Identification of a Nickel(II) Binding Site on Hemoglobin Which Confers Susceptibility to Oxidative Deamination and Intramolecular Cross-linking*

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Transition metal-catalyzed oxidative damage to proteins has been implicated in a variety of adverse physiological processes including aging (1), atherosclerosis (2), and ischemic reperfusion injury (3). The metal binding site-specific nature of metal-catalyzed oxidation of proteins has been well established (4, 5). Oxidative modification of proteins in vivo has been postulated to produce carbonyl groups on amino acid side chains, including the formation of γ-glutamyl semialdehyde from arginine (6), and 2-amino adipic semialdehyde from lysine (7). Investigators have used model systems employing Fe(II) and H₂O₂ to study oxidative modification of proteins and peptides in vitro, and the physiological relevance of such Fenton chemistry models in elucidating mechanisms of in vivo oxidation of proteins is widely accepted (7, 8).

An application of this type of chemistry to intentionally effect oxidative intermolecular cross-linking specific to complexed proteins in vitro has been recently reported (9–12). That technique typically employs the addition of an exogenous tripeptide GGH, nickel(II), and strong oxidant (peracid), which together form a high valent metal complex that propagates covalent bond formation between associated proteins. Nickel(II) complexes of histidyl peptides have also been reported to exhibit Fenton reaction activity with hydrogen peroxide (13).

Previously, the investigation of metal-catalyzed protein oxidation of hemoglobin and other heme proteins has been complicated by the interaction of the heme iron with peroxides. This reaction can produce significant heme oxidation and protein degradation through the formation of the highly unstable ferryl-heme species (14). We report here a reaction of hemoglobin and oxidants under conditions in which oxidative cross-links between protein subunits can be generated without causing appreciable heme iron oxidation, protein denaturation, or aggregation. A metal binding site on hemoglobin appears to promote covalent bond formation between the β globin subunits upon addition of oxone in the presence of nickel. This reaction also results in a substantial yield of a modification that we have identified as oxidative deamination of the β globin amino terminus, generating in its place an α-ketoamide. Results from our studies indicate that the β globin His-2 is required for redox active nickel complexation, oxidative deamination, and intramolecular cross-linking.

MATERIALS AND METHODS

Recombinant Hemoglobins—Expression of recombinant hemoglobin in Escherichia coli has been previously reported (15, 16). Recombinant human hemoglobin rHb69 was genetically engineered with the replacement of the wild type valine residues at all NH₂ termini with methionine, and a fusion of two α globins with a single glycine amino acid linker. All other recombinant hemoglobins were β globin variants of rHb69 and were expressed in the same Escherichia coli background. In the expression system used, the initiating methionine residue is quantitatively removed by endogenous E. coli methionyl-aminopeptidase when the second amino acid expressed was alanine but was fully retained when the second residue was histidine or leucine (17). All mutants used in this study had an identical dι-a globin and the differences between them occurred only in the amino- and carboxyl-terminal segments of the β globin, which are listed in Table I. Directed mutagenesis by polymerase chain reaction was used to create the β globin mutants with amino acid substitutions after the initiating methionine codon. Separate oligonucleotides were used to introduce these changes to the β subunit by amplification from a rHb69 β globin gene template, and subsequent cloning of the polymerase chain reaction fragments into a high copy number vector for expression (18). Candidates were screened by restriction digestion and confirmed by DNA sequencing. All recombinant hemoglobins were purified from E. coli expression strains by a procedure previously reported (19). The hemoglobins were sparged with carbon monoxide and buffer exchanged into 0.1 M sodium phosphate, pH 8.3, by Sephadex G25 chromatography. Final primary sequence of each mutant was confirmed by both automated Edman sequencing and LC-MS analysis.

Cross-linking Reaction Conditions—Cross-linking reactions were carried out in 1.6-ml Eppendorf tubes in a total volume of 100 μl in 0.1 M sodium phosphate, pH 8.3, with final hemoglobin concentration of 0.4 mM and a final nickel chloride (Aldrich) concentration of 0.8 mM. Hemoglobin solutions were retreated with carbon monoxide by a brief
head space sparge, after which a 16 mM solution of oxone (from Sigma) in 0.1 M sodium phosphate, pH 8.2, was added to a final concentration of 1.6 mM, and the tubes were placed on ice for 1 h under an argon head space. Reactions were quenched by addition of EDTA to a final concentration of 10 mM. Where noted, quenched reactions were treated with reductant by addition of 100 µM 1 M DTT (Bio-Rad) or 100 µM 1 M sodium cyanoborohydride (Sigma) both in 0.1 M sodium phosphate, pH 8.2. The reactions with DTT and sodium cyanoborohydride were placed under argon at room temperature for 10 min or 2 h, respectively, prior to precipitation of the globins with acidified acetone (20).

