

# Determining Protein–Protein Interactions by Oxidative Cross-Linking of a Glycine-Glycine-Histidine Fusion Protein<sup>†</sup>

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**ABSTRACT:** The Ni(II) complex of the tripeptide NH<sub>2</sub>-glycine-glycine-histidine-COOH (GGH) mediates efficient protein–protein cross-linking in the presence of oxidants such as oxone and monoperoxyphthalic acid (MMPP). Here we demonstrate that GGH fused to the amino terminus of a protein can still support cross-linking. The tripeptide was expressed at the amino terminus of ecotin, a dimeric macromolecular serine protease inhibitor found in the periplasm of *Escherichia coli*. In the presence of Ni(OAc)<sub>2</sub> and MMPP, GGH–ecotin is cross-linked to give a species that has an apparent molecular mass of a GGH–ecotin dimer with no observable protein degradation. The cross-linking reaction occurs between two ecotin proteins in a dimer complex. Furthermore, GGH–ecotin can be cross-linked to a serine protease target, trypsin, and the reaction is specific for proteins that interact with ecotin. The cross-linking reaction has been carried out on small peptides, and the reaction products have been analyzed by matrix-assisted laser desorption/ionization mass spectrometry. The target of the reaction is tyrosine, and the product is bityrosyl cross-links. The yield of the cross-linking is on the order of 15%. However, the reaction efficiency can be increased 4-fold by a single amino acid substitution in the carboxy terminus of ecotin that places an engineered tyrosine within 5 Å of a naturally occurring tyrosine. This cross-linking methodology allows for the protein cross-linking reagent to be encoded for at the DNA level, thus circumventing the need for posttranslational modification.

The ability of a protein to bind site-specifically to another protein or peptide is essential for most cellular processes. In addition, many biological functions are controlled by large macromolecular assemblies of proteins that must bind in a spatially correct orientation to create a functional system (1). The development of new tools to study the organization of protein complexes constitutes a major methodological problem in chemistry and molecular biology. One technique that has proven to be a highly effective method for studying protein–protein interactions in solution is protein cross-linking.

Most cross-linking reagents are based on the reactivity of a specific functional group found in an amino acid side chain (2). For example, the most commonly targeted amino acids contain side chains with nucleophilic functionalities that react with an electrophilic cross-linking reagent. Functional group-specific cross-linking requires that the reactive amino acids be within close proximity along the protein–protein interface. In addition, several nonspecific cross-linking reagents have been developed. Upon activation, these reagents generate reactive species, such as carbenes and nitrenes, that react rapidly with proteins in the immediate vicinity, irrespective of their amino acid sequence. A variety of different cross-

linking reagents have been synthesized by linking two or more reactive groups through a spacer arm. Despite the large number of commercially available cross-linking reagents, there is a continuing need for the development of new reagents that target additional amino acids.

While the formation of covalent protein adducts is very useful in detecting protein–protein interactions in solution, current methods of protein cross-linking suffer from important limitations. Mainly, addition of an exogenous cross-linking reagent to complex protein assemblies often leads to multiple cross-linked products that are difficult to characterize. One way to circumvent this problem is to covalently attach the cross-linking reagent to the protein of interest, thus delivering the reactive moiety site-specifically. Unfortunately, this approach is limited due to poor coupling yields and the need for large quantities of purified protein. Furthermore, the regiochemistry of these protein modification reactions shows little selectivity; consequently, modifying a specific amino acid within the context of an entire protein can be extremely difficult.

It would be of great value if the cross-linking reagent could be constructed at the genetic level, thus obviating the need for chemical modification. This approach has been used by synthesizing modified amino acids that contain a reactive cross-linking group (3, 4). The reactive moiety can then be incorporated into a protein by employing a coupled in vitro transcription–translation system that is primed to accept the modified amino acid (5–7). Recently, Kanamori and co-workers have used this approach to incorporate a photo-reactive benzophenone at several locations of two cyto-

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chrome  $b_2$ -dihydrofolate reductase fusion proteins (8). Upon photolysis, the fusion proteins cross-link to the mitochondrial membrane proteins Tom40, Tim44, and Ssc 1p with cross-linking efficiencies of 10% or less.

While this technique is powerful, it would be advantageous if the cross-linking reagent was composed solely of naturally occurring amino acids. Toward this goal, the tripeptide,  $\text{NH}_2\text{-Gly-Gly-His-COOH}$  (GGH) has been shown to mediate efficient protein-protein cross-linking in the presence of nickel acetate  $[\text{Ni}(\text{OAc})_2]$  and an oxidant such as monoperoxyphthalate (MMPP) or oxone (9). The reaction is specific for proteins that are known to associate in solution, but very little is known about the mechanism of action. This cross-linking reagent has proven to be useful for the detection of protein-protein interactions that are not observed by commonly used cross-linking reagents. For example, the tripeptide GGH-Ni(II)-MMPP system was employed to probe the hydrophobic interface of the F-actin and skeletal myosin subfragment complex (10). More recently, Fancy and co-workers demonstrated that His<sub>6</sub>-tagged proteins form covalent cross-links when treated with Ni(II) salts and oxidant (11, 12). The reaction requires the His<sub>6</sub>, indicating that the hexapeptide is serving as an encoded cross-linking reagent that is activated upon addition of metal and oxidant. The reaction mechanism for the His<sub>6</sub>-mediated cross-linking is believed to be similar to that of the tripeptide GGH-Ni(II) system. Both of these peptide-based cross-linking reagents have potential as useful tools for the study of macromolecular protein assemblies.

Because the GGH cross-linking reagent is a naturally occurring peptide, the reactive moiety can be encoded at the DNA level. To show that the GGH moiety can be used as an efficient cross-linking reagent when expressed at the amino terminus of a protein, ecotin was chosen as a model system. Ecotin, a macromolecular serine protease inhibitor from the periplasm of *Escherichia coli*, was selected for several reasons (13, 14). First, biochemical and crystallographic evidence suggest that ecotin exists as a dimer (15–17). Second, the amino terminus is distal from the dimer interface and the protease binding sites, suggesting that the addition of a tripeptide to this location would not disrupt dimer formation or protease inhibition (18). Furthermore, this remote location of the reactive moiety provides a method to determine if the metal binding site is intimately involved in the covalent modification or if the cross-linking reaction can occur at a site distant from the GGH-Ni(II) complex. Third, ecotin can be expressed in large quantities and is amenable to mutagenesis (19). Last, ecotin is a unique inhibitor in that it displays a broad inhibitory specificity against serine proteases with a chymotrypsin-like structure. GGH-ecotin cross-linking could serve as a useful approach for understanding the nature of ecotin's panspecificity as well as providing a valuable reagent for identifying novel serine proteases.

We demonstrate here that GGH expressed at the amino terminus of a protein can still support cross-linking in the presence of  $\text{Ni}(\text{OAc})_2$  and an oxidant. The reaction is highly specific for proteins that interact in solution. Mass spectrometry was used to identify the products of the oxidative cross-linking using small peptides as model systems, and our data demonstrate that the cross-linking reagent reacts with tyrosine, an amino acid not currently targeted by com-

mercially available cross-linking reagents. By a combination of mechanistic chemistry and rational protein design, the cross-linking efficiency was dramatically increased by the introduction of a single point mutation in the targeted protein.

## MATERIALS AND METHODS

**Materials.** All peptides were purchased from Sigma, and the concentrations were based on dry weight.  $\text{Ni}(\text{OAc})_2$ , MMPP, and sodium sulfite were purchased from Aldrich and used without further purification. Enzymes for DNA manipulations were purchased from New England Biolabs, Promega, or Boehringer Mannheim and were used according to the manufacturer's instructions. Cloned Pfu polymerase was obtained from Stratagene. DNA oligonucleotides were synthesized by standard phosphoramidite chemistry on a Perkin-Elmer/Applied Biosystems 391 DNA synthesizer and purified with a NENSORB column from DuPont NEN. Wild-type ecotin was expressed and purified according to previously published procedures from the protease-deficient *Escherichia coli* strain 27C7 (19). Ecotin concentrations were determined by absorbance with an extinction coefficient of  $1.36 \text{ mL mg}^{-1}$  (OD at 280 nm) (20). Cruzain was a kind gift from Dr. Martha Laboissiere and was expressed and purified according to the previously published procedure (21). Trypsin D102N was expressed and purified from *Saccharomyces cerevisiae* according to the previously published procedure (22) and was a kind gift from Ms. Jennifer Harris and Dr. Toshi Takeuchi. Trypsin D102N, which displays low proteolytic activity, was used to avoid the problem of autoproteolysis while still maintaining the binding affinity of ecotin for trypsin. Computer modeling was performed using the program InsightII (23) and was based on the crystal structure of the ecotin-collagenase complex (24).

