, and oxone stocks were made up in 0.2 M sodium bicarbonate, pH 8.2. Additionally, under optimized conditions, the oxone was added in five equal increments 2 min apart, rather than as a single bolus.

Amino Acid Analysis—Peptides were subjected to gas phase hydrolysis at 165 °C for 1 h in the presence of HCl containing 1% phenol, using a Savant AP100AminoPrep Hydrolyzer. Amino acids were analyzed using precolumn derivatization with AQC as described previously (16). Derivatization with 2,4-Dinitrophenyhydrazine—Derivatizations were performed as described previously (15).

1 The abbreviations used are: oxone, potassium peroxymonosulfate; AQC, 6-aminoninolinyl-N-hydroxysuccinimidyl carbamate; DNP, 2,4-di-nitrophenyhydrazine; HPLC, high performance liquid chromatography; LC-MS, HPLC interfaced directly to mass spectrometry.

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LC-MS—Electrospray mass spectrometry was performed using a Finnigan Mat LCQ™ ion trap mass spectrometer interfaced with an HP1090M HPLC. The LCQ™ spectrometer was calibrated as per the manufacturer’s recommendations, which provide for a specified accuracy of 0.01% for m/z masses of 100–2000 Da. Peptides were separated at room temperature using a Zorbax 300SB C18 column with a gradient of 1–40% acetonitrile in 0.1% trifluoroacetic acid/water (v/v) in 20 min at 1 ml/min, following an initial 4-min isocratic (1%) hold.

NMR Analysis—1H and 13C NMR spectra were collected on a Varian VXR-300S instrument. Peptides were dissolved in either deuterium oxide or deutero-Me2SO. Chemical shifts are reported relative to DSS for deuterium oxide samples or tetramethylsilane for deutero-Me 2SO samples. 1H chemical shift versus pH studies were performed using a D2O-phosphate system. No corrections of pH measurement for D 2O content were made.

RESULTS AND DISCUSSION

Reaction of the Ni(II)-complexed Tripeptide Ala-His-Ala with Peroxymonosulfate Produces N-terminal Oxidative Deamination—Reaction of the tripeptide Ala-His-Ala with Ni(II) and oxone under initial standard conditions resulted in >60% modification of the peptide. The modified peptide eluted as a broad, asymmetric peak several minutes later than the parent peptide peak when analyzed by reversed phase C18 HPLC (Fig. 1, A and B). The modified peptide displayed significantly increased absorption at 245 nm. Also, the modified peptide displayed a mass of 296.1 Da when analyzed by in-line mass spectrometry. This represents a discrete loss of 1 Da when compared with the parent peptide (296.1 versus 297.1 Da for the parent peptide). MS² fragmentation mapped the 1-Da loss to the A2, B2, and B3 fragment ions (Fig. 2, A and B). Y-type ions were not detected in either case. The ion of m/z 227, which can be attributed to Y2 structure, was instead assigned to loss of the C-terminal Ala residue from the AHA tripeptide (17). Ion fragment assignments were confirmed by MS³. A significant recovery of the histidine immonium fragment ion was observed (of m/z 110), which further indicated that the histidine residue was unaffected. These findings all suggested that the 1-Da mass loss was located on the N-terminal alanine, and the histidine residue remained unchanged.

The modified peptide was blocked to Edman protein sequencing, suggesting that a chemical modification had occurred at the N terminus. This finding was corroborated by a lack of reactivity with AQC reagent, which was used to probe for the presence of primary or secondary amines. Amino acid analysis of the modified peptide showed approximately one-half of the yield of alanine relative to histidine that was observed for the parent peptide. Also in contrast to the parent peptide peak, the modified peptide peak reacted quantitatively with DNP, yielding a derivatized peptide with 400 nm absorbance and mass of 476.1 Da (Fig. 3). This represents a mass gain of 180 Da, which is characteristic of a dinitrophenylhydrazone product and therefore indicative of the presence of a free carbonyl on the modified peptide. The presence of a free carbonyl, the apparent loss of free amino group reactivity, and the 1-Da mass decrease all indicated that the modified peptide was oxidatively deaminated at the N terminus, thus resulting in

![Fig. 1](#) Reversed phase C18 HPLC separation of Ala-His-Ala reactions under standard conditions in 0.1 M phosphate buffer, pH 8.2. A, parent peptide; B, after reaction with NiII/oxone; C, NiII/oxone oxidation followed by treatment with NaCNBH₃; D, after reaction with oxone, without NiII.)
an α-ketoamide peptide (Reaction I).