Analytical C3 HPLC Separation—Recombinant hemoglobin was diluted to 2 mg/ml in HPLC grade water. Where specified, heme was removed from globins by precipitation with 20 volumes of cold 0.6% HCl in acetone and resolubilized in 2% (v/v) formic acid to a final concentration of 2 mg/ml. Approximately 100 µg of protein was injected onto an HP 1090IM using a Zorbax 300SB-C3 HPLC analytical column (0.46 × 25-cm) at 40 °C. Solvent A was 0.1% (v/v) trifluoroacetic acid in water and solvent B was 0.1% (v/v) trifluoroacetic acid in acetonitrile. The flow rate was maintained at 1 ml/min. Gradient conditions were as follows: isocratic 35% solvent B for 5 min, then the percentage of solvent B was increased to 49% over 45 min. Eluant was monitored at 215, 280, and 400 nm. In some cases the eluant was also monitored for detection of di-tyrosine by the method of Heinecke et al. (21) using a Hewlett-Packard 1046A programmable fluorescence detector employing excitation and emission wavelengths of 325 and 410 nm, respectively.

SDS-PAGE Analysis—Hemoglobin from native state reactions (approximately 15 mg/ml) were diluted 1:5 with water and then 1:1 with NOVEXTM SDS sample buffer. Where noted, the reducing (2 ×) sample buffer contained 100 mM DTT. Sample loads were about 20 µg/well. Nondenaturing Size Exclusion Chromatography of Native State Hemoglobin—Hemoglobins, including those taken directly from reactions, were diluted to 1 mg/ml in phosphate-buffered saline, pH 7.8. Injection volumes of 50 µl were analyzed using a Pharmacia Superose 12 HR
column (1 x 30-cm) with a flow rate of 0.5 ml/min in phosphate-buffered saline, pH 7.8. Chromatography was performed using an HP1090M (Hewlett-Packard) HPLC and monitored at 280 and 400 nm.

Electrospray Mass Spectrometry—Electrospray mass spectrometry (LC-MS) was performed using a Finnigan MAT LCQ 228 interfaced with an HP1090M as described previously (22).

Tryptic Mapping—Tryptic mapping was performed using Poroszyme trypsin cartridges (22). Separation was performed using a Zorbax 300SB-C18 column (0.46 x 25-cm). The eluant was monitored at 215, 280, and 400 nm.

Pepsin Mapping—Pepsin mapping was performed using immobilized enzyme from Pierce. Dried globins were resolubilized to approximately 2 mg/ml in 2% formic acid, and enzyme suspension was added to approximately 1:4 v/v. Digests were incubated at 30 °C with gentle shaking for 2 h after which the immobilized enzyme was removed by microcentrifugation. 50 μl of the reaction supernatant was injected for reversed phase HPLC chromatography using a Vydac C18 column. Separation conditions were as follows: isocratic 1% B for 5 min, then buffer B was linearly increased to 70% B over 69 min at a flow rate of 1 ml/min. Buffer A was 0.1% trifluoroacetic acid (v/v) in water and buffer B was 0.1% trifluoroacetic acid (v/v) in acetonitrile. The eluant was monitored at 215, 280, and 400 nm.

Detection of Ni(II) Complexation—The stoichiometry of Ni(II)-rHb binding in phosphate-buffered saline at pH 7.4, 25 °C, rHb (4 μM) was determined by titrating with Ni(II)SO4 (Sigma). The absorbance change at 240 nm was monitored using a Shimadzu-2101 UV-visible spectrophotometer and plotted as normalized change versus the ratio of Ni(II):Hb.

Derivatization with 2,4-Dinitrophenylhydrazine—Using a modified procedure of Levine et al. (23), the reaction mixture for derivatization of lyophilized reversed phase HPLC separated peptides was prepared as follows: a room temperature saturated solution of DNP (Aldrich) was prepared fresh in neat methanol and centrifuged briefly at 10,000 x g.

The supernatant was diluted 1:1 with deionized water and centrifuged again. The DNP supernatant was added to substrate using 50 μl/100 μg of peptide. Concentrated HCl was then added at 1 μl per 50 μl, and the reaction mixture placed at room temperature for 1 h. Reactions were diluted 1:1 with 2% formic acid in water prior to reversed phase HPLC-MS analysis.

In the case of reactions with native state hemoglobin, the DNP supernatant was mixed 1:1 with the EDTA-quenched hemoglobin/nickel/oxone reaction mixtures and the combined mixture was placed at room temperature for 1 h. Hemoglobin was then precipitated with acidified acetone prior to peptide mapping (22).

