Highly Specific Oxidative Cross-Linking of Proteins Mediated by a Nickel−Peptide Complex†

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ABSTRACT: The Ni(II) complex of the tripeptide NH_2-Gly-Gly-His-COOH is shown to mediate efficient protein−protein cross-linking in the presence of oxidants such as oxone and monoperoxyphthalic acid. Only proteins that associate specifically in solution are cross-linked under these conditions. Preliminary probes of the mechanism of the reaction suggest that the active intermediate may be a high-valent metal complex that attacks aromatic amino acids.

Chemical cross-linking constitutes a powerful method to study the organization of multiprotein complexes in vitro. Most bifunctional cross-linkers include tethered groups that react with nucleophilic amino acid side chains. For example, glutaraldehyde contains two reactive carbonyl groups that can form Schiff base adducts with the amino groups of proximal lysines. Less common are monofunctional reagents that chemically activate side chain moieties for coupling, but even in these cases a nucleophilic side chain is generally involved. For example, diimides such as 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC) activate Asp and Glu carboxylates for amide formation with Lys side chains.

In order to increase the utility of chemical cross-linking as a technique for the study of multiprotein complexes, there is a continuing need to develop new reagents, particularly species that do not require the presence of nucleophilic groups. We report here that the tripeptide, NH_2-Gly-Gly-His-COOH (GGH), in the presence of nickel acetate (Ni$(OAc)_2$) and monoperoxyphthalic acid (MMPP) is an efficient cross-linking reagent. Proteins that interact in solution are acted upon by this reagent to give covalently bound dimers and in some cases higher order complexes. Proteins that are monomers in solution do not become covalently cross-linked even at high concentrations, indicating that the metallopeptide complex mediates the cross-linking of only closely associated proteins.

MATERIALS AND METHODS

Proteins and Reagents. The UvsX (Hinton & Nossal, 1986) and gene product 32 (Giedroc et al., 1990; Bittner et al., 1979) proteins and the GAL4 (1−140) fragment (Carey et al., 1989) were purified according to literature procedures from *Escherichia coli* expression strains. All proteins were dialyzed from their storage buffers into 50 mM sodium phosphate (pH 7.0) and 150 mM NaCl. Protein concentrations were determined using the Bradford reagent (Bio-Rad) with bovine serum albumin (BSA) as the standard. All other concentrations were based on dry weight. BSA was purchased from United States Biochemical. Ubiquitin, horseradish superoxide dismutase, catalase from bovine liver, and GGH were purchased from Sigma. Magnesium monoperoxyphthalic acid hexahydrate (MMPP), Ni$(OAc)_2$, sodium ascorbate salt, oxone, and $(NH_4)_2Fe(SO_4)_2$ were purchased from Aldrich. $CuCl_2$ was purchased from Baker.

GGH−Ni(II) Cross-Linking Reactions. Cross-linking reactions were carried out in a total volume of 15 μL. Final concentrations were 50 mM phosphate (pH 7.0), 150 mM NaCl, 10 μM protein (unless otherwise indicated in the figure legends), and 100 μM GGH−Ni(II). The nickel−peptide complex was formed by mixing a 1:1 molar ratio of nickel acetate and GGH in unbuffered H_2O. Following a 5 min equilibration, the solution was diluted to the desired concentration with the phosphate buffer. This protocol was adopted due to the tendency of nickel to precipitate in phosphate buffer in the presence of only the acetate ligand. The GGH−Ni(II) complex does not precipitate from phosphate buffer even after long periods of time. The reactions were initiated by the addition of MMPP to 100 μM and incubated at room temperature for 1 min (unless indicated otherwise). The reaction was then quenched by the addition of 5 μL of 4× loading buffer (0.24 M Tris, 8% SDS, 2.88 M β-mercaptoethanol, 40% glycerol, 0.4% bromophenol blue, and 0.4% xylene cyanol). The samples were heated at 100 °C for 5 min and then analyzed electrophoretically using a 10% tricine−SDS polyacrylamide gel (Schagger & von Jagow, 1987). The protein bands were visualized by staining with Coomassie Brilliant Blue.

Protein Cleavage by GGH−Cu(II). The same conditions as those described earlier were employed, except that sodium ascorbate and H_2O_2 (unless indicated in the figure) were used as the oxidizing agents. Sodium ascorbate was added to a final concentration of 5 mM, and the reaction was initiated by the addition of H_2O_2 to 5 mM. The reactions were carried out at room temperature for the times indicated in the figures. The reactions were quenched by the addition of 5 μL 4× loading buffer. The samples were analyzed by electrophoresis as described earlier.