1H and 13C NMR analyses of the modified peptide peak were used to further verify this structural assignment. The 1H spectrum of the (putative) oxidatively deaminated peptide displayed a shift of the N-terminal alanine methyl group downfield from 1.23 to 2.32 ppm along with a resonance splitting change from a doublet to a singlet, consistent with formation of a ketone at the α-carbon of Ala-1 (Table I). The C-1 vinyl proton of the histidine imidazole ring was observed to be significantly deshielded relative to the parent peptide. However, an additional study that examined the effect of pH versus 1H chemical shift of the histidine C-2 vinyl proton demonstrated no significant change in pKₐ of the imidazole group following Ni(II)/oxone oxidation, yielding further evidence that the histidine side chain was not modified in the reaction (Fig. 4).

13C NMR also indicated the presence of an intact histidine imidazole ring, two amide carbons, one carboxylic acid carbon, and a downfield ketone carbonyl resonance at 196 ppm, all in agreement with oxidative deamination (Table II).

Sodium cyanoborohydride treatment of the modified peptide peak yielded quantitative reduction of the Ni(II)/oxone-oxidized AHA peptide, resulting in a species that eluted at 6.4 (solid line) and 7.3 (dotted line) min as shown in Fig. 1C; D, modified peptide from oxone reaction without Ni(II) (oxime). Annotation of fragment ions refers to parent peptide.

**Fig. 2.** MS² fragmentation spectra. A, parent AHA peptide; B, Ni(II)/oxone-reacted peptide (α-ketoamide); C, oxidized and NaCNBH₃ reduced peptide (α-hydroxamide) eluting at 6.4 (solid line) and 7.3 (dotted line) min as shown in Fig. 1C; D, modified peptide from oxone reaction without Ni(II) (oxime). Annotation of fragment ions refers to parent peptide.
and B3 fragment ions (Fig. 2D). From these findings, we postulated that this modification was likely the result of chemical oxidation of the terminal amine nitrogen to an oxime (18). ¹H proton NMR analysis confirmed this assignment. The N-terminal methyl resonance was shifted downfield from 1.23 to 1.85 ppm and resonated as a singlet. Also observed was the appearance of a single, sharp resonance far downfield at 11.86 ppm, which is consistent with an oxime proton.

**Reaction of the Ni(II)-complexed Tripeptide Ala-His-Ala with Sulfite and Oxygen Produces Identical N-terminal Oxidative Deamination—**Muller et al. (13) have postulated the in situ formation of HSO₅⁻ from SO₃²⁻ in the presence of ambient oxygen, Ni(II), and the tripeptide Lys-Gly-His-amide. Although Ni(II) complexes of this sequence motif have been reported to exhibit catalytic properties different from that of histidine at position 2 (7), we decided to test for indications of peroxymonosulfate formation from sulfite autooxidation on a peptide with histidine at position 2.

The tripeptide Ala-His-Ala was incubated with sodium sulfite in the presence of nickel under the standard conditions described above. This treatment was observed to produce modification of >60% of the peptide. The modified species displayed the same elution time, 1-Da mass loss, and MS² fragmentation pattern by LC-MS analysis as the oxidatively deaminated a-ketoamide species produced using oxone. Additional chemical tests, including Edman sequencing, DNP, and AQC reactivity, as described above for the modified product of AHA using Ni(II) and oxone (excepting proton NMR analysis), confirmed that an identical oxidatively deaminated AHA peptide was produced using Ni(II), sulfite, and ambient oxygen.

The sulfite reaction with AHA in the absence of added nickel was tested. It was found that in contrast to the oxone reaction, no oxime formation was observed. In fact, when nickel was omitted from the reaction of the AHA tripeptide with sulfite, no detectable modification of any kind was observed.

**Reaction of the Ni(II)-complexed Tripeptide Ala-Ala-Ala Shows No Evidence of Oxidative Deamination with Either SO₅²⁻ / O₂ or Oxone—**We have previously postulated that histidine at position 2 is an essential part of an oxidatively reactive Ni(II) binding site on the β-chain of human hemoglobin (15). Therefore, as a comparison with the AHA peptide, we examined the reactivity of the tripeptide AAA. Reaction of the AAA tripeptide with Ni(II) and oxone under standard conditions also resulted in >60% modification of the peptide. This modified peptide eluted primarily as a sharp, symmetrical peak several minutes later than the unmodified parent peptide peak when analyzed by reversed phase C18 HPLC (Fig. 5, A and B). The modified peptide displayed increased absorption at 250 nm and a discrete mass gain of 14 Da when analyzed by LC-MS compared with the parent peptide (245.1 versus 231.1 Da for the parent peptide.) This species was found to be blocked to Edman sequencing. MS² fragmentation also indicated that the modification was located on the N-terminal amino acid. Based on these findings, we concluded that this modification was an oxime on the N-terminal nitrogen. When nickel was omitted from the oxone reaction with the Ala-Ala-Ala tripeptide, an identical product was observed (Fig. 5C). Therefore, oxime formation appears to be entirely non-nickel-dependent.

