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Protein Cross-linking Mediated by Metalloporphyrins

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Abstract—A biomimetic protein cross-linking reaction is described which employs oxidatively-activated manganese and iron porphyrins as the reactive species. A wide range of proteins cross-link under these conditions, but only if they are intimately associated in solution. The reaction is rapid, efficient, and will be useful for the suprastructural analysis of multiprotein complexes.

Introduction

Most biological processes are mediated by macromolecular assemblies comprised of many polypeptides that associate in a highly specific manner.[1] A crucial goal in elucidating the mechanism of action of these machines is to map the pattern of protein–protein contacts at various functional stages. The development of new protein cross-linking reagents that can be delivered to a specific target protein within a given complex is an important step towards facilitating this aim. Synthesis of chimeric molecules which encompass both a peptide-binding domain and a reactive chemical moiety allows for selective binding of the cross-linking reagent to a peptide receptor. The reactive group can then be activated, leading to highly specific cross-linking of proteins that interact with the tagged protein (Fig. 1). A site-directed system of this type minimizes the extensive covalent modification of many amino acids throughout the complex that occurs when common cross-linkers are used which hampers interpretation of the data and can lead to artifactual results.

A first generation system of this type was introduced recently.[2,3] In this case, the chimeric molecule was a nickel ion and the receptor was a nickel-binding peptide, the six histidine tag. This design was based on a previous observation that the peptide–metal complex, NH_2-GGH·Ni(II), mediates the cross-linking of closely associated proteins when treated with a peracid.[4] The GGH-Ni(II) and His_6-Ni(II) cross-linking reactions are believed to proceed by similar mechanisms. The reactive intermediate is thought to be a high-valent Ni(III) species which is rapidly reduced by a tyrosine residue. The resultant tyrosyl radical then couples with another tyrosine leading to the formation of a stable bityrosyl adduct.[5] This mechanistic hypothesis (see ref. 5 for a more detailed discussion) is supported by the observation that cross-linking proceeds much more efficiently if a tyrosine residue is engineered into the target protein, either at the protein–protein interface[5] or near the His_6 tag (D. Fancy and T. Kodadek, submitted). While bityrosines have been demonstrated to be a major product of the oxidative cross-linking reaction, it is possible that other covalent cross-links could be formed by the attack of a nearby nucleophilic residue, such as cysteine or lysine, on the resultant tyrosyl radical. A major advantage of this chemistry is that the cross-linking reagent is not incorporated into the product, but merely initiates direct bond formation between two residues at the protein–protein interface. Thus, only proteins that are in intimate contact with one another can form cross-linked products. This is in contrast to most common cross-linking reagent which contain two reactive groups separated by a linker arm of variable length (Fig. 2).

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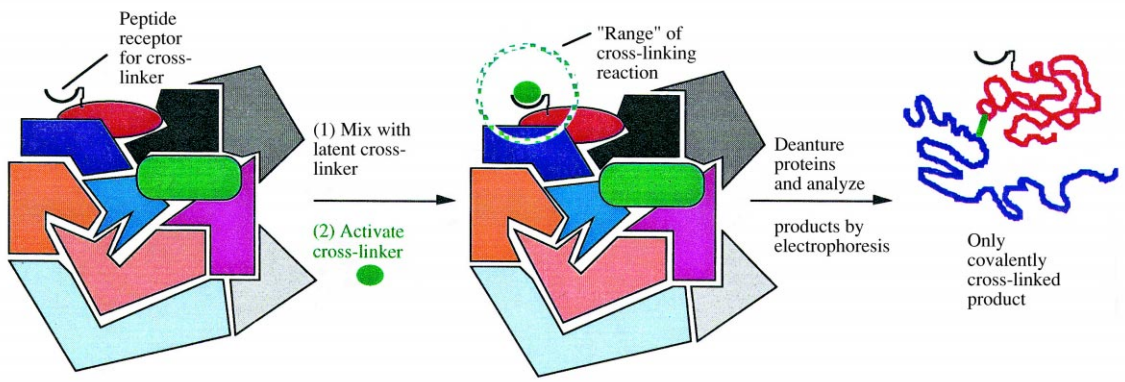

Figure 1. Schematic representation of a site-directed cross-linking strategy to analyze the architecture of multiprotein complexes. A small peptide tag is attached to the protein of interest (orange) at the genetic level. This peptide binds specifically to a latent cross-linking reagent, directing this species to a particular point in a large complex. Activation of the cross-linking reagent then initiates a localized chemical reaction that results in the cross-linking of the tagged protein to closely associated species. The products can be analyzed by denaturing the complex and analyzing the products by denaturing gel electrophoresis, using an antibody to identify the tagged protein and any covalent adducts that contain it.