Protein Cleavage by EDTA−Fe(II). Cleavage reactions were carried out in a total volume of 15 μL. Final concentrations were 50 mM phosphate (pH 7.0), 150 mM

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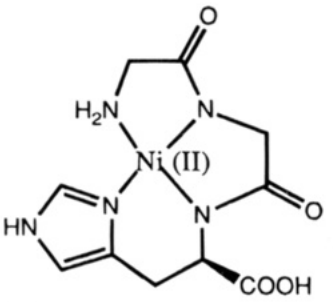

FIGURE 1: The GGH−Ni(II) complex.

NaCl, 10 μM protein, 100 μM EDTA−Fe(II), and 5 mM sodium ascorbate. The EDTA−Fe(II) complex was prepared by mixing equimolar amounts of EDTA (pH 8.0) and $(NH_4)_2$-$Fe(SO_4)_2$, as described previously (Celander & Cech, 1990), and then diluting to the appropriate concentration with phosphate buffer (pH 7.0). Reactions were initiated by the addition of H_2O_2 to a final concentration of 5 mM. After 10 min at room temperature, the reactions were quenched by the addition of 5 μL 4× loading buffer. The samples were analyzed by electrophoresis as described earlier.

Effect of Superoxide Dismutase on the Cross-Linking Reaction. Cross-linking reactions were carried out in a total volume of 15 μL. Final concentrations were 50 mM phosphate (pH 7.0), 150 mM NaCl, 11 μM gp32, and 1.1 mM GGH−Ni(II). Horseradish superoxide dismutase (40 kDa) was added to a final concentration of 0.22 mg/mL (99 units). The reaction was initiated by the addition of MMPP to a final concentration of 1.25 mM. The reaction was carried out for 1 min at room temperature before quenching with 1 μL of 0.2 M thiourea and 5 μL of 4× loading buffer. The samples were analyzed by electrophoresis as described earlier.

Effect of Catalase on the Cross-Linking Reaction. The same conditions as those described for the reactions containing superoxide dismutase were employed, except that catalase from bovine liver was added to a final concentration of 0.25 mg/mL (93 units).

Effect of Amino Acids on the Cross-Linking Reaction. These experiments followed the basic protocol given earlier, except that an amino acid (tryptophan, tyrosine, or lysine, concentration given in the figure caption) was added to the solution prior to the introduction of the metal−peptide complex and the oxidant.

RESULTS

GGH−Ni(II) Is an Efficient Protein Cross-Linking Reagent. The complex formed by the tripepetide GGH and Ni(II) salts (Figure 1) is known to mediate the oxidative cleavage of both DNA (Mack & Dervan, 1990, 1992) and proteins (Cuenoud et al., 1992) in the presence of peracids. We show here that treatment of certain proteins with GGH−Ni(II) under oxidative conditions results in efficient protein−protein cross-linking.

Gene product 32 (gp32) of bacteriophage T4 is a single-stranded DNA (ssDNA) binding protein involved in replication and recombination (Alberts & Frey, 1970; Karpel, 1990; Chase & Williams, 1986). The protein binds to ssDNA cooperatively and also self-associates in solution in the absence of nucleic acids. In the presence of GGH−Ni(II) and MMPP, gp32 is converted to discrete higher molecular weight forms as determined by denaturing gel electrophoresis

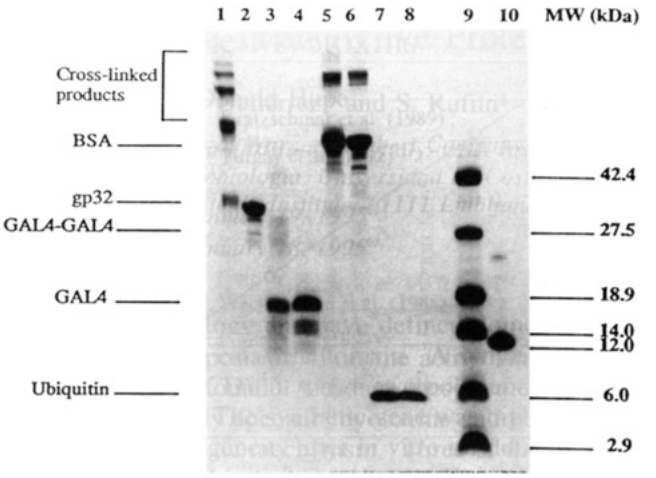

FIGURE 2: GGH−Ni(II) cross-links only proteins that bind specifically to one another: lane 1, 10 μM gp32, 100 μM GGH−Ni(II), 130 μM MMPP; lane 2, 10 μM gp32; lane 3, 20 μM GAL4 (1−140), 100 μM GGH−Ni(II), 130 μM MMPP; lane 4, 20 μM GAL4 (1−140); lane 5, 11 μM BSA, 100 μM GGH−Ni(II), 130 μM MMPP; lane 6, 11 μM BSA; lane 7, 20 μM ubiquitin, 100 μM GGH−Ni(II), 130 μM MMPP; lane 8, 20 μM ubiquitin; lane 9, molecular weight markers; lane 10, cytochrome *c*. Note that the higher molecular weight bands in the BSA reaction (lane 5) are also in the control reaction (lane 6), indicating that these bands are due to an impurity in the BSA preparation and are not the result of cross-linking.