**Expression of Ecotin Mutants:** (A) *GGH-Ecotin.* Site-directed mutagenesis was performed by the method of Kunkel using the plasmid pBS-ecotin (25). The codons encoding the tripeptide GGH were inserted between the signal sequence and Ala-1 of ecotin using the primer GCC TGG GCG GGT GGC CAT GCA GAA AGC GTC CAG. The underlined region represents the Gly-Gly-His codons. The GGH-ecotin gene was then subcloned into the expression vector pTacTac between the *Bam*HI and *Hind*III sites. The mutation was confirmed by DNA sequencing of the gene. Expression and purification of the tagged protein were performed using the same procedure established for wild-type ecotin (19). The average yield of purified protein was 20 mg/L of expression medium. Protein solution concentrations were determined by absorbance based on an extinction coefficient of  $1.36 \text{ mL mg}^{-1}$  (OD at 280 nm) (20). The presence of the GGH tag on the amino terminus was confirmed by protein sequencing.

(B) *GGH-Ecotin D137Y.* The Asp to Tyr mutation at residue 137 was accomplished by PCR mutagenesis. The ecotin fragment from nucleotide 64 to nucleotide 489 was amplified from the vector pTacTac GGH-ecotin using the 5' primer GGA AGC GTC CCA CTG GAA AAC (contains a *Pf*/MI site) and the 3' primer TAT TGC ACT AAG CTT TTA GCG AAC TAC CGC GTT GTA AAT TTT CTC (contains a *Hind*III site). The base change is noted by an underline. The PCR amplifications were performed

using 125 pmol of 5' primer, 125 pmol of 3' primer, 125  $\mu$ M dNTPs, 100 ng of cDNA, and 5 units of cloned Pfu polymerase. The thermocycler conditions were as follows: five cycles at 94 °C for 1 min, 45 °C for 2 min, and 72 °C for 3 min, followed by 20 cycles of 94 °C for 1 min, 60 °C for 2 min, and 72 °C for 3 min. The fragment was purified using a Promega PCR wizard kit. The PCR fragment was subcloned into pBS GGH-ecotin between the *Pfl*MI and *Hind*III sites. The entire gene was then subcloned into the expression vector pTacTac between the *Bam*HI and *Hind*III sites. The mutation was confirmed by DNA sequencing. Protein expression and purification was accomplished using the same procedure described for wild-type ecotin (19). Approximately 15 mg of purified protein were isolated from 1 L of expression medium. Protein solution concentrations were determined by absorbance with a calculated extinction coefficient of 1.44 mL mg<sup>-1</sup> (OD at 280 nm) (20).

**Cross-Linking Reactions.** The reactions were performed such that the final concentrations were 50 mM sodium phosphate, pH 7.0, 150 mM NaCl, 10  $\mu$ M GGH-ecotin (unless otherwise noted in the figure legends), 100  $\mu$ M Ni(OAc)<sub>2</sub>, and 100  $\mu$ M MMPP in a final volume of 15  $\mu$ L. The Ni(OAc)<sub>2</sub> and MMPP solutions were prepared immediately before use. GGH-ecotin and Ni(OAc)<sub>2</sub> were mixed together in H<sub>2</sub>O to avoid precipitation of Ni(II) salts and equilibrated at room temperature for 15 min. The protein-Ni(II) complex was then diluted with the appropriate volume of buffer. Reactions were initiated by the addition of MMPP. After 1 min at room temperature, 5  $\mu$ L of 4 $\times$  loading buffer (0.24 M Tris, 8% SDS, 2.88 M  $\beta$ -mercaptoethanol, 40% glycerol, and 0.4% bromophenol blue) was added to quench the reaction. The samples were boiled for 5 min and analyzed by SDS-PAGE employing 10% Tricine gels (26). Visualization of the protein bands was accomplished using Coomassie brilliant blue. If the reactions were performed in the presence of another protein, that protein was added to the reaction mixture 5 min prior to the addition of MMPP. Similarly, if the reaction was performed in the presence of tyrosine, the free amino acid was added before the addition of MMPP. Yields were determined by scanning the gels against a reflective background using a Umax S-6E scanner and the computer program Magiscan (Umax). The amount of dimer formed was compared to the total amount of protein in a lane by integrating the band intensity using the publicly available program NIH 1.61 (<http://usersupport.smsu.edu/noninet/graphics.htm>).

**Reaction of Small Peptides with GGH-Ni(II) and MMPP:** (A) *Reaction and Isolation Conditions.* The reactions were performed such that the final concentrations were 50 mM sodium phosphate, pH 7.0, 150 mM NaCl, 1.0 mM GGH-Ni(II), 1.0 mM peptide, and 1.0 mM MMPP. The total volume of the reaction mixture was kept at 2 mL. Ni(OAc)<sub>2</sub> and GGH were mixed in a small volume of H<sub>2</sub>O and allowed to equilibrate for 5 min. The metal complex was diluted with the appropriate volume of buffer and added to the peptide of interest. Reactions were initiated by the addition of MMPP, and an immediate color change to yellowish-brown was observed. The reaction was quenched after 1 min by the addition of thiourea to a final concentration of 1.5 mM. The reaction mixture was loaded onto a Vydac C18 reverse-phase high-performance liquid chromatography column (0.46  $\times$  25 cm). The column was washed with 20%

acetonitrile/0.1% trifluoroacetic acid at a flow rate of 1 mL/min followed by a linear gradient of 20–40% acetonitrile/0.1% trifluoroacetic acid over 20 min. The elution of peptides was monitored by UV absorbance at 220 nm. All new peptide peaks were collected and the solvent was removed under vacuum.

(B) *Mass Spectrometry.* The molecular weights of the new peptide peaks were measured by matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) on a VG ToFSpec SE mass spectrometer (Micromass Ltd, Manchester, England) equipped with a nitrogen laser and operated in reflectron mode. Peptides were cocrystallized in a matrix consisting of 100 mM  $\alpha$ -cyano-4-hydroxycinnamic acid. The sequence of the peptides was determined either by MALDI-MS on the VG ToFSpec instrument employing the post source decay (PSD) technique, or by MALDI-MS-MS on a VG AutoSpec 5000 TOF tandem mass spectrometer (27, 28).

*Amino-Terminal Protein Sequencing.* Cross-linking reactions were performed as described, and the samples were blotted onto poly(vinylidene fluoride) (PVDF) plus transfer membrane from Micron Separation Inc. (Westborough, MA). The proteins were visualized by staining with Coomassie brilliant blue. Amino-terminal sequencing was performed by the Biomolecular Resource Center at UCSF. Samples containing 1–10 pmol of protein were subjected to Edman degradation using an Applied Biosystems 470A gas-phase sequencer. The phenylthiohydantoin (PTH) derivatives were identified and quantitated by reverse-phase HPLC using an on-line Applied Biosystems 120A PTH analyzer.