Reaction of the tripeptide Ala-Ala-Ala with Ni(II) and sodium sulfite under standard (SO₅²⁻) conditions produced no detectable modification of any kind. This finding suggests that in the case of the Ala-Ala-Ala tripeptide, no peroxymonosulfate-like species is generated by that peptide in the presence of Ni(II), SO₅²⁻, and ambient oxygen. Histidine at position 2 therefore appears to be required for both susceptibility to oxidative deamination, as well as the apparent ability to generate a peroxymonosulfate-like species from sulfite and oxygen.

**Sequence Effects Studies Using Small Peptides Reacted with Ni(II) and Either SO₅²⁻ / O₂ or Oxone Show an Absolute Requirement for Histidine at Position 2 for Oxidative Deamination—**To further delineate sequence effects and requirements for susceptibility to oxidative deamination, we studied reac-

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**FIG. 3. Reversed phase separation of DNP-reacted peptides tested for the presence of a free carbonyl group.** A, blank; B, parent AHA peptide; C, Ni(II)/oxone-reacted peptide (α-ketoamide). Results indicate that oxidized Ala-His-Ala peptide contains a reactive carbonyl group. The observed mass of 476.1 Da is consistent with a dinitrophenylhydrazone derivative of the oxidized peptide (α-ketoamide).
tions of Ni(II) and a series of small peptides with either SO$_3$$^2$-$/$O$_2$ or oxone as the oxidant. We examined the reactions for any evidence of modification of the parent peptide. The results of these experiments are shown in Table III.

Almost all of the peptides with histidine at position 2 demonstrated significant susceptibility to oxidative deamination under both sets of conditions, whereas none of the peptides lacking histidine at position 2 demonstrated any oxidative deamination under either condition. It should be noted that in the cases of both AHA and GHG, a significant amount of the deaminated product from reaction at 30 mM peptide was found to be in the form of apparently diastereomeric pairs of relatively stable dimers (exhibiting a mass of 592 Da in the case of AHA peptide). These dimers were not observed when the SO$_3$$^2$-$/$O$_2$ reaction was performed at a peptide concentration of 3 mM, indicating that their formation was (peptide) concentration-dependent. Further characterization of the dimer by $^1$H NMR indicated it to be a product of aldol condensation of two mole-
cules of $\alpha$-ketoamide (data not shown). These dimers were not observed when oxone was used as the oxidant.

Unlike Ala-His, the dipeptides carnosine ($\beta$-alanyl-histidine) and homocarnosine ($\gamma$-amino butyryl-histidine) were found to be nonreactive to Ni(II)/SO$_3$$^2$-$/$O$_2$-promoted deamination. These results suggest that only $\alpha$-amines are susceptible to this pathway of deamination. Interestingly, these peptides showed very little oxime formation in the reaction with oxone. Peptides with proline as the first residue also produced noticeably different results. N-terminal proline, being a second-
ary amine, is not likely to be as susceptible to the oxidative release of ammonia as other amino acids. No released ammonia in either reaction with PHA was detected, and in both cases, a modified peptide that was formed demonstrated a loss of 2 Da (321 versus 323 Da for the parent peptide). The modified peptide was unreactive with AQC, indicating that the secondary amine was no longer present. This result would be consistent with formation of a cyclic imine species that was stable against spontaneous hydrolysis.