The His_6 system works well for the analysis of purified complexes containing a single tagged protein, as well as in crude *Eschericia coli* extracts.[2] Unfortunately, attempts to cross-link His_6-tagged proteins to their binding partners in eukaryotic extracts have met with little success, even in extracts where small molecule reducing reagents have been removed by dialysis (D. Fancy and T. Kodadek, unpublished observations). A possible explanation for this limitation is that eukaryotic cells are much richer in metal-binding proteins than are bacteria, thus hampering the selective delivery of the nickel ion to the peptide receptor in eukaryotic cell extracts. This is a serious limitation since it is often difficult and time-consuming to purify multiprotein complexes in their native form.

Two possible solutions to this problem can be envisioned. One is to explore the use of peptide receptors other than His_6 that have much higher affinity for Ni(II).[5] The other is to move beyond simple peptide–metal ion complexation and employ more sophisticated cross-linking conjugates for which there will be few, if any, high affinity sites in naturally occurring eukaryotic proteins. A step in this direction would be to encase the oxidatively active metal in a stable ligand that would block nonspecific binding to chelating sites on protein surfaces. Once a metal–ligand complex with suitable chemical properties is found, a cognate peptide receptor for it might be found through combinatorial methods.

Metalloporphyrins represent a class of oxidatively active ligand–metal complexes that have the characteristics described above. Furthermore, heme-containing proteins can act as potent oxidants when treated with two-electron oxidants or oxygen and a reducing agent. For example, sperm whale myoglobin undergoes covalent coupling through tyrosine dimer formation when exposed to hydrogen peroxide.[6–8] In addition, *Arthromyces* peroxidase has been shown to mediate the oxidative polymerization of calmodulin through tyrosine cross-links[9–12] and bityrosine adducts are found in atherosclerotic lesions as a result of tyrosine oxidation mediated by myeloperoxidase.[13,14] Bityrosine formation has been catalyzed by other peroxidases as well.[15,16] Since many reactions of heme-containing proteins can be reproduced with synthetic models,[17,18] these literature precedents suggested that simple, water-soluble metal porphyrin complexes might serve as the type of reagents we require. We show here that simple, water-soluble manganese and iron porphyrins mediate cross-linking of closely associated proteins in the presence of oxidants such as peroxide and peracids. However, proteins that are not bound stably are not cross-linked. Therefore, metalloporphyrins may serve as useful reagents in the development of a second generation site-directed cross-linking system.

Results and Discussion

A series of purified proteins were treated with chloromanganese(III)-tetra(*p*-sulfanato)phenylporphyrin (Mn TPPS) and the oxidant $KHSO_5$ in a buffered aqueous solution. After a one minute incubation at room temperature, the oxidations were quenched by the addition of excess thiourea, and the results were analyzed by denaturing polyacrylamide gel electrophoresis (SDS–PAGE) followed by staining with Coomassie Brilliant Blue (Fig. 3). Under these conditions, the dimeric protein glutathione-*S*-transferase (GST) is cross-linked to