## RESULTS

**GGH-Fusion Protein Supports Cross-Linking.** GGH-ecotin was expressed at equivalent levels as the wild-type protein. In the presence of Ni(OAc)<sub>2</sub> and MMPP, GGH-ecotin forms slower migrating species as determined by SDS-PAGE (Figure 1, lane 1). The molecular weight of the major product correlates to approximately 32 000, demonstrating the formation of covalently bound GGH-ecotin. Minor amounts of higher order species are formed and the molecular weights of these species correspond to GGH-ecotin tetramers and hexamers. The yield of dimer formed in the reaction is 15–20% when compared to the monomer. The reaction demonstrates a strict requirement for both Ni(II) and MMPP, as either reagent alone does not support cross-linking (Figure 1, lanes 2 and 3). The requirement for metal in the reaction suggests that nickel is not complexed to GGH during the expression and purification procedure. MMPP is required for cross-linking; however, other oxidants such as oxone and sodium sulfite will support cross-linking as well (data not shown). Wild-type ecotin does not cross-link in the presence of Ni(II) and MMPP, indicating that GGH is required for nickel binding to ecotin (Figure 1, lane 4). However, cross-linking of wild-type ecotin is observed when the protein is treated with GGH-Ni(II) and MMPP (Figure 1, lane 5). It is important to note that when GGH-ecotin is treated with Ni(II) and oxidant or oxidant alone, the protein bands observed by SDS-PAGE are diffuse (compare lanes 1 and 3 to lane 6 in Figure 1). This suggests that multiple protein modifications are incurred, leading to a heterogeneous sample.

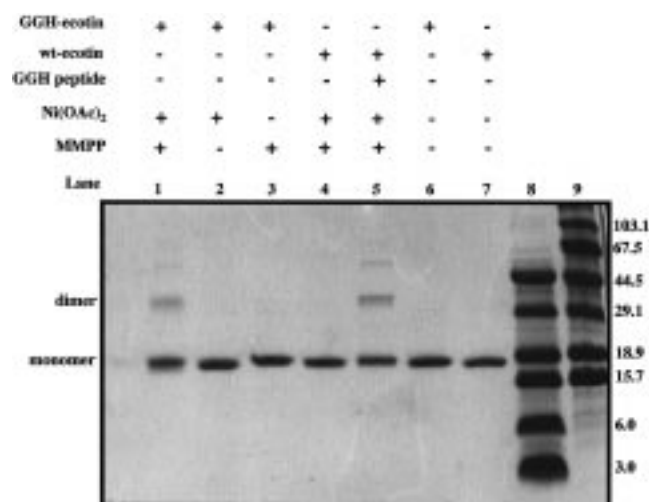


FIGURE 1: Cross-linking reaction requires metal, oxidant, and the metal binding peptide. Lane 1: 10  $\mu$ M GGH-ecotin, 100  $\mu$ M Ni(OAc)<sub>2</sub>, and 100  $\mu$ M MMPP. Lane 2: 10  $\mu$ M GGH-ecotin and 100  $\mu$ M Ni(OAc)<sub>2</sub>. Lane 3: 10  $\mu$ M GGH-ecotin and 100  $\mu$ M MMPP. Lane 4: 10  $\mu$ M wild-type ecotin, 100  $\mu$ M Ni(OAc)<sub>2</sub>, and 100  $\mu$ M MMPP. Lane 5: 10  $\mu$ M wild-type ecotin, 100  $\mu$ M GGH-Ni(II), and 100  $\mu$ M MMPP. Lane 6: 10  $\mu$ M GGH-ecotin. Lane 7: 10  $\mu$ M wild-type ecotin. Lanes 8 and 9: molecular weight markers.

The yield of the dimer product is approximately 15%, even though the protein concentrations used in these experiments (10  $\mu$ M or greater) are above the dissociation constant of the ecotin dimer (1 nM; Yang and Craik, unpublished results). Several experiments were performed to test if the oxidizing capabilities of the cross-linking system were being destroyed with time. To determine if the amino terminus of the GGH-ecotin fusion protein was intact after cross-linking, the remaining monomeric GGH-ecotin was isolated and subjected to Edman degradation. The protein isolated under these conditions had the amino-terminal sequence GGHAET, indicating that the fusion protein was intact (data not shown). However, the yield of histidine was only 2% relative to those of the two previous glycine residues. While the PTH-His signal is often low in comparison to other amino acids when the protein is isolated from PVDF, the possibility that the histidine is modified under the reaction conditions cannot be ruled out with this experiment.

To further determine if the cross-linking reagents were being either destroyed or exhausted during the reaction, wild-type ecotin was treated with free GGH-Ni(II) and MMPP. The reaction was quenched with thiourea, and the reaction mixture was exhaustively dialyzed against reaction buffer to remove all reagents. The mixture of monomeric and dimeric ecotin was then treated to another round of cross-linking under the same conditions. No additional covalently bound dimeric ecotin was observed by repetitive cross-linking (data not shown). These data suggest that chemistry other than protein cross-linking may be occurring and that these side reactions render the protein unable to cross-link.

**The Cross-Linking Reaction Occurs within the Dimeric Ecotin Complex.** Evidence suggested that the cross-linking reaction occurs between two ecotin molecules in a dimeric complex, i.e., intracomplex. Cross-linking is observed using 10  $\mu$ M GGH-Ni(II)-ecotin while no reaction is observed when wild-type ecotin is treated with 10  $\mu$ M GGH-Ni(II) (data not shown). This indicates that the effective molarity

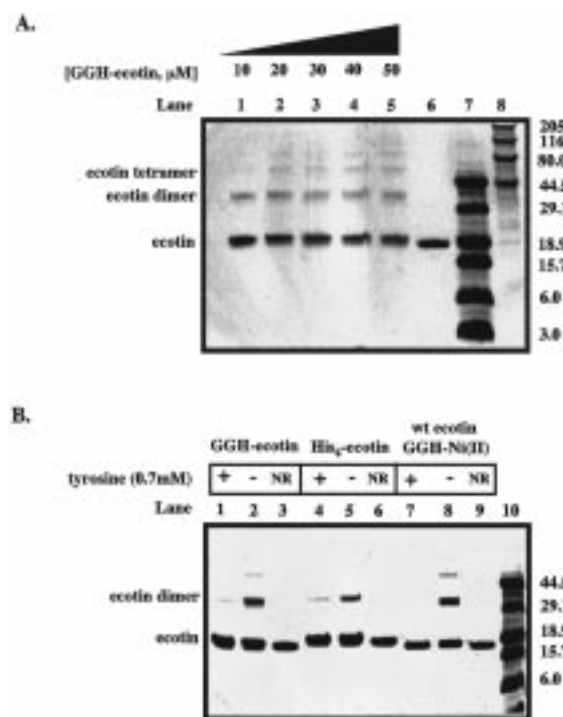


FIGURE 2: Cross-linking reaction occurs intracomplex. (A) The reaction was performed with increasing concentrations of GGH-ecotin. The ratio of protein to Ni(OAc)<sub>2</sub> and MMPP was kept constant. Samples were diluted prior to SDS-PAGE such that the total amount of protein loaded was the same in each lane. Lane 1: 10  $\mu$ M GGH-ecotin, 100  $\mu$ M Ni(OAc)<sub>2</sub>, and 100  $\mu$ M MMPP. Lane 2: 20  $\mu$ M GGH-ecotin, 200  $\mu$ M Ni(OAc)<sub>2</sub>, and 200  $\mu$ M MMPP. Lane 3: 30  $\mu$ M GGH-ecotin, 300  $\mu$ M Ni(OAc)<sub>2</sub>, and 300  $\mu$ M MMPP. Lane 4: 40  $\mu$ M GGH-ecotin, 400  $\mu$ M Ni(OAc)<sub>2</sub>, and 400  $\mu$ M MMPP. Lane 5: 50  $\mu$ M GGH-ecotin, 500  $\mu$ M Ni(OAc)<sub>2</sub>, and 500  $\mu$ M MMPP. Lane 6: 10  $\mu$ M GGH-ecotin. Lanes 7, and 8: molecular weight markers. (B) The reaction was performed in the presence or absence of tyrosine. Lane 1: 20  $\mu$ M GGH-ecotin, 100  $\mu$ M Ni(OAc)<sub>2</sub>, 100  $\mu$ M MMPP, and 0.7 mM tyrosine. Lane 2: 20  $\mu$ M GGH-ecotin, 100  $\mu$ M Ni(OAc)<sub>2</sub>, and 100  $\mu$ M MMPP. Lane 3: 20  $\mu$ M GGH-ecotin. Lane 4: 20  $\mu$ M His<sub>6</sub>-ecotin, 100  $\mu$ M Ni(OAc)<sub>2</sub>, 100  $\mu$ M MMPP, and 0.7 mM tyrosine. Lane 5: 20  $\mu$ M His<sub>6</sub>-ecotin, 100  $\mu$ M Ni(OAc)<sub>2</sub>, and 100  $\mu$ M MMPP. Lane 6: 20  $\mu$ M His<sub>6</sub>-ecotin. Lane 7: 20  $\mu$ M wild-type ecotin, 100  $\mu$ M GGH-Ni(II), 100  $\mu$ M MMPP, and 0.7 mM tyrosine. Lane 8: 20  $\mu$ M wild-type ecotin, 100  $\mu$ M GGH-Ni(II), and 100  $\mu$ M MMPP. Lane 9: 20  $\mu$ M wild-type ecotin. Lane 10: molecular weight markers.