Peptides GGH and KGH-amide with a histidine residue in position 3 were also tested. Under similar conditions, these peptides have been shown by Muller et al. (13) and others (12, 14, 19) to produce radicals capable of causing DNA damage and protein cross-linking. No deamination of either the GGH or the KGH-amide peptides were detected in reactions with Ni(II)/
SO$_3$$^2$-$/$O$_2$. The KGH-amide peptide was modified in the reaction to an earlier eluting product exhibiting a mass gain of 80 Da, consistent with the displacement of one proton by an SO$_3$H group. This product was not further characterized. In the reactions with oxone, decarboxylation of GGH and other peptide degradation of both peptides were observed, none of which appeared to include deamination. By contrast, the tripeptide KHG-amide (bursin) appeared to readily N-terminally deami-
nate with both Ni(II)/SO$_3$$^2$-$/$O$_2$ and Ni(II)/oxone. However, the ketoamide product appeared to spontaneously form an intramolecular Schiff’s base with the $\epsilon$-amine of the lysyl side chain (Reaction II). This was confirmed by LC-MS, Edman sequencing, and reaction with DNP. Under these conditions, no evidence of $\epsilon$-amino side chain deamination was found.

Peptides with methionine and tyrosine as the N-terminal residues also appeared to present exceptions to susceptibility to oxidative deamination. The lack of reactivity of MH using sul-
fite is possibly due to sulfoxide formation acting as a “sink” for oxidative modification, although no substantial difference in the amount of methionine sulfoxide formed was seen relative to ML with either oxidant (20, 21). It is also conceivable that other chemical and electronic properties of the sulfur atom of the methionine side chain could affect the reaction pathway. The lack of deamination of the dipeptide YH is less clear. Stable tyrosine radicals have been reported in proteins (22, 23). However, no evidence of dityrosine, a known product of oxidative degradation of tyrosine involved in protein cross-linking, was detected (12, 22), and no evidence of tyrosine radical was found by EPR or found to be trappable by the method of Fenwick and English (24). However, with an increased excess of sulfite, we noticed significant accumulation of a product of the YH reac-
Nickel-catalyzed N-terminal Oxidative Deamination

1H NMR of parent AHA in Me2SO 1.22 ppm (3H, d, J = 7.32 Hz); 1.23 ppm (3H, d, J = 6.84 Hz); 2.94 ppm (2H, d, J = 5.86 Hz); 3.61 ppm (1H, q, J = 6.84 Hz); 4.00 ppm (1H, dq, J = 7.32, 0.87 Hz); 4.44 ppm (1H, dt, J = 7.81, 5.86 Hz); 6.75 ppm (1H, s); 7.54 ppm (1H, s); 8.16 ppm (1H, d, J = 7.08 Hz); 8.43 ppm (1H, d, J = 7.61 Hz).

1H NMR of oxidized AHA (Ni) in Me2SO (α-ketoamide) 1.29 ppm (3H, d, J = 7.33 Hz); 2.32 ppm (3H, s); 3.06 ppm (1H, dd, J = 15.14, 8.79 Hz); 3.21 ppm (1H, dd, J = 15.38, 4.88 Hz); 7.33 ppm (1H, s); 4.39 ppm (1H, dt, J = 8.79, 8.55 Hz); 7.33 ppm (1H, s); 8.39 ppm (1H, d, J = 7.08 Hz); 8.57 ppm (1H, d, J = 8.79 Hz); 8.96 ppm (1H, s).

1H NMR of oxidized AHA (no Ni) in Me2SO (oxime) 1.30 ppm (3H, d, J = 7.33 Hz); 1.85 ppm (3H, s); 3.07 ppm (1H, dd, J = 7.94, 15.75 Hz); 3.17 ppm (1H, dd, J = 5.25, 15.26 Hz); 4.23 ppm (1H, dq, J = 7.08, 7.32 Hz); 4.71 ppm (1H, m); 7.31 ppm (1H, s); 7.83 ppm (1H, d, J = 8.30 Hz); 8.49 ppm (1H, d, J = 7.08 Hz); 8.95 ppm (1H, s); 11.86 ppm (1H, s).

1H NMR of oxidized and reduced AHA (6.8 min) in Me2SO (α-hydroxyamide) 8.98 ppm (1H, s); 8.37 ppm (1H, d, J = 8.78 Hz); 7.83 ppm (1H, d, J = 9.03 Hz); 7.34 ppm (1H, s); 4.66 ppm (1H, dt, J = 8.48, 8.78 Hz); 4.23 ppm (1H, m); 3.95 ppm (1H, q, J = 6.84 Hz); 3.15 ppm (1H, dd, J = 15.26, 4.88 Hz); 2.98 ppm (1H, dd, J = 15.14, 8.79 Hz); 1.30 ppm (3H, d, J = 7.33 Hz); 1.10 ppm (3H, d, J = 6.84 Hz).