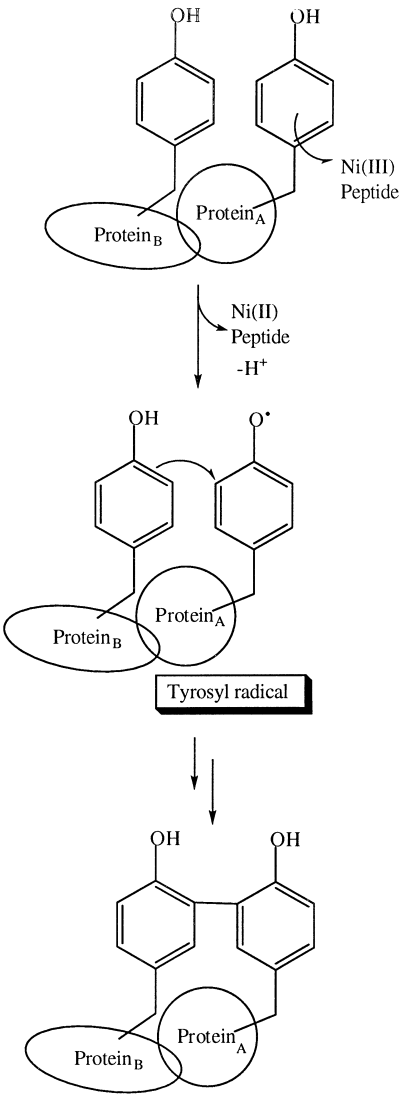

Figure 2. Postulated chemical mechanism of oxidative cross-linking mediated by peptide·Ni(II) complexes in the presence of a peracid. A high-valent Ni(III) species is postulated to be formed by reaction of the Ni(II) complex with the peracid. This then oxidizes a tyrosine residue, leading to production of a radical cation (not shown) which would deprotonate rapidly to provide a tyrosyl radical. This radical could then couple to another tyrosine, or possibly other nucleophiles such as cysteine or lysine side chains, to provide a covalent cross-link (see ref. 5 for a more detailed discussion of the mechanism). The tyrosine residues shown would be present at the protein–protein interface.

form a new species that corresponds to the expected molecular mass of a GST homodimer (lane 8). The TATA-binding protein (TBP), which is known to form stable dimers[19] as well as higher-order oligomers[20] is also reactive. When treated with MnTPPS and $KHSO_5$, TBP is cross-linked to yield a mixture of oligomers (lane 10). The phage T4 UvsY protein, which forms long filaments that are important in homologous DNA recombination[21,22] was also treated with the oxidative cross-linking system. Under these conditions, UvsY was cross-linked in almost quantitative yield, forming large covalent multimers whose molecular weights could not be precisely determined by SDS–PAGE (lane 6). No cross-linked products were observed when either the porphyrin or the oxidant was omitted from the reaction (data not shown). Since SDS–PAGE was performed under reducing conditions, the cross-linked products were not the result of disulfide bond formation.

The same reaction conditions failed to cross-link ubiquitin, a rigorously monomeric protein (Fig. 3, lane 4). This indicates that the MnTPPS-mediated reaction does not couple proteins randomly, but rather cross-links only polypeptides that are intimately associated with one another. This conclusion was also supported by experiments in which TBP, GST and UvsY were mixed together, and then treated with porphyrin and oxidant. Only homooligomeric products were observed (data not shown). Lysozyme represents an interesting intermediate case. This protein is monomeric, but has hydrophobic patches on its surface that lead to transient self-association in solution. The MnTPPS-mediated reaction is able to trap these interactions, producing dimeric and trimeric products (Fig. 3, lane 2).

The reaction is not limited to the production of homodimers and higher oligomers. This was demonstrated by incubating a ^{32}P-labeled polypeptide containing the activation domain of the Gal4 transcription factor[3] with TBP (Fig. 4). These polypeptides form a stable protein complex that may be involved in transcriptional activation in yeast.[23] When treated with MnTPPS and $KHSO_5$, a new protein species with the apparent molecular mass of the cross-linked activation domain-TBP product was observed using a PhosphorImager (lane 4). The yield is on the order of 10%. A fainter band of even higher apparent molecular mass was also observed. The size of this species suggests it is a TBP_2-activation domain product, which is expected if the activation domain binds to the TBP dimer.