Protein Sequencing—The sequence of proteins and peptides was determined by automated Edman degradation chemistry using a Porton 2090 gas phase sequencer. Beckman peptide supports were used as recommended by the manufacturer. PTH-amino acids were identified by reversed phase chromatography on a modified Hewlett-Packard 1090L HPLC using a Hewlett-Packard AminoQuant column.

Amino Acid Analysis—Protein and peptides were subjected to gas phase hydrolysis at 165 °C for 1 h in the presence of HCl containing 1% phenol, using a Savant AP100 AminoPrep hydrolyzer. Amino acids were analyzed using precolumn derivatization with 6-aminoquinolinol-N-hydroxysuccinimidyl carbamate as described previously (24).

RESULTS

Reaction of Human Hemoglobin A0 and Ozone in the Presence of Nickel(II) Produces Nonreducible, Intramolecularly Cross-linked Globin Dimers—The reaction of carbon monoxide liganded human hemoglobin A0, Ni(II), and ozone at pH 8.2, produced a substantial amount of dimerized globins as measured by denaturing SDS-PAGE analysis (Fig. 1, lanes 2 and 3). Addition of Ni(II) only showed no change from the control.
Following treatment with DTT, the reacted hemoglobin still contained a similar amount of dimeric globin species, which were not observed in the DTT-treated control reaction (Fig. 1, lanes 4 and 5). In contrast, nondenaturing size exclusion chromatography of the Ni(II)/oxone-treated hemoglobin showed less than 2% in total content of higher molecular weight species (i.e., greater than 64 kDa), indicating that relatively little intermolecular protein cross-linking was caused by the reaction (data not shown). Spectrophotometric analysis of hemoglobin following the reaction with Ni(II)/oxone did not indicate an increase in methemoglobin content. CD spectroscopy revealed small but significant changes in molar ellipticity in the Soret region with an isoelectric point at 430 nm (data not shown). These results suggest that the reaction of human hemoglobin and oxone in the presence of Ni(II) produces mainly intramolecular cross-linking of the protein, which is not disulfide mediated. Interestingly, the hemes remain ferrous throughout the cross-linking reaction. The changes detected in the Soret region of the CD spectrum suggest some change in protein structure, possibly indicative of changes in chain-chain interactions or other structural perturbations influencing aromatic residues surrounding the heme pockets (25, 26). These minor spectral changes did not reflect the dramatic change in oxygen affinity observed for hemoglobin A0 following the reaction with Ni(II)/oxone. Oxygen affinity increased with p50 dropping from 10.0 to 4.1 torr, and cooperativity (Hill nmax) decreased from 2.8 to 1.0.

**Reaction of Recombinant Hemoglobin and Oxone in the Presence of Nickel(II) Produces Intramolecular Dimerization of the β Globins**—The recombinant hemoglobin rHb67 is structurally distinct from human hemoglobin A0 in that the wild type NH2-terminal valine of β globin is substituted by alanine. Additionally, the α globins of rHb67 are genetically fused by a single glycine amino acid linker and the wild type NH2-terminal valine of the first α globin domain is substituted by methionine. The reversed phase HPLC chromatograms of both human hemoglobin A0, and the recombinant hemoglobin rHb67, before and following the reaction with Ni(II) and oxone, are shown in Fig. 2. It can be seen that the genetic fusion of the α globins in rHb67 affords considerably increased separation between elution of the two globin types. This allows for discrete separation of a new peak formed in the reaction, which elutes between them. Area integration of the chromatogram indicates approximately 50% of the β globin in rHb67 was converted to this form, as shown in Fig. 2. This peak was not resolved from oxidized α globin peaks in the case of (reacted) human hemoglobin A0. Fluorescence monitoring of this new peak by the method of Heinecke et al. (21) yielded no evidence of di-tyrosine formation. When 50 mM EDTA was included in the reaction with Ni(II)/oxone, this modified globin peak was not observed (data not shown).
analyzed by reducing SDS-PAGE and showed greater than 80% of the protein migrated as a 32-kDa protein, apparently as a dimeric $\beta$ globin (Fig. 4).

Consistent with the results seen with human hemoglobin $A_0$, the reaction of rHb67 also did not result in appreciable formation of multimeric hemoglobin (<2%), as evidenced by native state size exclusion chromatography. In addition, the reaction of recombinant hemoglobin in the presence of Ni(II)/oxone did not cause any significant change in the visible absorbance spectrum. These results indicate that the reaction of rHb67 and oxone in the presence of nickel(II) produces intramolecularly cross-linked $\beta$ globin, most of which does not appear to be disulfide bond mediated, and that just as in the case of human hemoglobin $A_0$, these cross-links can be formed without appreciable oxidation of the ferrous hemes.