1H NMR of oxidized and reduced AHA (7.3 min) in Me2SO (α-hydroxyamide) 8.98 ppm (1H, s); 8.41 ppm (1H, d, J = 7.33 Hz); 7.83 ppm (1H, d, J = 8.79 Hz); 7.34 ppm (1H, s); 4.63 ppm (1H, m); 4.22 ppm (1H, m); 3.99 ppm (1H, q, J = 6.71 Hz); 3.13 ppm (1H, dd, J = 15.14, 5.37 Hz); 3.01 (1H, dd, J = 14.77, 7.81 Hz); 1.30 ppm (3H, d, J = 7.33 Hz); 1.16 ppm (3H, d, J = 6.71 Hz).

Table I

| 1H NMR results |
|----------------|
| 1H NMR of parent AHA in Me2SO | 1.22 ppm (3H, d, J = 7.32 Hz); 1.23 ppm (3H, d, J = 6.84 Hz); 2.94 ppm (2H, d, J = 5.86 Hz); 3.61 ppm (1H, q, J = 6.84 Hz); 4.00 ppm (1H, dq, J = 7.32, 0.87 Hz); 4.44 ppm (1H, dt, J = 7.81, 5.86 Hz); 6.75 ppm (1H, s); 7.54 ppm (1H, s); 8.16 ppm (1H, d, J = 7.08 Hz); 8.43 ppm (1H, d, J = 7.61 Hz). |
| 1H NMR of oxidized AHA (Ni) in Me2SO (α-ketoamide) | 1.29 ppm (3H, d, J = 7.33 Hz); 2.32 ppm (3H, s); 3.06 ppm (1H, dd, J = 15.14, 8.79 Hz); 3.21 ppm (1H, dd, J = 15.38, 4.88 Hz); 7.33 ppm (1H, s); 4.39 ppm (1H, dt, J = 8.79, 8.55 Hz); 7.33 ppm (1H, s); 8.39 ppm (1H, d, J = 7.08 Hz); 8.57 ppm (1H, d, J = 8.79 Hz); 8.96 ppm (1H, s). |
| 1H NMR of oxidized AHA (no Ni) in Me2SO (oxime) | 1.30 ppm (3H, d, J = 7.33 Hz); 1.85 ppm (3H, s); 3.07 ppm (1H, dd, J = 7.94, 15.75 Hz); 3.17 ppm (1H, dd, J = 5.25, 15.26 Hz); 4.23 ppm (1H, dq, J = 7.08, 7.32 Hz); 4.71 ppm (1H, m); 7.31 ppm (1H, s); 7.83 ppm (1H, d, J = 8.30 Hz); 8.49 ppm (1H, d, J = 7.08 Hz); 8.95 ppm (1H, s); 11.86 ppm (1H, s). |
| 1H NMR of oxidized and reduced AHA (6.8 min) in Me2SO (α-hydroxyamide) | 8.98 ppm (1H, s); 8.37 ppm (1H, d, J = 8.78 Hz); 7.83 ppm (1H, d, J = 9.03 Hz); 7.34 ppm (1H, s); 4.66 ppm (1H, dt, J = 8.48, 8.78 Hz); 4.23 ppm (1H, m); 3.95 ppm (1H, q, J = 6.84 Hz); 3.15 ppm (1H, dd, J = 15.26, 4.88 Hz); 2.98 ppm (1H, dd, J = 15.14, 8.79 Hz); 1.30 ppm (3H, d, J = 7.33 Hz); 1.10 ppm (3H, d, J = 6.84 Hz). |
| 1H NMR of oxidized and reduced AHA (7.3 min) in Me2SO (α-hydroxyamide) | 8.98 ppm (1H, s); 8.41 ppm (1H, d, J = 7.33 Hz); 7.83 ppm (1H, d, J = 8.79 Hz); 7.34 ppm (1H, s); 4.63 ppm (1H, m); 4.22 ppm (1H, m); 3.99 ppm (1H, q, J = 6.71 Hz); 3.13 ppm (1H, dd, J = 15.14, 5.37 Hz); 3.01 (1H, dd, J = 14.77, 7.81 Hz); 1.30 ppm (3H, d, J = 7.33 Hz); 1.16 ppm (3H, d, J = 6.71 Hz). |

Table II

| 13C NMR results |
|----------------|
| 13C NMR of oxidized AHA in Me2SO (α-ketoamide) | 16.84, 24.61, 26.40, 47.64, 51.37, 116.79, 129.12, 133.70, 160.60, 168.73, 173.74, 195.96 ppm. |

Fig. 4. pH dependence of the 1H chemical shift of the C-2 vinyl proton of His-2 imidazole side chain of parent AHA and Ni(II)/oxone-reacted peptide (α-ketoamide).