(Figure 2, lane 1). These slower mobility bands have apparent molecular masses that correspond to gp32 dimers, tetramers, and so on (see also Figure 7, which includes higher molecular weight standards). The fact that trimers are not observed suggests that the fundamental unit of gp32 is a dimer and that these dimers then associate into higher order structures. The gels were run under denaturing conditions, arguing that the observed products are covalently bound. Furthermore, it is exceedingly unlikely that the cross-linked species are held together by disulfide bonds, since the samples were boiled in the presence of high concentrations of reducing agent (β-mercaptoethanol) prior to electrophoresis. While no degradation products were observed, the remaining monomeric gp32 had a slightly reduced mobility relative to the input gp32 (compare lanes 1 and 2 in Figure 2), suggesting that covalent modifications other than those that led to cross-linking had occurred.

GGH−Ni(II) Cross-Links Only Proteins That Bind Specifically to One Another. These results led us to explore the reaction of GGH−Ni(II) and MMPP with a variety of different proteins. We found that, in addition to gp32 (Figure 2, lane 1), a DNA binding fragment of the yeast GAL4 protein (residues 1−140, Figure 2, lane 4) could also be covalently cross-linked (Figure 2, lane 3). GAL4 is a transcriptional activator that contains a DNA binding and dimerization domain within the 140 amino acids of the N-terminus (Carey et al., 1989). The 1−140 fragment exists as a discrete dimer in solution. In the presence of GGH−Ni(II) and MMPP, this fragment formed covalent dimers. A very small amount of trimer was also observed. However, in this case the cross-linking was not as efficient as it was for gp32, and the cross-linked species ran as a diffuse smear rather than as a sharp band, indicating that other chemical modifications had occurred. This is not surprising since the GAL4 DBD is a very Cys-rich, and therefore easily oxidized, polypeptide.

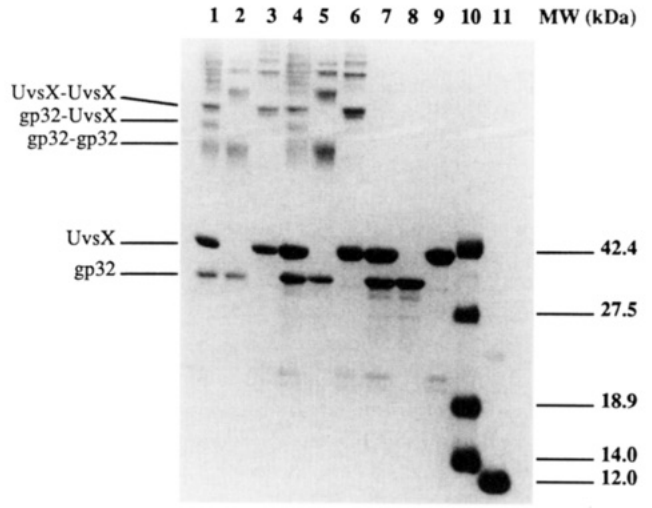

FIGURE 3: Formation of a gp32−UvsX cross-link. The GGH−Ni(II) and MMPP concentration is 100 μM for lanes 1−6. Lanes 7−9 contain only protein. Proteins were equilibrated for 5 min prior to the addition of MMPP: lane 1, 5.0 μM gp 32, 5.0 μM UvsX; lane 2, 5.0 μM gp32; lane 3, 5.0 μM UvsX; lane 4, 10.0 μM gp32, 10.0 μM UvsX; lane 5, 10.0 μM gp32; lane 6, 10.0 μM UvsX; lane 7, 10.0 μM gp32, 10.0 μM UvsX; lane 8, 10.0 μM gp32; lane 9, 10.0 μM UvsX; lane 10, molecular weight markers; lane 11, cytochrome *c*.