Characterization of Hemoglobins Oxidatively Modified by Oxone in the Presence of Nickel(II) Evidences Oxidative Deamination of the $\beta$ Globin Amino Terminus—The monomeric and dimeric $\beta$ globin fractions separated by reversed phase HPLC from Ni(II)/oxone-reacted rHb67 were analyzed by peptide mapping using an in-line immobilized trypsin cartridge method (22) and monitored by UV and LCQ™ mass spectrometry. $\beta$ globin purified from unreacted rHb67 by reversed phase HPLC was also analyzed as a control. The peptide maps of these three fractions are shown in Fig. 3. It can be seen that both fractions from hemoglobin reacted with Ni(II)/oxone show distinct differences compared with $\beta$ globin from the control sample. Since the control hemoglobin map was well characterized (22), LC-MS mass analysis allowed us to readily identify most of the major difference peptides produced in the reaction. These difference peaks included oxidized methionine and cysteine containing peptides that exhibited mass gains of 16 and 48 Da, respectively. These mass gains are consistent with oxidation of methionine to methionine sulfoxide and cysteine to cysteic acid, and these peptide assignments were confirmed by MS/MS fragmentation. Such side chain oxidations could be expected for the sulfur-containing amino acids of a protein exposed to these concentrations of the chemical oxidant oxone (28). More surprising was the finding that the level of NH$_2$-terminal $\beta$ globin tryptic peptide $\beta1$ (Ala$_1$-Lys$_8$), eluting at 34.6 min, was markedly decreased in the monomeric and dimeric $\beta$ globin fractions from Ni(II)/oxone-treated hemoglobin. In both cases a new, asymmetric peak appeared. This new peak eluted at approximately 41 min, and displayed a mass exactly 1 Da less than the nominal $\beta1$ peptide. MS/MS spectra of this new peak showed striking commonality of fragment masses with the nominal $\beta1$ peptide (Fig. 3). The 1-Da mass loss consistently mapped to the B$_2$ ion. Since the mass of the B$_1$ ion was outside the range of the LCQ™ spectrometer in these MS/MS experiments, further mapping of the 1-Da mass loss was not possible. This peptide was found to be blocked to automated Edman protein sequencing. Amino acid analysis of this peptide yielded the predicted
amino acid composition of the nominal β1 peptide, except for the NH2-terminal alanine, which was not recovered. Reaction of this new peptide with 2,4-dinitrophenylhydrazine analyzed by LC-MS yielded evidence of formation of an adduct (two stereoisomers) with characteristic absorbance at 400 nm and mass of 1102 Da, consistent with hydrazone formation, indicating the presence a free carbonyl group. No reactivity with DNP was seen with the nominal β1 peptide. Cyanoborohydride treatment of the oxidized peptide (41 min peak in trypsin map, Fig. 3) was seen to yield a doublet of peptides, both exhibiting a mass increase of exactly 2 Da, (i.e. 1 Da more than the nominal β1 peptide). These findings suggested that the β globin NH2 terminus had undergone an oxidative deamination in which the NH2-terminal amino group was replaced with a free carbonyl group. Reduction with cyanoborohydride created chirality at the α carbon of the former NH2-terminal alanine producing two diastereomeric alcohols.

Ni(II)/oxone reacted rHb67 hemoglobin treated with 2,4-dinitrophenylhydrazine prior to globin precipitation and trypsin mapping also yielded two peaks, which were absent in the control sample (Fig. 6). Masses of both isoforms were identical and consistent with stereoisomeric hydrazone adducts to deaminated globin. No evidence for oxidative deamination produced by the reaction at any other site on the hemoglobin was found.

Surprisingly, although baseline “noise” appeared to be increased, the map of dimeric β globin did not display any significant difference peaks that would correspond to identifiable peptide cross-links connecting the two globins. We also noted a marked decrease in the yield of the COOH-terminal peptides β15 (Val133-Lys144) and β15,16 (Val133-His146) in the dimeric β globin fraction, along with the loss of the β1 peptide.

Histidine at Position 2 of β Globin Is Required for Both β Globin NH2-terminal Dimerization and Intramolecular β Globin Dimerization—The LC-MS data from trypsin maps of Ni(II)/oxone-oxidized human hemoglobin A0 also showed a loss of the β1 (Val1-Lys8) tryptic peptide and a corresponding increase of oxidatively deaminated peptide. However, there was no indication that the yield of nominal α1 (Val1-Lys8) peptide of the α globin was diminished in the reaction. An extensive query for a peptide(s) exhibiting 1-Da loss from the nominal α1 peptide failed to yield any evidence of oxidative deamination of the α globin NH2 terminus. Since valine is the NH2-terminal residue for both the α and β globin chains of A0 human hemoglobin, we wondered if a primary sequence difference between α and β globin was conferring susceptibility to NH2-terminal deamination of valine specifically to the β globin.