Further experiments revealed that the conditions employed in Figure 3 provided optimal results. Incubations longer than a minute at these protein concentrations (μM) or an increase in the oxidant concentration did not provide better yields of cross-linked products. Addition of axial ligands such as imidazole did not noticeably stimulate the manganese porphyrin-mediated cross-linking reactions (data not shown). As shown in Figure 5, chloroprotoporphyrin IX iron (III) (heme) also mediates protein cross-linking when hydrogen

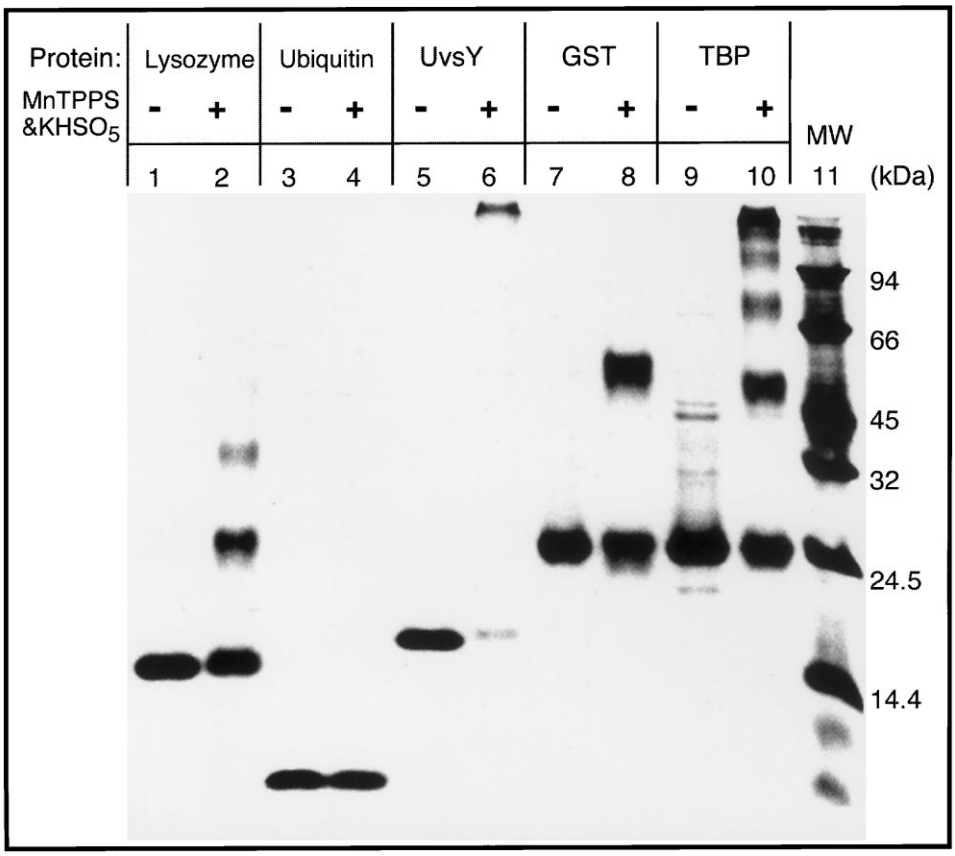

Figure 3. Manganese poprhyrin-mediated cross-linking of associated proteins in the presence of $KHSO_5$. The proteins indicated were incubated in the absence (odd numbered lanes) or presence (even numbered lanes) of MnTPPS and $KHSO_5$. UvsY, GST, and TBP are known to self-associate and were cross-linked under these conditions. Ubiquitin is rigorously monomeric and was not cross-linked. Lysozyme monomers associate transiently in solution and thus also provide some cross-linked products.

peroxide was used as the terminal oxidant. However, a direct comparison between these experiments and those employing MnTPPS/$KHSO_5$ reveals that the MnTPPS-mediated reaction is more efficient. Furthermore, the heme/peroxide-mediated cross-linking reaction often produces irreproducible results. The yields of cross-linked products can vary considerably and the reaction is sensitive to the quality of the hydrogen peroxide solution. Therefore, we recommend the MnTPPS/$KHSO_5$ system for routine use.

A thorough mechanistic analysis of this cross-linking reaction has not been undertaken. Based on literature precedent[24] however, it seems reasonable to assume that the reaction proceeds through a high-valent manganese–oxo complex,[25] which abstracts an electron from an accessible tyrosine residue on the target protein. This would be expected to lead to covalent cross-linking by coupling of the resultant tyrosyl radical with another tyrosine (see Fig. 2). This mechanistic hypothesis is consistent with the result shown in Figure 6. In these reactions various amounts of exogenous tyrosine, tryptophan, phenylalanine or lysine were added to a standard MnTPPS/$KHSO_5$-mediated cross-linking of GST. Tyrosine and, to a slightly lesser extent, tryptophan inhibited the production of GST dimers. Lysine and phenylalanine had little effect. This is the result expected if the cross-linking indeed proceeds through the oxidation of a tyrosine or tryptophan residue.[5,26] Most of the high-valent manganese–oxo complexes formed in solution will encounter free amino acid rather than GST, leading to inhibition of cross-linking if that amino acid is oxidizable.