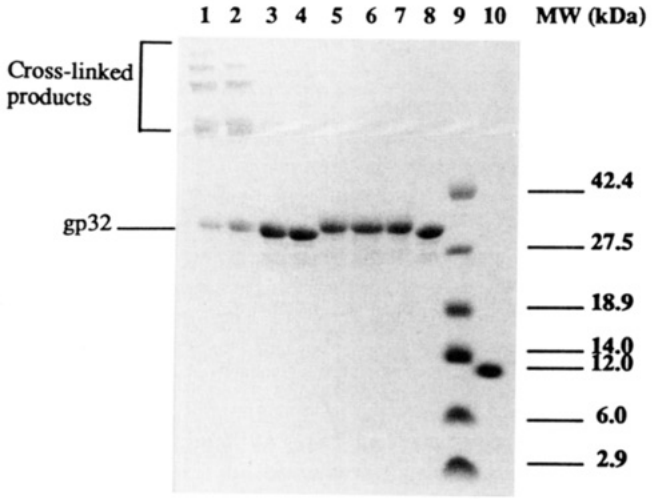

FIGURE 4: Cross-linking reaction requires an excess of peptide, metal, and oxidant. The gene product 32 concentration is 10 μM in all lanes. All reactions were carried out for 5 min: lane 1, 100 μM GGH−Ni(II), 110 μM MMPP; lane 2, 52 μM GGH−Ni(II), 54 μM MMPP; lane 3, 10 μM GGH−Ni(II), 11 μM MMPP; lane 4, 100 μM GGH−Ni(II) alone; lane 5, 110 μM MMPP alone; lane 6, 110 μM GGH without Ni, 110 μM MMPP; lane 7, 104 μM Ni(OAc)2, 110 μM MMPP; lane 8, gp32 alone; lane 9, molecular weight markers; lane 10, cytochrome *c*.

The phage T4 UvsX gene product (Figure 3, lane 9), a RecA-like strand transferase involved in genetic recombination (Formosa & Alberts, 1986; Kodadek et al., 1988; Yonesaki & Minagawa, 1985), is also cross-linked efficiently by GGH−Ni(II) and MMPP (Figure 3, lanes 3 and 6). Like gp32, the UvsX protein self-associates in solution and on ssDNA (Yu & Egelman, 1993). Previous affinity chromatography studies have shown that UvsX and gp32 bind to one another specifically (Formosa et al., 1983; Formosa & Alberts, 1984). Therefore, it was of interest to determine whether these proteins could be cross-linked to one another. As shown in Figure 3 (lanes 1 and 4), when UvsX and gp32 were mixed together and treated with GGH−Ni(II) and MMPP, a variety of higher molecular weight bands were observed. A species with an apparent molecular mass corresponding to a UvsX−gp32 heterodimer was produced, in addition to the ladders of UvsX and gp32 self-cross-linking bands. Western blotting demonstrated that this product contained the gene 32 protein (data not shown). Antibodies that recognize UvsX are not available, so that the presence of UvsX in this product could not be demonstrated directly. However, the fact that this species was not produced in reactions containing only one of the two proteins argues strongly that it is the gp32−UvsX cross-linked product. It is noteworthy that treatment of these two proteins with EDC, a carbodiimide cross-linking reagent, did not produce heterodimers (Jiang et al., 1993).

The gp32−gp32, UvsX−UvsX and gp32−UvsX associations that we detect by oxidative cross-linking are known to be specific in nature. To test the selectivity of our cross-linking protocol, we examined the reaction of GGH−Ni(II)/MMPP with proteins that do not self-associate in solution. As shown in Figure 2 (lanes 5−8), ubiquitin and BSA, which are strictly monomeric in solution (Schlesinger et al., 1975; Goldstein et al., 1975; Gavilanes et al., 1982; Squire et al., 1968), are not cross-linked even at protein concentrations as high as 100 μM. Furthermore, the addition of ubiquitin to a concentration of 170 μM did not interfere with gp32−gp32 cross-linking, nor did it result in gp32−ubiquitin cross-linked species (data not shown). The higher molecular weight bands seen in the BSA-containing lanes are impurities in the protein preparation and are not the result of cross-linking (compare lanes 5 and 6).

Cross-Linking Reaction Requires Peptide, Metal, and Oxidant. The peptide GGH, $Ni(OAc)_2$, and MMPP are all required for cross-linking of the gene 32 protein (see Figure 4). Neither MMPP (lane 5) nor GGH−Ni(II) (lane 4) alone mediated cross-linking of gp32, although treatment of gp32 with MMPP alone did result in a slight broadening of the protein band. The Ni(II) must be complexed by GGH in order for cross-linking to occur. Neither $Ni(OAc)_2$ and MMPP (lane 7) or GGH and MMPP (lane 6) were able to cross-link gp32. For efficient cross-linking to occur, the metal−peptide complex and oxidant must be present in excess, as the efficiency of cross-linking was observed to decline drastically when the concentration of GGH−Ni(II) and MMPP was reduced 10-fold (compare lanes 1−3).