**Table II**

| Ions ID | Observed m/z | Predicted m/z | Fragment type |
|---------|-------------|---------------|---------------|
| 1       | 100.1       | β15,16_B1     |
| 2       | 199.1       | β15,16_B2     |
| 3       | 270.2       | β15,16_B3     |
| 4       | 326.9       | β15,16_B4     |
| 5       | 497.6       | β15,16_B5     |
| 6       | 611.4       | β15,16_B6     |
| 7       | 681.9       | β15,16_B7     |
| 8       | 795.7       | β15,16_B8     |
| 9       | 866.4       | β15,16_B9     |
| 10      | 1004.2      | β15,16_B10    |
| 11      | 1003.6      | β15,16_B11    |
| 12      | 751.6       | (β15,16-17)   |
| 13      | 820.8       | (β15,16-17) + β11,12 |
| 14      | 877.5       | (β15,16-17) + β13,14 |
| 15      | 928.5       | (β15,16-17) + β15,16 |
| 16      | 975.8       | (β15,16-17) + β17,18 |
| 17      | 1041.1      | (β15,16-17) + β19,20 |
| 18      | 1105.8      | (β15,16-17) + β21,22 |
| 19      | 147.1       | β1, Y1        |
| 20      | 276.2       | β1, Y2        |
| 21      | 404.9       | β1, Y3        |
| 22      | 502.1       | β1, Y4        |
| 23      | 603.3       | β1, Y5        |
| 24      | 717.3       | β1, Y6        |
| 25      | 853.5       | β1, Y7        |
| 26      | 1353.6      | (β15,16-17) + β15,16-Y8 |
| 27      | 1490.6      | 1491.4, 1492.6 (+2) (β15,16-17) + β15,16-Y8 |
| 28      | 1562.6      | 1562.6 (β15,16-17) + β15,16-Y9 |
| 29      | 838.1       | 838.3 (+2) (β15,16-17) + β15,16-Y10 |
| 30      | 873.5       | 873.8 (+2) (β15,16-17) + β15,16-Y11 |
| 31      | 930.6       | 930.8 (+2) (β15,16-17) + β15,16-Y12 |
| 32      | 986.0       | 986.3 (+2) (β15,16-17) + β15,16-Y13 |
| 33      | 1015.3      | 1015.9 (+2) (β15,16-17) + β15,16-Y14 |
| 34      | 1044.2      | 1044.4 (+2) (β15,16-17) + β15,16-Y15 |
| 35      | 1079.7      | 1079.9 (+2) (β15,16-17) + β15,16-Y16 |
| 36      | 1129.7      | 1129.4 (+2) (β15,16-17) + β15,16-Y17 |

* (+2) indicates double charged ions.
phase HPLC (Fig. 8). These results appeared to confirm that the presence of histidine at position 2 of globin was monitored by in-line LCQ mass spectrometer to confirm peptide assignments. Loss of NH$_2$- and COOH-terminal peptides of the β globin was observed. Oxidation of sulfur-containing peptides was also detected.

One potentially significant difference between α and β globins was the presence of histidine at position 2 of β globin. Histidine is known to form transition metal coordination sites in proteins (29), and the oxidative deamination of the β globin amino terminus appeared to require Ni(II). We postulated that this histidine might be required for deamination of the adjacent amino acid on the β chain. To test this, we constructed and expressed a recombinant hemoglobin mutant designated rHb66, which was identical to rHb67 except that the histidine at position 2 of the β globin was substituted with alanine. rHb66 and rHb67 were identically prepared and reacted with oxone in the presence of nickel(II). Trypsin maps of the two reactions revealed that the β globin of rHb66 was not oxidatively deaminated by the reaction (Fig. 7), nor was any cross-linking of the β globins in reacted rHb66 observed by reversed phase HPLC (Fig. 8). These results appeared to confirm that the histidine at position 2 of β globin is a key component of both the nickel-dependent oxidative deamination and intramolecular β globin cross-linking.

To further test this hypothesis, another pair of recombinant hemoglobin mutants were constructed both with methionine substituted for alanine at position 1 of the β globin and engineered with either leucine or histidine at position 2 of the β globin. This pair, designated rHb68 and rHb69, respectively, along with rHb66 and rHb67, were identically prepared and subjected to the reaction with oxone in the presence of Ni(II). Reversed phase HPLC analysis of these reactions showed that only the hemoglobins with histidine at position 2 were observed to form the peak identified as intramolecularly cross-linked β globin dimer (Fig. 8). The Met-1 of rHb69 was found to be quantitatively oxidized to methionine sulfoxide in the reaction. Very little peptide with a mass consistent with both NH$_2$-terminal deamination and methionine oxidation was found. Although the His-2 was seen to be required for cross-link formation, we were unable to find any evidence that the His-2 was itself modified in the reaction with either rHb67 or rHb69.