of GGH-Ni(II) is much higher within the context of the ecotin dimer than it is free in solution, and thus the chemistry is localized within the protein complex. To further address this issue, a detailed concentration study was performed (Figure 2A). The cross-linking reaction was performed under increasing GGH-ecotin concentrations. After the reactions were quenched, concentrations were adjusted such that constant amounts of protein were loaded onto the gel. Under all concentrations, the amount of dimer formed as determined by SDS-PAGE remained constant (Figure 2A, lanes 1–5). Since all reactions were performed at concentrations above the nanomolar dissociation constant of the ecotin dimer, this result strongly supports the idea that the cross-linking occurs intracomplex (15, 16). As expected, some nonspecific cross-linking is observed at higher protein concentrations as indicated by the formation of tetramer and hexamer complexes of ecotin (Figure 2A, lanes 2–5). The experiment was performed three times to ensure that the results were

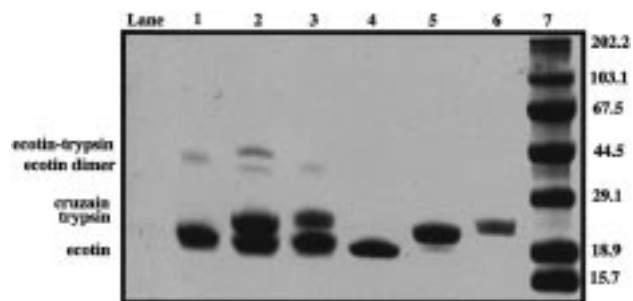


FIGURE 3: GGH-ecotin cross-links specifically to its protease target. All reactions were performed in the presence of 250 mM tyrosine. This helped to reduce nonspecific cross-linking due to trypsin aggregation at high concentrations. Lane 1: 20  $\mu$ M GGH-ecotin, 100  $\mu$ M Ni(OAc)<sub>2</sub>, and 100  $\mu$ M MMPP. Lane 2: 20  $\mu$ M GGH-ecotin, 20  $\mu$ M trypsin D102N, 100  $\mu$ M Ni(OAc)<sub>2</sub>, and 100  $\mu$ M MMPP. Lane 3: 20  $\mu$ M GGH-ecotin, 20  $\mu$ M cruzain, 100  $\mu$ M Ni(OAc)<sub>2</sub>, and 100  $\mu$ M MMPP. Lane 4: 20  $\mu$ M GGH-ecotin. Lane 5: 20  $\mu$ M trypsin D102N. Lane 6: 20  $\mu$ M cruzain. Lane 7: molecular weight markers.

not due to subtle differences in the loading of protein onto the gel.

To further probe this issue, the effects of the addition of free tyrosine to the reaction mixture were examined. Relatively low concentrations of tyrosine are known to quench the GGH-Ni(II)-mediated cross-linking reaction, presumably because tyrosine reacts with the reactive species and/or because tyrosine couples to the protein under the oxidative cross-linking conditions (9, 29). In the presence of 250  $\mu$ M tyrosine, treatment of wild-type ecotin with free GGH-Ni(II) and MMPP results in no dimer formation as determined by SDS-PAGE (data not shown). As is seen in Figure 2B, the reaction of wild-type ecotin with free GGH-Ni(II) and MMPP is completely quenched when the reaction is performed in 0.7 mM tyrosine (Figure 2B, lane 7). However, when GGH-Ni(II)-ecotin is treated with MMPP and a 70-fold excess of tyrosine over the GGH protein, dimer formation is still observed (Figure 2B, lane 1). The dimer band is reduced in intensity, suggesting that the reaction yield is somewhat diminished, but this is not surprising under such large excesses of tyrosine. Importantly, all higher order species resulting from nonspecific cross-linking are absent. As a comparison, His<sub>6</sub>-ecotin was cross-linked under identical conditions (Figure 2B, lanes 4 and 5). His<sub>6</sub> fusion proteins support cross-linking in the presence of Ni(II) salts and oxidant, and the reaction occurs within a protein complex (11). The cross-linking of His<sub>6</sub>-ecotin proceeds with an efficiency of 15%, similar to that of GGH-ecotin, and the effects of tyrosine are identical for both reactions. These data taken together are strong support for specific, intra-complex cross-linking.

**GGH-Ni(II)-Ecotin Can Be Cross-Linked to Its Protease Targets.** Since ecotin is a serine protease inhibitor, the ability of GGH-ecotin to cross-link to its protease target was explored. Ecotin binds to trypsin in a dimeric fashion (18). Each inhibitor-protease complex contains two ecotin molecules arranged in a contralateral fashion and two protease molecules, one at each end of the ecotin dimer. In the presence of trypsin, GGH-Ni(II)-ecotin is cross-linked to yield species with apparent molecular masses of 32 kDa, corresponding to an ecotin-ecotin dimer, and 40 kDa, the expected mass for an ecotin-trypsin heterodimer (Figure 3,

lane 2). The reaction was performed in the presence of 250  $\mu$ M tyrosine to ensure that nonspecific cross-linked products were not formed. Immunoblotting with antibodies against ecotin confirmed the identity of the two species. Interestingly, no ecotin<sub>2</sub>-trypsin<sub>2</sub> complex was observed by SDS-PAGE. The specificity of this reaction is demonstrated when one compares the cross-linking of GGH-tagged versus nontagged proteins. When the ecotin-trypsin complex was treated with free GGH-Ni(II) and MMPP, the reaction pattern became very complicated and many nonspecific protein complexes, such as a trypsin-trypsin dimer, were observed (data not shown).

To confirm that GGH-Ni(II)-ecotin is cross-linking specifically to a protease target, the reaction was performed in the presence of cruzain, a cysteine protease that is not inhibited by ecotin. Indeed, no product corresponding to an ecotin-cruzain cross-link is observed as demonstrated by Coomassie staining (Figure 3, lane 3) or immunoblotting (data not shown). Only ecotin-ecotin cross-links are observed. These data show that a GGH-tagged protein can be cross-linked to a nontagged target and the reaction is highly specific.

**The Target of the Cross-Linking Reaction Is Tyrosines.** Several pieces of data suggest that aromatic amino acids are the targeted residues in the GGH-Ni(II) oxidation reaction (9). First, proteins that are rich in aromatic amino acids cross-link more efficiently than those rich in aliphatic amino acids. Second, the cross-linking reaction can be quenched in the presence of tyrosine and to a lesser extent tryptophan. Addition of free lysine does not affect the reaction. Last, bityrosines are known to form when proteins are subjected to oxidative conditions (30-33).