We found that MMPP is not the only oxidant with the ability to support GGH−Ni(II)-mediated cross-linking. Oxone can also be used (Figure 5, lanes 3 and 4), but H_2O_2 and sodium ascorbate do not support cross-linking (Figure 5, lanes 5 and 6).

The use of GGH−Cu(II) instead of the corresponding nickel complex gave rise to different chemistry. In the presence of GGH−Cu(II), H_2O_2, and sodium ascorbate, no cross-linking was observed. As can be seen in Figure 5 (lanes 11 and 12), there is a reduction in the intensity of the gp32 band, suggesting that the protein may have been randomly cleaved and degraded, although we cannot rule out other less likely possibilities such as copper-induced precipitation. Under the same conditions, no reaction at all was observed using GGH−Ni(II) (lanes 5 and 6). When much longer incubation periods (30−60 min) were employed, the treatment of gp32 with GGH−Cu(II) and MMPP or oxone did produce a very small amount of cross-linked

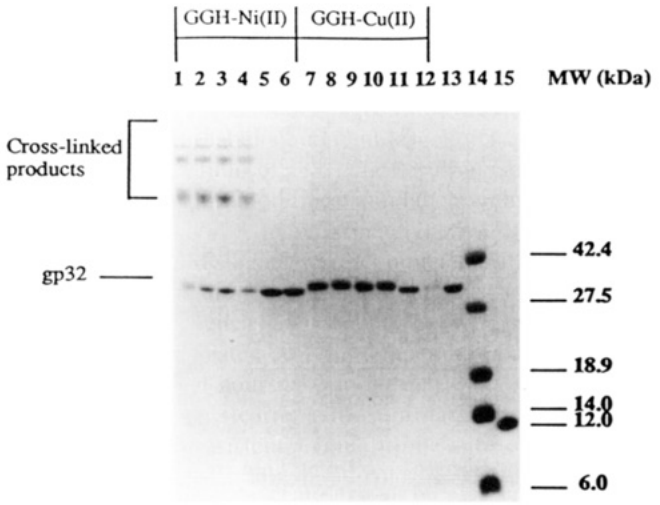

FIGURE 5: Effect of different oxidants. Lanes 1−6 are reactions performed with 100 μM GGH−Ni(II), and lanes 7−12 are reactions performed with 100 μM GGH−Cu(II): lanes 1 and 7, 110 μM MMPP, incubated for 1 min; lanes 2 and 8, 110 μM MMPP, incubated for 10 min. lanes 3 and 9, 100 μM oxone, incubated for 1 min; lanes 4 and 10, 100 μM oxone, incubated for 10 min; lanes 5 and 11, 5 mM sodium ascorbate, 5 mM H_2O_2, incubated for 1 min; lanes 6 and 12, 5 mM sodium ascorbate, 5 mM H_2O_2, incubated for 10 min; lane 13, gp 32 alone; lane 14, molecular weight markers; lane 15, cytochrome *c*.

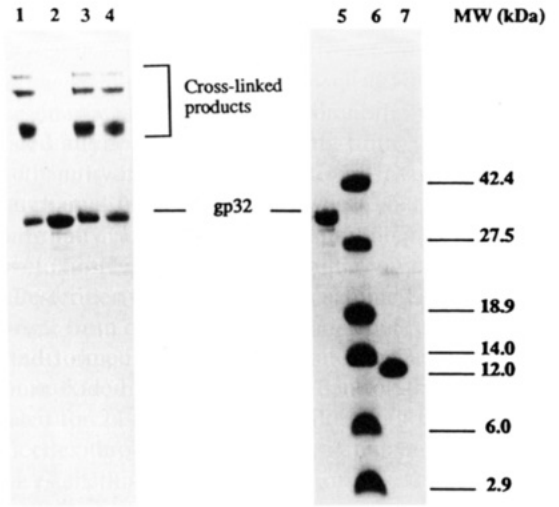

FIGURE 6: Effect of radical scavengers on the cross-linking reaction. All reactions contain 10 μM gp32, 110 μM GGH−Ni(II), and 125 μM MMPP. Reactions were performed for 1 min at room temperature: lane 1, no radical scavenger; lane 2, 10 mM thiourea; lane 3, 500 mM mannitol; lane 4, 500 mM *tert*-butyl alcohol; lane 5, gp32 alone; lane 6, molecular weight markers; lane 7, cytochrome *c*.

species, but protein degradation was the major result (data not shown).