The same four recombinant hemoglobins described above were titrated with nickel sulfate and absorbance changes at 240 nm were monitored spectrophotometrically. The two mutants containing histidine at position 2 of β globin showed a saturable absorbance change indicative of two metal binding sites (as did human hemoglobin A$_0$, data not shown), whereas the two mutants lacking a histidine at position 2 showed no change at 240 nm, indicating the absence of similar Ni(II) binding (Fig. 9). This finding suggests the presence of two unique binding sites for Ni(II) on hemoglobin, which are absent in mutants lacking β globin His-2.

Characterization of β Globin Dimer Suggests Involvement of Heterogeneous Cross-links between the Amino- and Carboxy-terminal Regions of Adjacent β Globins—Comparative trypsin maps of oxone-treated human hemoglobin A$_0$ and rHb67 both displayed significant loss of the β15 (Val$_{113}$-Lys$_{84}$) and β15,16 (Val$_{113}$-His$_{146}$) tryptic peptides relative to the controls (data not shown). This was unexpected because the β15,16 peptide contains no methionines or cysteines, which might be oxidized by oxone. Comparative pepsin mapping of the reversed phase HPLC separated monomeric and dimeric fractions of reacted rHb67 β globin also showed a substantial decrease in the amount of a COOH-terminal peptide peak Lys$_{84}$-His$_{146}$ in the dimeric fraction relative to the monomeric fraction (data not shown). Pepsin mapping also revealed two disulfide cross-linked peptides in the dimeric fraction. These were identified as a homodimeric Val$_{111}$-Glu$_{121}$ peptide linked by a disulfide bridge between Cys-112, and a heterodimeric peptide of Val$_{111}$-Glu$_{121}$ and Ser$_{199}$-Asn$_{192}$ linked by a disulfide bond between Cys-112 and Cys-93. These disulfide cross-linked peptides disappeared from the maps when the reaction mixtures were treated with DTT (or cyanoborohydride) prior to peptide mapping. Because treatment with these reducing agents did not lower the yield of dimeric β globin shown by reversed phase HPLC analysis, it appears that these readily reducible disulfide cross-links are secondary to the oxidative dimerization of the β globins. No other significant difference peptides were found in either pepsin or trypsin maps that would correspond to an oxidative cross-link between two β globins.

The recombinant hemoglobin rHb67 was also subjected to the Ni(II)/oxone reaction after which sodium cyanoborohydride...
was added to the reaction prior to denaturation with acidified acetone. In this case, trypsin mapping yielded evidence of a low level of cross-linked peptide containing both the deaminated β1 (923 Da) and the β15,16 (1449.7 Da) tryptic peptides. This peptide exhibited an average mass of 2356.4 Da, which is consistent with the predicted mass (923 + 1449.7 – 18 + 2 = 2356.7) for a peptide cross-linked by a reduced Schiff’s base between an oxidatively deaminated β1 and the β15,16 tryptic peptides.

**A**<sup>*</sup>-H-L-T-P-E-E-K
| NH |
|---|
| V-V-A-G-V-A-N-A-L-A-H-K-Y-H |

**SEQUENCE 2**

A<sup>*</sup> represents deaminated NH<sub>2</sub>-terminal alanine residue.

Confirmation that this peptide (which eluted as a very minor doublet, possibly diastereomers) was the product of a reduced Schiff’s base condensation of these two peptides was made using orthogonal analyses. Protein sequencing of this peptide yielded an unusual double sequence. In the first cycle only valine was observed, as expected for the β15,16, but with no yield of alanine as would be expected from a β1 peptide. In the next cycles, however, we could clearly read the expected sequences of both the β15,16 and β1 peptides. In cycle 14, only a residual yield of the expected lysine (Lys-144) was observed, but cycle 15 showed a normal yield of tyrosine (Tyr-145). In the final cycle His-146 was not detected, which was inconclusive since hydrophilic COOH-terminal residues are often poorly recovered. These sequencing data suggested that the first residue (deaminated NH<sub>2</sub>-terminal alanine) was cross-linked to Lys-144 by a reduced Schiff’s base. Reduction would create a secondary amine at the (former) NH<sub>2</sub> terminus of the β1 peptide which could undergo Edman chemistry, cleavage and then allow further sequencing of the β1 peptide. This secondary amine would be linked to the side chain of Lys-144, which in consequence would be absent in cycle 14 of the Edman sequencing. MS/MS fragmentation spectra of the triply charged cross-linked peptide (m/z 786.3), although complicated, also appeared to confirm a cross-link between deaminated β1 and the β15,16 peptide. The spectra showed a series of B ions from the β15,16 peptide with no corresponding Y ions. However, an apparent Y ion series from the β15,16 peptide cross-linked to oxidatively deaminated β1 peptide was observed (ions 26–36, Fig. 10 and Table II). Also, a series of Y ions from the β1 peptide was seen, along with corresponding B ions of a deaminated β1 peptide cross-linked to the β15,16 peptide (ions 12–18, Fig. 10 and Table II). Such an ion series is consistent with fragmentation of the β1 and β15,16 peptides cross-linked by a secondary amine formed between the deaminated Ala-1 and Lys-144 after cyanoborohydride reduction of their Schiff’s base condensation product.