The MnTPPS/$KHSO_5$-mediated protein cross-linking reaction provides results similar to those obtained using NH_2-GGH·Ni(II) and peracids,[4] providing rapid and efficient cross-linking of many tightly associated proteins. Less stable interactions, such as the transient self-association of lysozyme, can also be trapped. However, the porphyrin-mediated reaction is not promiscuous, and there is no evidence that this system will cross-link

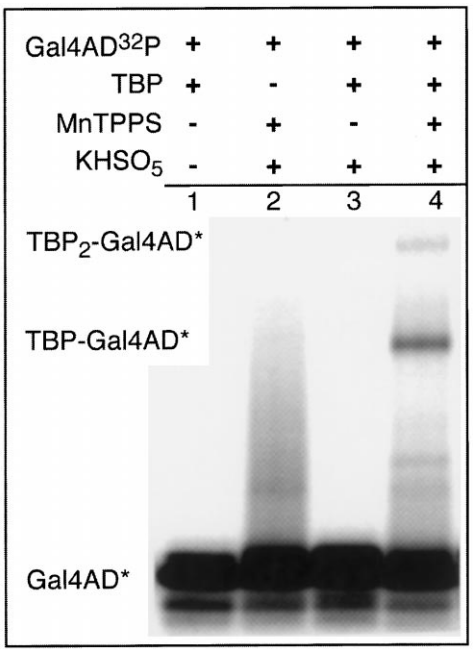

Figure 4. The manganese prophyrin-mediated cross-linking reaction can produce covalently coupled heterodimers. A ^{32}P-labeled 50mer peptide containing the Gal4 activation domain was mixed with TBP at a concentration well above the reported K_D of the complex (2×10^{-7} M). The complex was then treated with the reagents indicated. Products were analyzed by denaturing gel electrophoresis and phosphorimagery. Therefore, only activation domain-containing species are visible. Covalently cross-linked bands were observed that had the expected apparent molecular mass for a Gal4 AD-TBP product and a Gal4 AD-$(TBP)_2$ species. These products were produced only when TBP, the Gal4 AD, MnTPPS, and $KHSO_5$ were present. Quantitation on the phosphoimager revealed that the yield of the AD·TBP product was about 10%, based on the amount of AD present.

proteins that do not associate with one another specifically. While rigorous product analysis has not been undertaken, this reaction is expected to be of the 'zero Å' type in which nearby side chains of the interacting proteins couple to one another directly without the incorporation of an intervening linker arm into the product. Therefore, we anticipate that porphyrin-mediated cross-linking will find utility in the analysis of multiprotein complexes. More importantly, manganese or iron porphyrins can serve as stable, but highly active reagents in a second generation site-directed cross-linking system of the type shown in Figure 1 if appropriate peptide receptors can be isolated, perhaps from combinatorial libraries (for reports of naturally occurring heme-binding peptides, see refs 27, 28). Alternatively, the porphyrin could be appended to other molecules with known peptide or polypeptide ligands. Efforts along these lines are underway.