Fig. 5. Reversed phase C18 HPLC separation of Ala-Ala-Ala tripeptide reacted under standard conditions in 0.1 M phosphate buffer, pH 8.2. A, parent peptide; B, after reaction with Ni(II)/oxone; C, after reaction with oxone, without Ni(II).
Nickel-catalyzed N-terminal Oxidative Deamination

TABLE III
Amino acid sequence effects on oxidative modifications

| Peptide(s) | Ni/SO_4^2-/O_2 | Primary product                        | Ni/oxone                        |
|-----------|----------------|----------------------------------------|---------------------------------|
| AAA       | No reaction    | N-terminal oxime                       |                                |
| AHA       | Oxidative deamination | Oxidative deamination |                                |
| AHK       | Oxidative deamination | Oxidative deamination |                                |
| EHG       | Oxidative deamination | Oxidative deamination |                                |
| GH        | Oxidative deamination | Oxidative deamination |                                |
| GHG       | Oxidative deamination | Oxidative deamination |                                |
| GGH*      | No deamin./other modif. | Degradation |                                |
| KGH*      | Deamination leading to intramolecular Schiff's base | Deamination leading to intramolecular Schiff's base |                                |
| Carnosine | No reaction    | Trace of oxime                         |                                |
| Homocarnosine | No reaction | Trace of oxime                        |                                |
| HL        | No reaction    | N-terminal oxime                       |                                |
| IH        | Oxidative deamination | Oxidative deamination |                                |
| LH, LHL   | Oxidative deamination | Oxidative deamination |                                |
| LH + AAA  | LH, oxidative deamination; AAA, no reaction | LH, oxidative deamination; AAA, N-terminal oxime |                                |
| LL, LLL   | No reaction    | N-terminal oxime                       |                                |
| MH        | Methionine sulfoxide | Methionine sulfoxide and oxidative deamination |                                |
| ML        | Methionine sulfoxide | Methionine sulfoxide |                                |
| PHA       | Cyclic imine   | Cyclic imine                           |                                |
| VH        | Oxidative deamination | Oxidative deamination |                                |
| VHLTP     | Oxidative deamination | Oxidative deamination |                                |
| VL        | No reaction    | N-terminal oxime                       |                                |
| YH        | No oxidative deamination, sulfo-peptide formation | Trace of oxidative deamination/degradation |                                |

* C-terminal amide.

FIG. 6. Yield of oxidative deamination versus Ni(II) to LH peptide ratio for oxone and SO_4^2-/O_2 reactions under optimized conditions as described under "Experimental Procedures" (30 mM dipeptide and 30 mM oxone or sulfite).

A similar Ni(II) titration study using the LH dipeptide was conducted with oxone. In contrast to the SO_4^2-/O_2 system, under initial conditions, maximal oxidative deamination required nearly 1:1 Ni(II):peptide when oxone was used. However, under optimized conditions for the oxone reaction, the oxone was added to the reaction in five equal increments, rather than as a single bolus. In this case, 30% deamination was achieved using approximately 1:5 Ni(II):peptide (catalytic turnover of 1.5), suggesting that redox cycling of Ni(II) can be achieved in the oxone reaction as well (Fig. 6). Chromatograms of this “optimized” oxone reaction, along with a standard condition sulfite reaction, both with the LH dipeptide at 1:5 Ni(II):peptide, are shown in Fig. 7. It can be seen from these that the sulfite reaction results in significantly decreased degradative side products compared with oxone. It appeared that when nickel was limiting, oxime formation was a potentially competing side reaction. We therefore purified the LH oxime species and re-reacted it with Ni(II) and oxone under standard conditions. No evidence of oxidative deamination was observed, although significant heterogeneous, uncharacterized peptide degradation occurred. This finding suggested that oxime formation may be a competitive and kinetically relevant “dead-end” side reaction when Ni(II) is limited during reaction with oxone.