To further establish the target amino acid and identify the product of the cross-linking reaction, a series of small peptides were treated with GGH-Ni(II) and MMPP. After quenching, the reaction mixture was separated by HPLC on a C<sub>18</sub> column. The products were isolated and subjected to MALDI-MS for molecular weight determination. The sequences were then determined by MALDI-PSD-MS or MALDI-MS-MS. In the case of Arg-Val-Tyr-Ile-His-Pro-Ile, the product isolated has a MH<sup>+</sup> value of 1791.7, which is equal to the mass of a peptide dimer minus 2 mass units. This suggests that the peptide formed a covalent dimer adduct, losing two protons in the process. MALDI-MS-MS resulted in a spectrum with extensive fragmentation (Figure 4). The spectrum showed the anticipated *m/z* values for the carboxy-terminal y<sub>2</sub>-y<sub>4</sub> fragments and the amino-terminal fragments a<sub>1</sub>, a<sub>2</sub>, b<sub>1</sub>, and b<sub>2</sub>. This suggests that a modification has occurred at the tyrosine, which lies at residue 3. To further support this, the a<sub>3</sub>, a<sub>4</sub>, a<sub>5</sub>, and b<sub>3</sub>, b<sub>4</sub>, b<sub>5</sub> fragments have the mass of the expected fragment plus the mass of the intact peptide minus 2 mass units. Similarly, the y<sub>5</sub> and y<sub>6</sub> fragments follow this pattern. Furthermore, a fragment that corresponds to a peptide-tyrosine adduct is seen in the spectrum. As further support for a tyrosine cross-link being formed, a *m/z* of 325.2 is observed, which corresponds to an ion of a tyrosine dimer. Thus, interpretation of the spectrum unambiguously reveals that the cross-linking is localized on the tyrosines that couple to form a species with the molecular weight of bityrosine. It is important to note that the mass spectral data do not identify which geometric isomer of bityrosine is formed. Indeed,

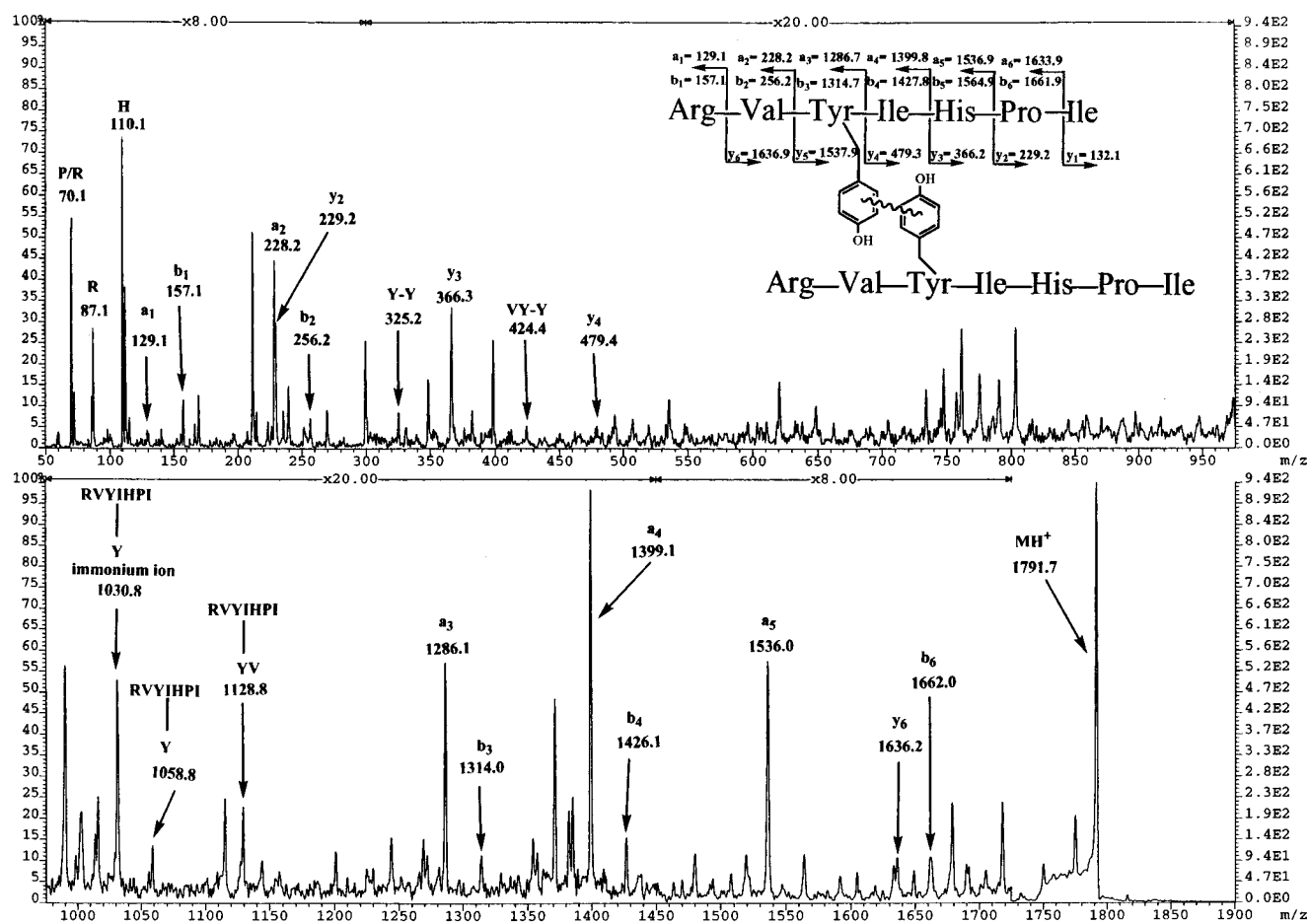


FIGURE 4: MALDI-MS-MS spectrum for the Arg-Val-Tyr-Ile-His-Pro-Ile peptide treated with GGH-Ni(II) and MMPP. The inset shows the predicted masses resulting from a, b, and y ion fragmentation.

the HPLC peak for this peptide was broad, suggesting that multiple isomeric forms of bityrosine are produced.

The heptapeptide, Ser-Met-Glu-Val-Arg-Gly-Trp, also reacts with the GGH-Ni(II) and MMPP system. However, the reaction product is not a cross-linked species but a peptide with a  $MH^+$  value of 896.4, a molecular weight that is 32 mass units higher than that of the parent peptide (data not shown). Peptide sequencing by MALDI-PSD yields a spectrum in which the  $b_2$ – $b_6$  ions can clearly be identified. All of the  $y_2$ – $y_5$  ions have the predicted  $m/z$  values plus 32 mass units. These data clearly demonstrate that the modification occurs at tryptophan. Interestingly, no methionine oxidation was observed in this peptide. The aliphatic peptide, Ala-Gly-Ser-Glu, was completely unreactive to the oxidation conditions. In all three cases, none of the peptides formed covalent cross-links with the GGH tripeptide. These data taken together demonstrate that the covalent protein cross-links formed by the GGH-Ni(II) oxidation system are the result of bityrosine formation.

**Rational Protein Engineering Can Dramatically Increase the Efficiency of the Cross-Linking Reaction.** The data indicate that tyrosine is the target amino acid of the cross-linking reaction. However, other protein modifications such as tryptophan oxidation can occur. This suggests that cross-linking efficiencies may be increased if two tyrosine residues are in close proximity within the protein complex. Examination of the crystal structure revealed that Tyr-127 found in the carboxy terminus is the only tyrosine residue that is located at the protein–protein interface of the ecotin dimer

(Figure 5) (24). However, there is no tyrosine residue from the opposite ecotin molecule within 20 Å of Tyr-127. To address the issue of enhancing cross-linking efficiency, PCR mutagenesis was employed to change Asp-137 to a tyrosine. Asp-137 was chosen for several reasons. It is solvent-accessible and forms no hydrogen bonds within the protein complex. This suggests that the residue can be changed with little effect on the protein structure or the binding affinity of the ecotin dimer complex. In addition, Asp-137 lies within 5 Å of Tyr-127. The GGH-ecotin D137Y mutant was expressed and purified using the same protocol employed for wild-type ecotin. When GGH-ecotin D137Y was subjected to the same cross-linking conditions, the yield of dimeric protein was dramatically increased to approximately 60% (Figure 6, lane 1). This is a substantial improvement over cross-linking yields observed for GGH-ecotin (Figure 6, lane 2).