Experimental

Proteins and reagents. GST,[29] TBP,[30] and UvsY[21] were purified according to published protocols. Ubiquitin and lysozyme purchased from Sigma Chemical Co. (U6253, from bovine red blood cells, lyophilized and L6876, from chicken egg whites, crystallized, dialyzed and lyophilized). Both were used as received. The His_6-Gal4 AD ^{32}P peptide was purified and radiolabeled as described.[3] Prior to use, proteins were dialyzed from storage buffer into phosphate buffered saline (PBS, 50 mM phosphate, pH 7.2, 150 mM NaCl). For subsequent storage, protein solutions in PBS were frozen in N_2(liq.) and stored at −80 °C. Amino acids (Tyr, Trp, Phe, Lys) and EDTA (ethylenediamine tetraacetic acid) were purchased from Sigma and used as received. 5, 10, 15, 20-tetrakis(4-sulfonatophenyl)-21*H*,23*H*-porphine manganese (III) chloride (MnTPPS) and hemin (iron (III) protoporphyrin IX chloride) were purchased from Aldrich Chemical Co. and used as received. Porphyrins were stored in the dark at 4 °C as 10 mM solutions in dimethylformamide (DMF, Aldrich, 99%). These DMF solutions were diluted into PBS (50 mM phosphate, pH 7.2, 150 mM NaCl) just prior to use. Potassium monopersulfate ($KHSO_5$, oxone) was purchased from Fluka as 47% of a mixture including $KHSO_4$ and K_2SO_4. It was dissolved in double distilled water at 0 °C and used immediately. Hydrogen peroxide was purchased from Aldrich as a 30 wt% solution in water. It was stored at 4 °C in the dark and used within two weeks of purchase. Immediately prior to use, the hydrogen peroxide was diluted with double distilled water at 0 °C. The concentrations of purified proteins were calculated using the Bio-Rad Bradford reagent versus a standard solution of bovine serum albumin (BSA). Concentrations of other reagents were based on dry weights.

Porphyrin-mediated cross-linking reactions. Cross-linking reactions were carried out in a total volume of 20 μL. Final concentrations were 50 mM phosphate (pH 7.2), 150 μM NaCl, 10 μM protein, and 100 μM porphyrin, unless stated otherwise. Protein(s) and porphyrin were allowed to equilibrate on ice for the time necessary to prepare the oxidant solution (~5 min). The reactions were initiated by the addition of oxidant and quenched with 8 μL of 4×loading buffer (0.2 M Tris, 2.88 M β-mercaptoethanol, 8% SDS, 40% glycerol, 0.4% bromophenol blue, and 0.4% xylene cyanol). The samples were heated to 100 °C for 5 min and then separated by electrophoresis through a 10% tricine–SDS polyacrylamide gel. The proteins were visualized by staining with Coomassie brilliant blue, unless otherwise indicated.

For the manganese porphyrin-mediated reactions, cross-linking was initiated by addition of $KHSO_5$ to a

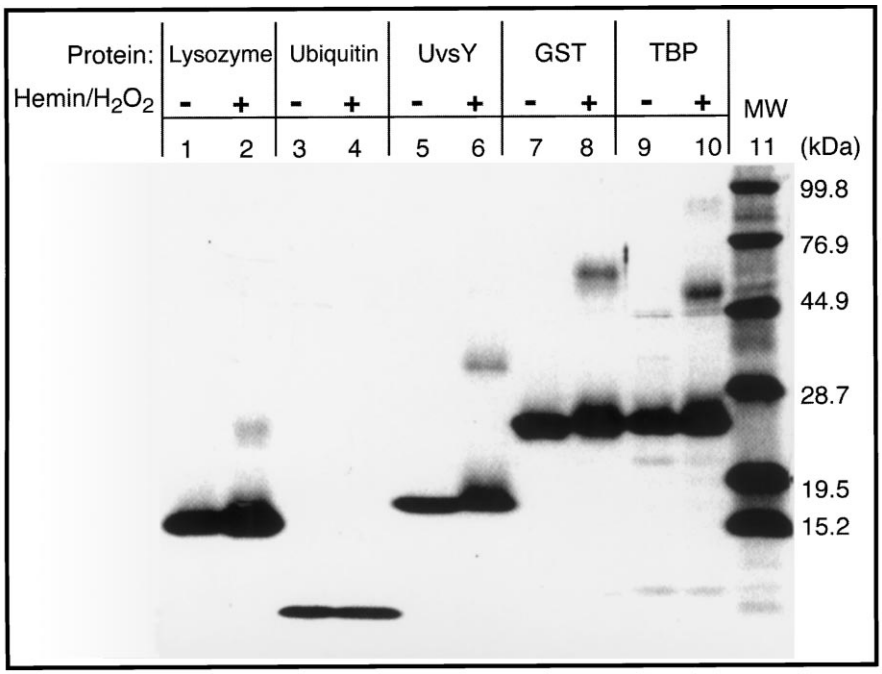

Figure 5. An iron porphyrin and hydrogen peroxide also mediate protein cross-linking. The proteins indicated were incubated in the absence (odd numbered lanes) or presence (even numbered lanes) of hemin and hydrogen peroxide. Cross-linking is observed for associating proteins, but the reaction is less efficient than the comparable MnTPPS/$KHSO_5$-mediated processes.