This reduced Schiff’s base cross-link, found only following cyanoborohydride reduction, was present in an amount too low to account for most of the cross-linking seen. This observation prompted us to test whether β globin dimerization was primarily dependent on Schiff’s base formation between deaminated β globin NH<sub>2</sub> terminus and lysine 144 on the other β globin. We produced a hemoglobin mutant, rHb98 (β globin K144A). This mutant formed an intramolecular β globin dimer under the defined Ni(II)/oxone conditions to the same extent as rHb67 (Table I), suggesting that Schiff’s base condensation between the newly formed free carbonyl at the NH<sub>2</sub> terminus and Lys-144 from the opposing β globin was not the predominant mechanism of the cross-linking.

The β globin COOH-terminal region is rich in aromatic residues, which are potential targets for oxidative cross-linking (5, 30). To test for specific involvement of these residues in cross-linking, several other variants of rHb98 were also tested (Table I). Two of these mutants contained one additional β globin substitution to rHb98, either H143A, or Y145A, (designated rHb95 and rHb97, respectively). Also tested was a variant of rHb89 with an additional substitution of Y145H (the Bethesda mutation), designated rHb96, as well as a variant of rHb67 with a deletion of the COOH-terminal His-146 residue, designated rHb80. These mutants were found to form intramolecular β globin dimers in the Ni(II)/oxone reaction to about the same extent as rHb67, except for rHb97, which formed less dimer under the standard reaction conditions. Trypsin mapping of these mutants showed a consistent decrease in the relative area of the NH<sub>2</sub>- and COOH-terminal peptides following reaction with Ni(II)/oxone (an example of maps of rHb95 is shown in Fig. 11). In the reaction with rHb95 an additional
peak eluting after the β15 peptide was observed. This peptide displayed a mass gain of 15 Da compared with the COOH-terminal β15 peptide. MS² fragmentation analysis of this peptide identified it as a modified β15 peptide with the 15 ± 0.2-Da mass gain located on the His-146 (data not shown). It appeared that the substitution Y145A diminished subunit cross-linking (whereas Y145H did not), which may indicate a special role for aromatic residues at position 145 in the formation of β-β cross-links. However, it appears that residues at positions 143–146 can also participate in the formation of heterogeneous cross-linking.

Two additional recombinant hemoglobin β globin mutants were also produced and tested, in which the last 3 or 4 of the COOH-terminal residues were deleted (designated rHb81 and rHb82, respectively). Although the amino termini of rHb81 and rHb82 were found to be oxidatively deaminated during Ni(II)/oxone treatment, rHb81 formed β globin dimer to a significantly lesser extent than rHb87, and rHb82 formed virtually none at all (Fig. 12, Table I). These findings suggested cross-linking was directed from the NH₂ terminus of one β globin to the aromatic region at the far COOH terminus of the second β globin.