Nature of the Reactive Intermediate. In an attempt to gain some insight into the nature of the reactive intermediate(s) involved in the cross-linking reaction, the effect of hydroxyl radical scavengers on the cross-linking reaction was examined (see Figure 6). Thiourea at concentrations of 10 mM or greater quenched the reaction completely (lane 2). Thiourea at this concentration did not inhibit the cross-linking of gp32 by EDC (Jiang et al., 1993), indicating that thiourea did not disrupt the protein−protein contact, but rather titrated the intermediate(s) generated by GGH−Ni(II) and MMPP. However, mannitol and *tert*-butyl alcohol, common hydroxyl radical scavengers (Rana & Meares, 1991), did not quench the GGH−Ni(II)-mediated cross-linking reaction, even at concentrations as high as 500 mM (lanes 3 and 4). These data suggest that hydroxyl radicals are not involved in the cross-linking reaction.

To further test this hypothesis, the reaction of gp32 with EDTA−Fe(II), H_2O_2, and sodium ascorbate was examined. EDTA−Fe(II) is known to generate hydroxyl radicals in the presence of peroxides and a reducing agent (Rush & Koppenol, 1988; Sigel, 1969; Walling, 1975; Stubbe & Kozarich, 1987). Protein degradation was the only reaction observed; no cross-linking was detected under these conditions (data not shown). The degradation was inhibited by mannitol and *tert*-butyl alcohol as well as by thiourea. Taken together, these results indicate that cross-linking mediated by GGH−Ni(II) and MMPP is not the result of hydroxyl radicals.

Neither superoxide dismutase nor catalase had any effect on the cross-linking of gp32 by GGH−Ni(II) and MMPP (data not shown). These results indicate that neither superoxide nor H_2O_2 is an important intermediate in the cross-linking reaction, since there was more than enough enzyme to rapidly destroy these oxidants if they had formed. Finally, flushing the solution with argon prior to cross-linking had no effect on GGH−Ni(II)-mediated cross-linking of gp32 (data not shown), suggesting that O_2 is not an important reaction component, or if it is, it is required only at very low concentrations.

Aromatic Amino Acids Are Targets of the Oxidized Metal Complex. In order to ascertain likely targets of the active intermediate in the protein, we carried out the experiment shown in Figure 7B. The gene 32 protein was treated with GGH−Ni(II)/MMPP in the presence of various concentrations of lysine, tryptophan, or tyrosine, and the effect on cross-linking was assessed. Increasing concentrations of tyrosine and tryptophan, particularly the former, strongly inhibited the formation of gp32−gp32 cross-links, but the addition of lysine had essentially no effect. The aromatic amino acids did not inhibit gp32 self-association, as evidenced by gradient sedimentation experiments performed in the presence of tyrosine (data not shown). We suggest that the inhibition is due to a direct reaction between the amino acids and the oxidatively activated metal complex, thus titrating the amount of active intermediate available to react with the gene 32 protein. Since tyrosine was the most efficient inhibitor of gp32 cross-linking, it seems reasonable to propose that this residue might be a primary target for the cross-linking reagent. Conversely, the fact that lysine did not interfere with cross-linking suggests that this residue is not involved in the formation of protein−protein cross-links, supporting the view that the GGH−Ni(II)/MMPP system utilizes fundamentally different chemistry from most cross-linking reagents.

DISCUSSION

We have demonstrated that the complex formed between Ni(II) and the tripeptide GGH (Bossu & Margerum, 1977; Sakurai & Nakahara, 1979) is an efficient protein cross-linking reagent, in the presence of a strong oxidant such as MMPP. Although only a handful of proteins have been examined so far, the results presented here indicate that only proteins that are closely associated in solution are cross-linked under these conditions. Furthermore, the reaction