Reaction Kinetics for Oxidative Deamination of LH Dipeptide Reacted with Ni(II) and Either SO_4^2-/O_2 or Oxone—Studies were conducted to examine the kinetics of oxidative deamination of the LH dipeptide comparing the use of either SO_4^2-/O_2 or oxone (with sequential addition) as oxidants (Fig. 8). As can be seen, the oxidative deamination using oxone as the oxidant was completed within (at most) several minutes of initiation. By contrast, the reaction using SO_4^2- and ambient oxygen displayed a distinctly lower initial rate of deamination, which appeared to be relatively constant during the first 40 min of reaction. No further increase in deamination was observed beyond 6 h (up to 1 week). When this reaction was repeated under an oxygen-enriched head space (>95% O_2 at approximately 2 atm), no difference in the rate of peptide modification was observed. This finding suggested that oxygen diffusion is not rate-limiting under the standard conditions with sulfite. However, when oxygen was strictly excluded from the sulfite reaction (under argon), no peptide modification was observed. By contrast, exclusion of oxygen from the oxone reaction did not inhibit oxidative deamination.

Quenching and Inhibition Studies of Deamination of LH Dipeptide Reacted with Ni(II) and Either Sulfite or Oxone—Further studies were conducted to examine the effect of the addition of various compounds on the yields of oxidative modification in the LH/Ni(II) reactions using either sulfite or oxone. These additives included known chelating agents, radical scavengers, and different anions. The results of these experiments tabulated as percentage of inhibition of LH deamination under the respective standard conditions are listed in Table IV.

The lack of inhibition observed in the presence of ethanol, methanol, tert-butyl alcohol, or mannitol argues strongly against the involvement of diffusible SO_4^2- or OH- radicals me-
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Fig. 7. Reversed phase C18 HPLC separation of oxidative deamination reactions of LH dipeptide under optimized conditions. Top panel, 24-h reaction with Ni(II)/sulfite/oxygen; bottom panel, 10-min reaction with Ni(II)/oxone.

Fig. 8. Time course of oxidative deamination of LH peptide for oxone and SO₃²⁻/O₂ reactions under optimized conditions as described under “Experimental Procedures” (30 mM dipeptide and 30 mM oxone of sulfite).

Fig. 9. Yield of oxidative deamination versus oxidant to LH peptide ratio for oxone and SO₃²⁻/O₂ reactions under optimized conditions as described under “Experimental Procedures” (30 mM peptide and 6 mM Ni).

Table IV

| Reaction conditions  | Inhibition (%) |
|---------------------|----------------|
| Ni(SSO₃⁻)²⁻/O₂        | Ni/oxone       |
| 30 mM EDTA          | 100            | 100            |
| 30 mM ethanol       | 0              | 0              |
| 30 mM methanol      | 0              | 0              |
| 30 mM t-butyl alcohol | 0            | <5             |
| 30 mM BHA           | 18             | 16             |
| 30 mM thiourea       | >95            | 100            |
| 100 mM mannitol     | 0              | 0              |
| 1 mg/ml catalase    | <5             | <5             |
| 100 mM HCO₃⁻ (Na⁺)  | 65             | 0              |
| 15 mM CN⁻ (K⁺)      | 95             | 95             |
| 10 mM DMPO          | 51             | 12             |

BHA, butylated hydroxyanisole; DMPO, 5,5-dimethyl-1-pyrroline N-oxide.

Diating the oxidative deamination in either reaction (2, 26). The inability of catalase to inhibit either reaction suggests that it is unlikely that oxidative deamination in either case proceeds through formation of diffusible H₂O₂ followed by a Fenton-type reaction, as in a mechanistic scenario for oxidative protein carbonyl formation proposed by Stadtman (27). However, no test was conducted to determine whether this enzyme was inactivated by the reaction conditions, as has been reported for sulfite inactivation of several copper-dependent monoxygenases (28). The complete inhibition of oxidative deamination by the metal chelating agent EDTA would suggest that sequestration of nickel away from the peptide is the mode of action by which it precludes the reactions. The mechanism for inhibition exhibited by thiourea is less clear, but the differential effects of CN⁻ and HCO₃⁻ on reactions using SO₃²⁻/O₂ versus using
HSO₅²⁻ begins to suggest that these may act by perturbing the coordination structure of the reactive nickel complexes (29). Such differential effects also begin to suggest that the reactive complex that produces an actively oxidizing species from SO₃²⁻ may differ from the one involved in the reaction with preformed HSO₅²⁻ (oxone).