## DISCUSSION

The tripeptide GGH is well-known to bind both Cu(II) and Ni(II) with a dissociation constant on the order of  $10^{-16}$  (34–36). GGH binds Cu(II) as a slightly distorted square planar complex with the metal coordinated by the terminal amine group, two deprotonated amide nitrogens, and the  $\epsilon$ -nitrogen of the histidine residue (34). The structure of the GGH-Ni(II) complex is believed to be similar by analogy (37, 38). An  $E_{1/2}(\text{III,II})$  of 0.96 V (vs normal hydrogen electrode) has been measured for GGH-Ni indicating that

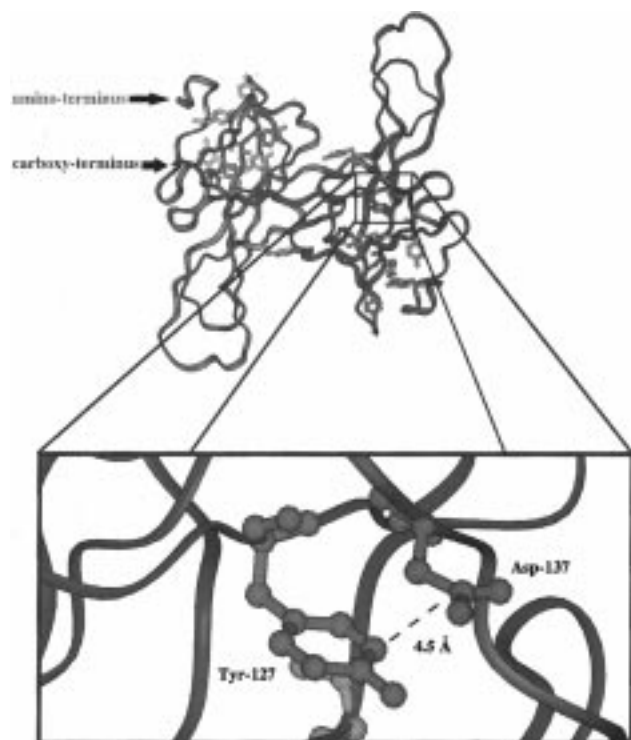


FIGURE 5: Structure of the ecotin dimer with one ecotin monomer shown in green and the other displayed in red. The amino terminus starts at residue 4 of wild-type ecotin. All tyrosines within the protein complex are shown. The inset shows the relationship between Tyr-127 and Asp-137 in the carboxy terminus of the ecotin.

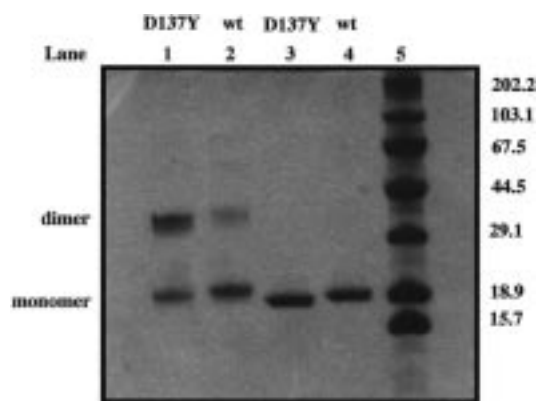


FIGURE 6: Cross-linking efficiency is improved by mutagenesis of a single residue, Asp-137, to a tyrosine. Lane 1: 20  $\mu$ M GGH-ecotin(D137Y), 100  $\mu$ M Ni(OAc)<sub>2</sub>, and 100  $\mu$ M MMPP. Lane 2: 20  $\mu$ M GGH-ecotin, 100  $\mu$ M Ni(OAc)<sub>2</sub>, and 100  $\mu$ M MMPP. Lane 3: 20  $\mu$ M GGH-ecotin (D137Y). Lane 4: 20  $\mu$ M GGH-ecotin. Lane 5: molecular weight markers.

the oxidation of the metal-peptide complex by peracids is a thermodynamically favorable reaction (35).

The Ni(II)-GGH complex has previously been shown to mediate oxidative cleavage of DNA (39-42) and proteins (43) when peracids are employed as oxidant. More recently, it was demonstrated that many peptides having the general form NH<sub>2</sub>-Xaa-Xaa-His-CONH<sub>2</sub> will cleave DNA when treated with Ni(II) salts and oxidant (44-46). The GGH-Ni(II) complex is also known to cross-link proteins under oxidizing conditions, and this reaction has been shown to be highly useful in detecting protein-protein interactions in solution (9). We demonstrate in this report that the tripeptide GGH can be expressed at the amino terminus of

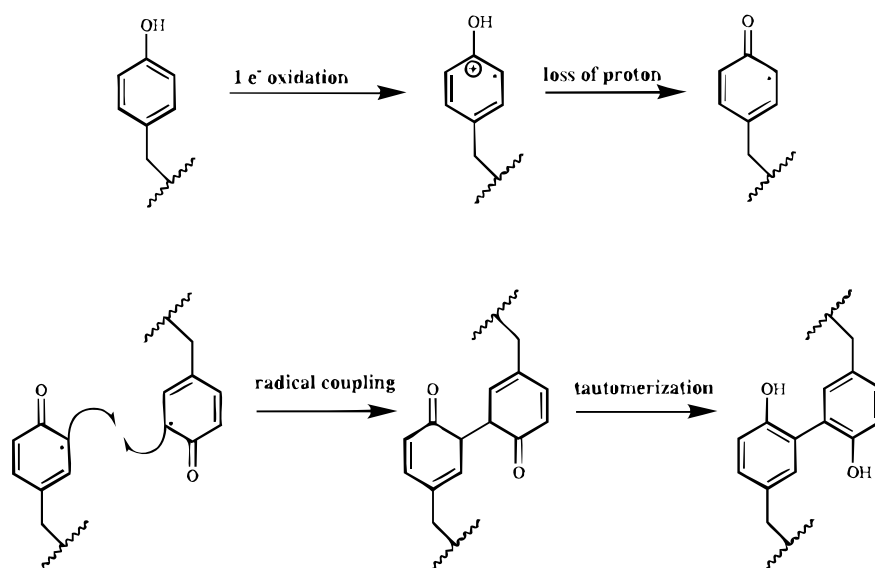
a protein, and in the presence of Ni(II) salts and oxidant, the protein will form covalent protein cross-links.

Under oxidative conditions, the GGH fusion protein of ecotin forms covalent cross-links to yield a GGH-ecotin dimer as determined by SDS-PAGE. The reaction displays a requirement for metal, oxidant, and the GGH metal binding domain. Similar reaction requirements are observed during cross-linking with the free peptide (9). This suggests that the chemistry of the cross-linking reaction is similar for the free peptide-Ni(II) complex and the GGH-Ni(II) fusion protein. It also indicates that the metal is bound in a similar fashion in both the tripeptide and the GGH fusion protein. Importantly, the GGH fusion protein is able to support cross-linking even though the reagent is held distant from the dimer interface. Modeling of the ecotin dimer suggests that the metal binding domain is, at a minimum, 11 Å away from a tyrosine at the protein-protein interface. This suggests that GGH can be used as a cross-linking reagent without disrupting the protein interface. This is not always true for cross-linking reagents that become covalently incorporated into the protein cross-link. While only the GGH-ecotin protein has been explored, the data suggest that GGH fusion proteins can find utility as probes to study protein-protein interactions.

One of the disadvantages of protein cross-linking is that the addition of free cross-linking reagent to a solution of proteins often yields data that are complicated to interpret, and many times false positive results are obtained. By physically associating the protein with the reactive cross-linking moiety by recombinant methods, many of these problems can be avoided. However, this is true only if the cross-linking occurs specifically within the protein complex. Intramolecular reactions are less sensitive to inhibition and, as expected, the addition of a large excess of tyrosine does not completely quench the reaction of GGH-ecotin. This strongly suggests that the reaction is occurring intracomplex. The cross-linking reaction mediated by free GGH-Ni(II) is completely quenched under the same conditions. The cross-linking yield does decrease, however, suggesting that the amino terminus is exposed to the solution and may react with very high concentrations of tyrosine. Concentration studies provide further support for the intramolecular nature of the GGH-ecotin cross-linking reaction. Thus GGH fusion proteins may prove to be very selective for proteins that interact in solution.