**DISCUSSION**

We have shown that a reaction of human hemoglobin A₀ with oxone in the presence of nickel(II) ions produces intramolecular cross-linking of the β globins and significant oxidative deamination of the β globin amino termini. Importantly, the oxidative conditions used did not oxidize the ferrous hemes of the carbon monoxide-ligated hemoglobin, and no catalytic activity of the heme centers appears to be involved. We did not observe any evidence that the α globins are similarly susceptible to NH₂-terminal oxidative deamination under the conditions used. Examination of different mutants of recombinant hemoglobin shows that the histidine at position 2 is required for Ni(II)-mediated oxidative deamination of the β globin amino terminus and intramolecular cross-linking of the β globins. Additionally, our experimental results suggest that Ni(II)-catalyzed oxidative intramolecular cross-linking of the β globins occurs between the NH₂ terminus of one and the COOH-terminal region of the other globin. Peptide mapping did not, however, provide identification of a primary cross-link but rather indicated the heterogeneous character of cross-links produced by the reaction. Characterization of a minor doublet of cross-linked peptides found following sodium cyanoborohydride treatment of the still native state hemoglobin reaction appears consistent with a Schiff's base reduction, resulting in a secondary amine bond between the oxidatively deaminated β globin terminus and the ε-amino group of Lys-144 of the opposing β globin within the same protein molecule. However, the yield of this cross-link was quite low, and recombinant hemoglobin mutants lacking lysine at position 144 remain susceptible to β globin dimerization. The reported structure of R-state hemoglobin indicates close spatial contact between the amino terminus and the carboxyl terminus of opposing β globins in the hemoglobin tetramer (Fig. 13) (31). Hemoglobin mutants containing substitutions: H143A, Y145H, or deletion of His-146 all exhibited comparable susceptibility to cross-linking, suggesting that no specific side chain of COOH-terminal residue is strictly required. The substitution Y145A, however, did appear to both diminish the amount of dimerization seen in the standard reaction and also displayed an oxidation of His-146 resulting in a mass gain of 15 Da, which was not seen in reactions of the other hemoglobin mutants. Two mutants of recombinant hemoglobin containing 3 or 4 amino acid deletions at the COOH terminus, showed significantly decreased or virtually no susceptibility to dimerization, respectively. This may be due to steric considerations, in that the new COOH-terminal region of these mutants is likely no longer in close enough proximity with the opposing β globin NH₂ terminus for cross-link formation.

From these findings, we postulate that histidine at β globin position 2 confers susceptibility under oxidizing conditions to the nickel-catalyzed formation of a carbon-centered radical at the α carbon of the β globin amino-terminal residue, analogous to that proposed by Stadtman (4) on the α carbon in the iron catalyzed deamination of lysine. The “activated” α carbon can react in one of three ways, which are depicted schematically (Fig. 14), A, the radical can be oxidized by the coordinated nickel forming an imino derivative that spontaneously hydrolyzes resulting in oxidative deamination analogous to mechanisms proposed by Stadtman (4) and Garrison (32); or B, the radical can attack a variety of sites on a spatially adjacent β COOH-terminal region leading to a heterogeneous set of car-
bon-carbon and/or carbon-nitrogen bonds. The numerous combinations of potential products resultant from this pathway may, in part, explain our difficulty in identifying specific NH₂-terminal to COOH-terminal region cross-linked peptides. A similar type of labile bond has recently been proposed to form between the β carbon of the essential tyrosine and a nitrogen of histidine in catalase HPII of E. coli (33); C, in the case where the NH₂-terminal residue is methionine, the radical can transfer to the side chain sulfur atom leading to the formation of methionine sulfoxide, effectively “quenching” oxidative deamination. This finding provides a unique example of methionine serving as an intrinsic antioxidant in a protein, a role recently postulated by Levine et al. (34). Recombinant hemoglobin with NH₂-terminal methionine may possess a superior antioxidative feature over wild type hemoglobin in this respect.

The formation of carbonyl derivatives in peptides and proteins by metal-catalyzed oxidation has been well documented (3, 5, 7, 35–37). The physiological relevance of such carbonyl formation in vivo with respect to aging (7, 35, 36) and certain pathological disorders (1, 3, 38) has been established for a variety of proteins including carbonic anhydrase isoenzyme III (1), glutamine synthetase (3), and human fibrinogen Aα chain (39). Further study of the mechanism of the nickel-catalyzed oxidation of hemoglobin may serve as a useful paradigm, providing insights into metal-catalyzed carboxyl formation, as well as oxidative cross-linking in proteins with metal binding sites less proximal to an amino terminus. Oxidative modification of proteins in vivo, including hemoglobin, is known to have a variety of physiologically significant consequences, including increased susceptibility to proteolysis (35, 37, 40). The physiological relevance of a redox-active nickel binding site on hemoglobin, as a factor in oxidative damage in vivo, may merit consideration.

Histidine in position 2 can be readily engineered into recombinant proteins and peptides to provide a redox-active Ni(II) binding site. Treatment with an oxidizing agent (e.g., oxone, peroxyxylphatic acid) can be intentionally used to site specifically introduce a free carbonyl. This terminal α-ketoamide group formed in the reaction could be an attractive “handle” for reaction with amines, hydrazides, and alkoxamines in aqueous solutions to form Schiff’s bases, hydrazones, and oximes respectively. These derivatives are stable under physiological conditions and could be used with a variety of biophysical or structural probes to stabilize proteins, deliver drugs, control enzymatic activity, and for generation of semisynthetic proteins (41).

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