Comparative Sulfite and Oxone Titration Studies with Ni(II)-complexed LH Dipeptide—Titration studies under standard conditions using oxone as the oxidant showed that approximately 1:1 oxone:peptide was sufficient to approach maximal deamination of the LH dipeptide (Fig. 9). It was also observed that ≥2:1 oxone:peptide resulted in significant, heterogeneous, uncharacterized degradation of the peptide (much worse than the 1:1 shown in Fig. 7). By contrast, titration with SO₃²⁻ under standard conditions reproducibly showed that significantly more than 1 equivalent of SO₃²⁻ to peptide was required to approach maximal deamination. With 3 equivalents of sulfite,

$\text{pH Effect Studies of LH Dipeptide Reacted with Ni(II) and Either Sulfite/O₂ or Oxone Show High Relative Yields of Oxidative Deamination throughout a Physiological pH Range—Reactions with the LH dipeptide/Ni(II) system using oxone or sulfite/O₂ were examined for effects of reaction pH, the results of which are shown in Fig. 10. Although the oxone reaction showed inhibition below pH 6.5, both reactions displayed a high relative yield of oxidative deamination over the physiologically relevant pH range of 6.5–8.5. This finding lends support to the possibility that this model system for oxidative peptide modification may be relevant to mechanisms of nickel and sulfite toxicities in vivo (1, 3).}$

**Conclusions**—We have shown that the reaction of peptides containing histidine at position 2 with Ni(II) and oxone produces oxidative deamination of the peptides with a minimum of other significant modifications of the peptide, and we found no evidence of modification of the histidine residue (up to 1 equivalent of oxone, above which other degradative modifications occur). When Ni(II) was omitted from the reaction, oxime formation on the N-terminal nitrogen was produced instead. In peptides lacking histidine at position 2, only oxime formation was observed, regardless of whether Ni(II) was present or not.

The identical oxidative deamination of peptides containing His-2 can be produced by using Ni(II), sulfite, and ambient oxygen. A substoichiometric amount of nickel can serve to deaminate the peptides in high yields, indicative of redox cycling of Ni(II) in the reaction. However, when either nickel, sulfite, or oxygen was excluded, no oxidative deamination or any other modification was observed. No oxidative deamination was observed in any peptides lacking histidine at position 2. Significant modifications other than deamination were observed in reactions with both GGH and KGH-amide peptides. These peptides have been reported to promote protein cross-
linking and DNA damage under similar conditions. Additionally, the GGH peptide is known to undergo oxidative degradaton, including decarboxylation in the presence of ambient oxygen or other oxidizers (6, 14, 30). These results, along with our finding of oxidative deamination only on α-carbon amines in His-2 peptides, indicate a unique structural specificity for a sequence motif that was first discovered in the β-chain of human hemoglobin (15).

The finding that both sulfite/O₂ and oxone produce an identical α-ketoamide suggests that both pathways may share a common reactive intermediate. The Ni(II)-catalyzed formation of peroxymonosulfate from sulfite and oxygen would appear to be consistent with these results (13). However, co-incubation of peptides lacking histidine at position 2 exposed to the Ni(II), sulfite, and oxygen system showed no evidence of oxime formation, suggesting that no diffusible peroxymonosulfate is produced during the deamination reaction. Additional quenching studies using well characterized radical scavengers appear to rule out diffusible OH⁻ or SO₄²⁻ as mediating any of the oxidative deamination observed (2, 26).

We postulate that the reactions of Ni(II) complexed His-2 peptides with either oxone or an in situ formed, nondiffusing, peroxymonosulfate-like species generated catalytically by sulfite autooxidation produce a common imine intermediate. All of our results would be consistent with a pathway involving imine formation at the N terminus, followed by spontaneous hydrolysis (with the exception of proline, which apparently forms a stable cyclic imine). The finding of ammonia release in all cases of oxidative deamination is consistent with such a pathway. This postulated pathway is shown in Fig. 11. Imine formation in oxidative deamination has been postulated by Stadtman (27) to be preceded by radical formation on the carbon adjacent to the departing amine group. If such is the case for Ni(II)-catalyzed N-terminal oxidative deamination, the radical appears inaccessible to quenching by common radical scavengers, i.e., a type of “caged” radical. Results with YH dipeptide suggest that the (tyrosyl) side chain can redirect the outcome of the reaction of such a complexed radical.

These findings are of interest from several perspectives. They provide a defined, potentially physiologically relevant model system with which to study the mechanism of a metal-catalyzed protein carbonyl formation, a modification known to be a frequent consequence of oxidative damage to proteins in vivo (31, 32). Such a defined system may provide an opportunity for further elucidating mechanisms underlying the known toxicities of sulfite, a ubiquitous environmental contaminant (2, 3). Additionally, our findings point toward a facile method for the intentional introduction of a carbonyl “handle” into natural and engineered proteins and peptides, under conditions that produce relatively few undesirable side reactions, a technology of significant potential utility (33, 34).

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