The reaction is not limited to the formation of homodimers; GGH-ecotin can be cross-linked to protease targets. Rat trypsin is inhibited by wild-type ecotin with a  $K_i$  of 0.93 nM (19). In the presence of MMPP, trypsin and GGH-Ni(II)-ecotin are cross-linked to form a trypsin-ecotin dimer. Similar results are seen when GGH-ecotin is cross-linked in the presence of elastase, another serine protease inhibited by ecotin (Brown and Craik, unpublished data). The reaction is highly specific for proteins that interact in solution, as ecotin-cruzin cross-links are not observed, even when probed with ecotin antibodies. Ecotin dimers are formed in the presence of cruzain, suggesting that the lack of a ecotin-cruzin cross-link is not the result of an impurity in the cruzain preparation that quenches the oxidation reaction. Despite the fact that ecotin binds to trypsin in a tetrameric fashion, the ecotin<sub>2</sub>-trypsin<sub>2</sub> species is not observed by chemical cross-linking using tagged ecotin (18).

Scheme 1



The reason for this is unknown; however, because the efficiency of cross-linking is on the order of 15%, it is likely that the yield of the tetramer cross-link is simply too small to detect. This is particularly relevant considering that three cross-linking events must occur in order to form the covalently linked tetrameric complex. However, the possibility that the first cross-linking event changes the tertiary structure of the complex cannot be ruled out, nor can the prospect that each GGH tag can support only one cross-linking event.

The GGH-Ni(II)-mediated cross-linking of tyrosine containing peptides clearly demonstrates that tyrosine is the substrate of the cross-linking system and the covalent cross-links are the result of bityrosine formation. Tyrosine is easily oxidized to form a long-lived tyrosine phenoxyl radical (33, 47). Upon recombination of two tyrosyl radicals, the major product is 2,2'-bityrosine. Bityrosines are well-known to form in proteins under oxidative conditions (31, 32, 48, 49). For example, bityrosines are found when the heme-containing protein sperm whale myoglobin is treated with H<sub>2</sub>O<sub>2</sub> (50, 51), and bityrosines are a major protein modification found in atherosclerotic lesions, the result of tyrosine oxidation mediated by myeloperoxidase (52, 53). More recently, it has been demonstrated that the treatment of bovine pancreatic ribonuclease A forms covalent tyrosine cross-links when treated with Ni(OAc)<sub>2</sub> and KHSO<sub>5</sub> (29). While the nature of the metal binding site is not known in this case, the chemistry of tyrosine coupling is most likely very similar.

The exact nature of the reactive species formed by the reaction of GGH-Ni(II) and oxidant is not known but a high valent nickel-oxo species has been previously proposed (9, 39, 40). Nickel-oxo species have also been suggested in the reaction of related polyamine-nickel complexes with oxygen atom donors (54–58). We propose a mechanism for protein cross-linking that involves oxidation of tyrosine by the reactive nickel species (Scheme 1). Tyrosine is oxidized to form a tyrosyl radical cation, thus reducing the nickel species. Loss of a proton from the radical cation results in a neutral species. Coupling of two tyrosyl radicals followed by tautomerization results in the formation of bityrosine. While the 2,2'-bityrosine isomer has been indicated, it should be

noted that the exact isomer of bityrosine resulting from the GGH-Ni(II) cross-linking has not been determined.

The cross-linking efficiency for the ecotin dimer is on the order of 15–20%. These yields are analytically useful; however, increasing the cross-linking efficiency would be beneficial toward efforts to identify protein–protein interactions in large complexes. Several reasons for incomplete cross-linking can be envisioned. Edman degradation of unreacted monomer suggests that the GGH tag remains on the amino terminus of the protein, but the results were ambiguous as to the nature of the histidine. Oxidized histidine residues are known, and preliminary mass spectrometry data suggests that oxidation of histidine is a side reaction of the oxidative cross-linking (Brown, Burlingame, and Craik, unpublished data). This nonproductive chemistry might lead to lowered cross-linking efficiencies by exhausting the oxidizing potential of the system or by lowering the affinity of the metal binding peptide for Ni(II). However, oxidation of histidine cannot completely account for the lowered reaction efficiencies. When wild-type ecotin is treated with an excess of free GGH-Ni(II) and MMPP or is repetitively treated with the cross-linking system, no increase in covalent dimer formation is observed. This suggests that other chemistry is occurring that renders the protein unable to form covalent dimers. Oxidation products of tryptophan, tyrosine, and methionine have all been observed when this chemistry is employed to cross-link GGH-ecotin (Brown, Burlingame, and Craik, unpublished data), and it is possible that these side products may affect the efficiency of the cross-linking reaction.

These data suggest that the cross-linking efficiencies may be increased if two reactive side chains were located in close proximity. Knowledge of the reaction product and mechanism has allowed for structure-based design to increase the cross-linking yield. Since the target amino acid of the cross-linking reaction is tyrosine, placing two tyrosines within close proximity in a protein complex should increase the cross-linking efficiency. Indeed, when Asp-137 in the carboxy-terminal arm of ecotin was replaced with a tyrosine residue, the yield of dimer formation increased from 15–20% to 60%. This suggests that if some information is known about a



protein recognition face, the possibility of detecting a protein-protein complex may be greatly enhanced by engineering a tyrosine close to or within the protein interface.

It should be noted that the cross-linked species within the wild-type GGH-ecotin complex is still unknown. Examination of the crystal structure of the ecotin dimer does not reveal an obvious site for bityrosine formation. The nearest possible tyrosine cross-link spans a distance of greater than 12 Å. It is possible that there is considerable flexibility within the protein complex that is not evident from the crystal structure. Alternatively, there may be oxidation products other than bityrosine that lead to the formation of covalent adducts. Recently, the copper amine oxidase, lysine oxidase, was found to contain a quinone cofactor that is the result of cross-linking of a lysine residue to an oxidatively modified tyrosine (59). This suggests that tyrosine oxidation can result in the formation of covalent adducts other than bityrosine, and efforts are underway to identify the cross-link product within the intact GGH-ecotin dimer.

The chemistry of oxidative cross-linking employing the His<sub>6</sub>-tagged proteins is believed to be very similar to that of GGH. Both reactions require the metal binding peptide, Ni(II) salts, and oxidant (11). The cross-linking occurs intracomplex for both systems as well. It is presumed that the amino acid targets for the reactions are similar (T. Kodadek, personal communication). The main difference between the two systems is the nature of metal binding. The binding constant for GGH-Ni(II) is on the order of 10<sup>3</sup>–10<sup>4</sup>-fold higher than that of His<sub>6</sub>-Ni(II), and the structure of the GGH complex is more defined. This may prove to be important if the cross-linking reaction is to be carried out in complicated protein mixtures such as cell lysates.

GGH fusion proteins have great potential as useful tools for the study of macromolecular protein assemblies. Because the reactive group is a naturally occurring peptide, the cross-linking moiety can be encoded at the DNA level, thus circumventing the need for posttranslational modification. Furthermore, the use of artificial amino acids is avoided. The cross-linking reaction is highly specific for proteins that interact in solution, suggesting that false positive results will be kept at a minimum. In addition, the oxidation reaction targets tyrosine, an amino acid that is not reactive with commercially available cross-linking reagents. Site-directed mutagenesis to introduce a tyrosine at the protein-protein interface can increase the cross-linking yields so that protein-protein interactions in large complexes that might have previously been undetected can be observed.