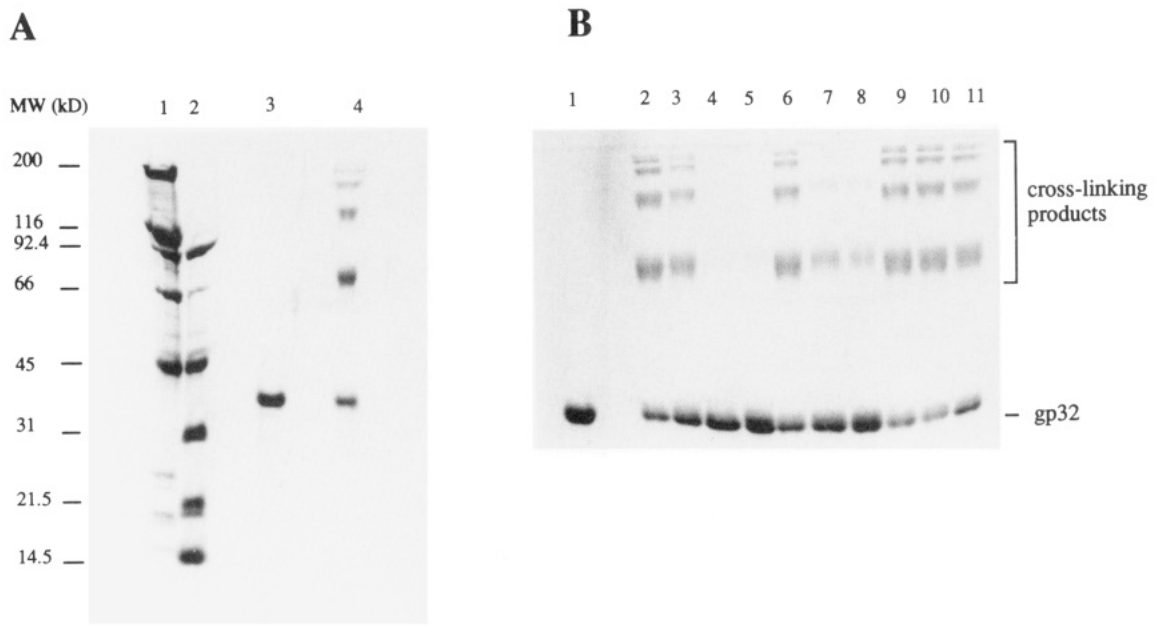

FIGURE 7: Tyrosine and tryptophan, but not lysine, interfere with the cross-linking of gp32, suggesting that aromatic residues are the targets of the oxidized nickel complex in the cross-linking reaction. (A) Lane 1, high molecular weight standards; lane 2, low molecular weight standards; lane 3, gp32; lane 4, gp32 + GGH−Ni(II) + MMPP. (B) Lane 1, gene 32 protein; lanes 2−11 contain the gene 32 protein, the GGH−Ni(II)/MMPP cross-linking reagents, and the following concentrations of amino acids, lane 2, no amino acid; lane 3, 65 μM tyrosine; lane 4, 250 μM tyrosine; lane 5, 450 μM tyrosine; lane 6, 65 μM tyrptophan; lane 7, 250 μM tryptophan; lane 8, 450 μM tryptophan; lane 9, 65 μM lysine; lane 10, 250 μM lysine; lane 11, 450 μM lysine.

appears to be fairly general. In addition to UvsX−UvsX, gp32−gp32, UvsX−gp32, and GAL4-GAL4 cross-linking, we have also recently found that the yeast TATA binding protein (TBP) and RAD51 protein can be cross-linked to themselves and that the phage T4 UvsY and gene 32 proteins can be cross-linked to one another. These have all been shown by alternative methods to represent specific interactions (S.-H. Yang, H. Jiang, and T. Kodadek, unpublished results).

Cross-linking requires the presence of Ni(II) and the peptide. One can infer the structure of the catalyst from a perusal of the crystal structure of the GGH−Cu(II) complex, which shows that the peptide serves as a tetradentate ligand in which the copper atom is bound in a mildly distorted square planar fashion by the N-terminal amino group, two amide nitrogens that have been deprotonated, and the ϵ-imidizole nitrogen of the histidine side chain (Camerman et al., 1976). While such detailed structural data are not available for the GGH−Ni(II) complex, Ni(II) and Cu(II) are bound in similar fashion to tetraglycine (Freeman & Taylor, 1965; Freeman et al., 1968) and, by analogy, are believed to have similar modes of complexation for the GGH ligand. It is not surprising that a Ni−peptide complex must be formed for cross-linking to occur, because the reactivity of nickel is known to be highly ligand dependent. For example, polyamine−Ni(II) complexes and GGH−Ni(II) are both known to cleave DNA (Mack & Dervan, 1990, 1992; Chen et al., 1991) and to epoxidize alkenes (Kinneary et al., 1988a,b; Koola & Kochi, 1987; Wagler & Burrows, 1988; Yoon & Burrows, 1988) in the presence of an oxidant, whereas free Ni salts are unreactive under the same conditions.