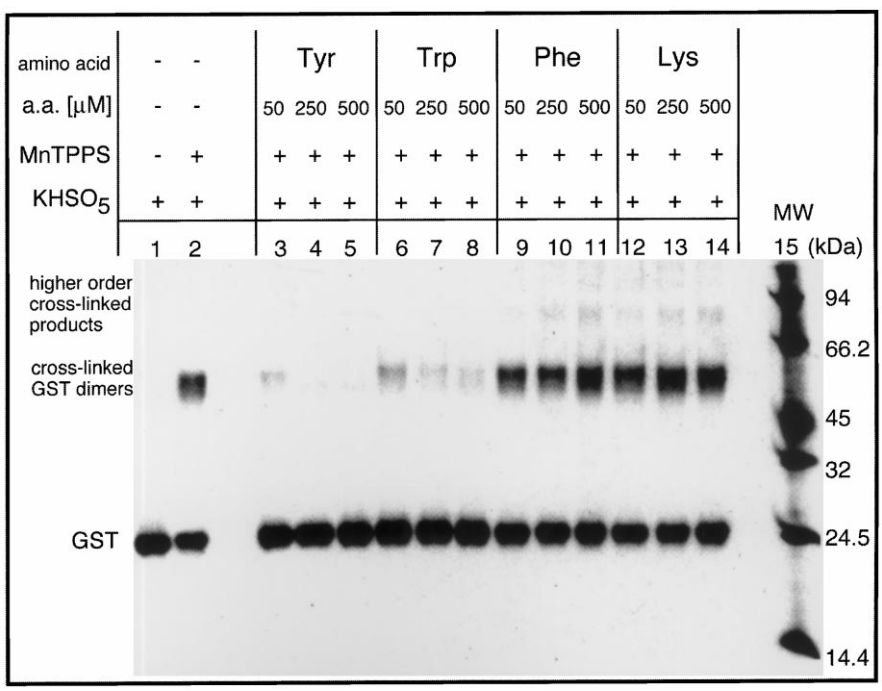

Figure 6. Competitive inhibition of MnTPPS/$KHSO_5$-mediated cross-linking by Tyr and Trp indicates that these side chains are the primary targets of the oxidized metalloporphyrin intermediate. The indicated amounts of either Tyr, Trp, Phe, or Lys were added to a solution containing 10 μg GST, 100 μM MnTPPS, and 100 μM $KHSO_5$. Tyr and Trp, the most easily oxidizable amino acids, strongly inhibited GST cross-linking, but the less easily oxidizable amino acids Phe and Lys did not.

final concentration of 100 μM. The solution was allowed to incubate at room temperature for 45 s, then quenched with 8 μL 4×loading buffer and analyzed as described above.

The hemin-mediated reactions were initiated by addition of H_2O_2 to a final concentration of 1 mM. The solution was allowed to incubate for 6 min at room temperature before quenching with 8 μL 4×loading buffer. The reactions were then evaluated using gel electrophoresis as described earlier.

Porphyrin mediated heterocross-linking of Gal4 AD ^{32}P and TBP. The total volume was 15 μL. Final concentrations were 50 mM phosphate (pH 7.2), 150 mM NaCl, 500 μM EDTA, 1 μM ^{32}P-labeled Gal4 AD, 1.6 μM TBP, and 100 μM MnTPPS. This mixture was allowed to equilibrate for 15 min. The cross-linking reaction was then initiated by addition of $KHSO_5$ to a final concentration of 100 μM. After 30 s at room temperature the 5.5 μL of 4×loading buffer was added. The samples were analyzed by electrophoresis through a 10% tricine–SDS polyacrylamide gel, and visualized by drying the gel on Whatman 3 mm paper, followed by phosphorimagery.

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