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## REFERENCES

- Sachs, A. B., and Buratowski, S. (1997) *Trends Biochem. Sci.* 22, 189–192.
- Wong, S. S. (1991) *Chemistry of Protein Conjugation and Cross-Linking*, CRC Press, Boca Raton, FL.
- Martoglio, B., Hofmann, M. W., Brunner, J., and Dobberstein, B. (1995) *Cell* 81, 207–214.
- High, S., Martoglio, B., Gorlich, F., Andersen, S. S. L., Ashford, A. J., Ginger, A., Hartmann, E., Prehn, S., Rapoport, T. A., Dobberstein, B., and Brunner, J. (1993) *J. Biol. Chem.* 268, 26745–26751.
- Noren, C. J., Anthony-Cahill, S. J., Griffith, M. C., and Schultz, P. G. (1989) *Science* 244, 182–188.
- Ellman, J. A., Mendel, D., and Schultz, P. G. (1991) *Methods Enzymol.* 202, 301–336.
- Roesser, J. R., Chorghade, S. M., and Hecht, S. M. (1986) *Biochemistry* 25, 6361–6365.
- Kanamori, T., Nishikawa, S.-I., Shin, I., Schultz, P. G., and Endo, T. (1997) *Proc. Natl. Acad. Sci. U. S. A.* 94, 485–490.
- Brown, K. C., Yang, S.-H., and Kodadek, T. (1995) *Biochemistry* 34, 4733–4739.
- Bertrand, R., Derancourt, J., and Kassab, R. (1997) *Biochemistry* 36, 9703–9714.
- Fancy, D. A., Melcher, K., Johnston, S. A., and Kodadek, T. (1996) *Chem. Biol.* 3, 551–559.
- Fancy, D. A., and Kodadek, T. (1997) *Tetrahedron* 53, 11953–11960.
- Chung, C. H., Ives, H. E., Almeda, S., and Goldberg, A. L. (1983) *J. Biol. Chem.* 258, 11032–11038.
- McGrath, M. E., Hines, W. M., Sakanari, J. A., Fletterick, R. J., and Craik, C. S. (1991) *J. Biol. Chem.* 266, 6620–6625.
- Pal, G., Szilagyi, L., and Graf, L. (1996) *FEBS Lett.* 385, 165–170.
- Seymour, J. L., Lindquist, R. N., Dennis, M. S., Moffat, B., Yansura, D., Reilly, D., Wessinger, M. E., and Lazarus, R. A. (1994) *Biochemistry* 33, 3949–3958.
- Shin, D. H., Song, H. K., Seong, I. S., Lee, C. S., Chung, C. H., and Suh, S. W. (1996) *Protein Sci.* 5, 2236–2247.
- McGrath, M. E., Erpel, T., Bystroff, C., and Fletterick, R. J. (1994) *EMBO J.* 13, 1502–1507.
- Wang, C.-I., Yang, Q., and Craik, C. S. (1995) *J. Biol. Chem.* 270, 12250–12256.
- Gill, S. G., and von Hippel, P. H. (1989) *Anal. Biochem.* 182, 319–326.
- Eakin, A. E., Mills, A. A., Harth, G., McKerrow, J. H., and Craik, C. S. (1992) *J. Biol. Chem.* 267, 7411–7420.
- Willett, W. S., Gillmor, S. A., Perona, J. J., Fletterick, R. J., and Craik, C. S. (1995) *Biochemistry* 34, 2172–2180.
- Dayringer, H. E., Tramontano, A., Sprang, S. R., and Fletterick, R. J. (1986) *Mol. Graphics* 4, 82–87.
- Perona, J. J., Tsu, C. A., Craik, C. S., and Fletterick, R. J. (1997) *Biochemistry* 36, 5381–5392.
- Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. U.S.A.* 82, 488–492.
- Schagger, H., and von Jagow, G. (1987) *Anal. Biochem.* 166, 368–379.
- Medzihradszky, K. F., Adams, G. W., Burlingame, A. L., Bateman, R. H., and Green, M. R. (1996) *J. Am. Soc. Mass Spectrom.* 7, 1–10.
- Medzihradszky, K. F., Maltby, D. A., Qiu, Y., Yu, Z., Hall, S. C., Chen, Y., and Burlingame, A. L. (1997) *Int. J. Mass Spectrom. Ion Processes* 160, 357–369.
- Gill, G., Richter-Rusli, A. A., Ghosh, M., Burrows, C. J., and Rokita, S. E. (1997) *Chem. Res. Toxicol.* 10, 302–309.
- Berlett, B. S., and Stadtman, E. R. (1997) *J. Biol. Chem.* 272, 20313–20316.
- Yamamoto, O. (1973) *Radiat. Res.* 54, 398–410.
- Yamamoto, O. (1977) in *Protein Cross-Linking, Part A* (Friedman, M., Ed.), pp 519–547, Plenum Press Publishing Co., New York.
- Prutz, W. A., Butler, J., and Land, E. J. (1983) *Int. J. Radiat. Biol.* 44, 183–196.
- Camerman, N., Camerman, A., and Sarkar, B. (1976) *Can. J. Chem.* 54, 1309–1316.
- Bossu, F. P., and Margerum, D. W. (1977) *Inorg. Chem.* 16, 1210–1214.
- Bannister, C. E., Raycheba, J. M. T., and Margerum, D. W. (1982) *Inorg. Chem.* 21, 1106–1112.
- Freeman, H. C., Guss, J. M., and Sinclair, R. L. (1968) *Chem. Comm.*, 485–487.

38. Freeman, H. C., and Taylot, M. R. (1965) *Acta Crystallogr.* 18, 939–952.
39. Mack, D. P., and Dervan, P. B. (1990) *J. Am. Chem. Soc.* 112, 4604–4606.
40. Mack, D. P., and Dervan, P. B. (1992) *Biochemistry* 31, 9399–9405.
41. Nagaoka, M., Hagihara, M., Kuwahara, J., and Sugiura, Y. (1994) *J. Am. Chem. Soc.* 116, 4085–4086.
42. Footer, M., Egholm, M., Kron, S., Coull, J. M., and Matsudaira, P. (1996) *Biochemistry* 35, 10673–10679.
43. Cuenoud, B., Tarasow, T. M., and Schepartz, A. (1992) *Tetrahedron Lett.* 33, 895–898.
44. Liang, Q., Denny Eason, P., and Long, E. C. (1995) *J. Am. Chem. Soc.* 117, 9625–9631.
45. Hartford, C., Narindrasorasak, S., and Sarkar, B. (1996) *Biochemistry* 35, 4271–4278.
46. Muller, J. G., Hickerson, R. P., Perez, R. J., and Burrows, C. J. (1997) *J. Am. Chem. Soc.* 119, 1501–1506.
47. Solar, S., Solar, W., and Getoff, N. (1984) *J. Phys. Chem.* 88, 2091–2095.
48. Huggins, T. G., Wells-Knecht, M. C., Detorie, N. A., Baynes, J. W., and Thorpe, S. R. (1993) *J. Biol. Chem.* 268, 12341–12347.
49. Giulivi, C., and Davies, K. J. A. (1993) *J. Biol. Chem.* 268, 8752–8759.
50. Tew, D., and Ortiz de Montellano, P. R. (1988) *J. Biol. Chem.* 263, 17880–17886.
51. Wilks, A., and Ortiz de Montellano, P. R. (1992) *J. Biol. Chem.* 267, 8827–8833.
52. Heinecke, J. W., Li, W., Francis, G. A., and Goldstein, J. A. (1993) *J. Clin. Invest.* 91, 2866–2872.
53. Marquez, L. A., and Dunford, H. B. (1995) *J. Biol. Chem.* 270, 30434–30440.
54. Kinneary, J. F., Albert, J. S., and Burrows, C. J. (1988) *J. Am. Chem. Soc.* 110, 6124–6129.
55. Kinneary, J. F., Wagler, T. R., and Burrows, C. J. (1988) *Tetrahedron Lett.* 29, 877–880.
56. Koola, J. D., and Kochi, J. K. (1987) *Inorg. Chem.* 26, 908–916.
57. Wagler, T. R., and Burrows, C. J. (1988) *Tetrahedron Lett.* 29, 5091–5094.
58. Yoon, H., and Burrows, C. J. (1988) *J. Am. Chem. Soc.* 111, 1933–1934.
59. Wang, S. X., Mure, M., Medzihradszky, K. F., Burlingame, A. L., Brown, D. E., Dooley, D. M., Smith, A. J., Kagan, H. M., and Klinman, J. P. (1996) *Science* 273, 1078–1084.

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