The Reactive Intermediate in the Cross-Linking Reaction Is Probably a High-Valent Nickel Species. The mechanism of the cross-linking reaction is currently unknown. However, it is likely to involve a high-valent nickel complex, possibly containing an oxo ligand derived from MMPP. A variety of experiments suggest that hydroxyl radicals, superoxide, and peroxides are not involved in the reaction. However, sulfur-containing compounds block cross-linking completely. Thiourea quenches the GGH−Ni(II) mediated cross-linking reaction at concentrations that do not disrupt protein−protein interactions. Furthermore, no cross-linking of gp32 was observed when the reaction was performed in HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) or PIPES (1,4-piperazinediethanesulfonic acid) buffer (50 mM, pH 7.0 (data not shown)). Thiourea, HEPES, and PIPES (Wang & Sayre, 1989; Grady et al., 1988) are all easily oxidized. Furthermore, it has been reported that GGH−Ni(II), in the presence of iodosylbenzene, epoxidized styrene (Mack & Dervan, 1990,1992), and a variety of chemically related polyamine−Ni(II) complexes will epoxidize olefins in the presence of various oxidants (Kinneary et al., 1988a,b; Koola & Kochi, 1987; Wagler & Burrows, 1988; Yoon & Burrows, 1988). A high-valent nickel−oxo species has been suggested to be the active intermediate in all of these reactions.

Cross-Linking May Involve Aromatic Amino Acids. Many cross-linking reagents react with nucleophilic functional groups in proteins. If the appropriate side chains are not present at the protein−protein interface, cross-linking does not occur. While the chemical nature of the GGH−Ni(II)/MMPP-generated cross-link(s) has not yet been identified, the oxidative nature of the reaction suggests that nucleophilic residues such as lysine are unlikely to be targets of the active species, consistent with our finding that exogenous lysine does not inhibit cross-linking. Rather, one would imagine that easily oxidized side chains would react rapidly with the high-valent nickel complex. While one would expect that cysteine side chains would be a prime target for the oxidized metal complex, it is very unlikely that disulfide bonds support the observed cross-linking since the samples were boiled in β-mercaptoethanol prior to electrophoresis, which was

conducted under denaturing and reducing conditions. However, other types of cysteine oxidation products, as well as internal cross-links and other types of irreversible oxidation reactions, could contribute to the electrophoretic inhomogeneity of the products observed in some cases. We speculate that covalent interprotein cross-linking might be the result of the oxidation of aromatic residues, creating protein-centered radicals or radical cations that then attack nearby unsaturated groups. Since the 140-residue GAL4 fragment contains only four aromatic residues, this could explain why it cross-links much less efficiently than the gene 32 or UvsX proteins, despite the fact that it is a stable dimer. There is precedent for this type of chemistry in the context of associated proteins, for example, the formation of 2,2′-bistyrosyl adducts (Solar et al., 1984; Yamamoto, 1973, 1977; Catalano et al., 1989; Tew & Ortiz deMontellano, 1988; Wilks & Ortiz deMontellano, 1992). An electron transfer mechanism would also rationalize the fact that both HEPES and PIPES buffers strongly inhibit cross-linking, since both are known to be oxidized by metal complexes possessing high redox potentials. However, we emphasize that these arguments are speculative, and more detailed hypotheses will have to await a rigorous mechanistic analysis, including the elucidation of the structure of a cross-linked species.

As mentioned previously, the treatment of certain proteins with the GGH−Ni(II) complex under oxidative conditions has been observed to result in extensive protein degradation. The data presented here are not in conflict with these results. Metallopeptide-mediated oxidative cross-linking occurs rapidly under our conditions and is observed only between proteins that are stably associated in solution. Previous studies employed monomeric proteins and longer incubation times. We also observed considerable protein degradation under these conditions. This is probably due to an alternate reaction pathway that involves hydrogen atom abstraction from the α-carbon of the amide backbone, a process known to result in cleavage of the peptide chain (Hoyer et al., 1990; Kurtz et al., 1978; Phelps et al., 1961; Ramer et al., 1988; Young & Tamburini, 1989; Bateman et al., 1985). We speculate that this reaction is intrinsically slower than the process that leads to cross-linking, thus favoring the latter reaction in the case of closely associated proteins.

This methodology is of interest in that it appears to represent a new type of cross-linking chemistry that does not involve nucleophilic residues, but rather targets aromatic side chains. It may therefore allow the detection of interactions that are missed using other cross-linkers. For example, GGH−Ni(II)/MMPP efficiently cross-links UvsX and gp32, while EDC does not (Jiang et al., 1993). In addition, GGH−Ni(II)/MMPP is, to the best of our knowledge, the first protein cross-linking reagent comprised of naturally occurring amino acids. Therefore, the GGH moiety could be tagged to the N-terminus of a protein of interest at the genetic level (Parks et al., 1994; Carter, 1990) and this construct introduced into the cell. If a multiprotein complex containing the tagged protein could be isolated, it might be possible to activate the fusion protein by the addition of Ni(II) and MMPP and to determine the identity of nearby factors in the complex by examination of the resultant cross-linking pattern.

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