New chemistry for the study of multiprotein complexes: the six-histidine tag as a receptor for a protein crosslinking reagent

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Background: To study very large macromolecular complexes, it would be useful to be able to incorporate probe molecules, such as fluorescent tags or photoactivatable crosslinkers, into specific sites on proteins. Current methods for doing this use relatively large amounts of highly purified protein, limiting the general utility of these approaches. The need for covalent posttranslational chemistry also makes it extremely difficult to use modified proteins in studies of native complexes in crude lysates or in living cells. We set out to develop a protein tag that would circumvent these problems.

Results: A very simple type of molecular recognition, metal–ligand complexation, can be used to deliver a nickel-based crosslinking reagent to proteins containing a six-histidine (His_6) tag. When activated with a peracid, the His_6–Ni complex mediates oxidative crosslinking of nearby proteins. The crosslinking reaction does not involve freely diffusible intermediates, and thus only those proteins in close proximity to the His_6-tagged polypeptide are crosslinked.

Conclusions: The His_6 tag, commonly used as an affinity handle for the purification of recombinant proteins, can also be used as an internal receptor for an oxidative protein-crosslinking reagent. No covalent protein modifications are necessary, since the His_6 tag is introduced at the DNA level. The crosslinking reaction is fast, efficient in most cases, and provides products that are easily separated from most other proteins present. This methodology should find widespread use in the study of multiprotein complexes.

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Introduction

Most important biological processes are mediated by very large complexes composed of many protein and/or nucleic acid molecules. Mapping the network of macromolecular contacts is centrally important to the study of these complicated assemblies. For very large complexes, such as ribosomes, multiprotein transcription assemblies, and spliceosomes, this is often difficult to accomplish if the probe molecules are not specific for a particular component of the complex. For example, addition of typical protein-crosslinking reagents, such as glutaraldehyde, to mega-dalton multiprotein complexes leads to the production of dozens or even hundreds of crosslinked products, and it is thus difficult to extract information on the particular factor under investigation. In the study of DNA–protein complexes containing multiple DNA-binding factors, probes such as hydroxyl radical or DNase I provide information regarding the region of DNA blanketed by the complex, but rarely distinguish between the contacts made by different proteins in the complex.

To obtain higher resolution data, several investigators have explored the development of modified protein or nucleic acid molecules that contain covalently-bound probe molecules, allowing the interactions of a particular component of a complex to be studied in a complicated background. Fluorescent labels are commonly used as probe molecules in these studies, but, more recently, reactive species have also been used. For example, iron–EDTA derivatives have been attached to proteins, usually through engineered cysteine residues, and used as affinity cleavage reagents for DNA and proteins [1–5].

Some work has been done to study affinity delivery of protein crosslinkers [6,7]. For example, Ebright and co-workers [8] attached a photoactivatable azide crosslinker, bearing a radioactive iodine atom, to *Escherichia coli* catabolite gene activator protein (CAP) via disulfide-bond formation between the crosslinker and an engineered cysteine in the protein. A CAP–RNA polymerase complex was formed and subjected to photolysis, resulting in CAP–polymerase crosslinking. The disulfide bond was then reduced, transferring the radioactive tag to the polymerase. This allowed identification of the subunit of RNA polymerase contacted by CAP.

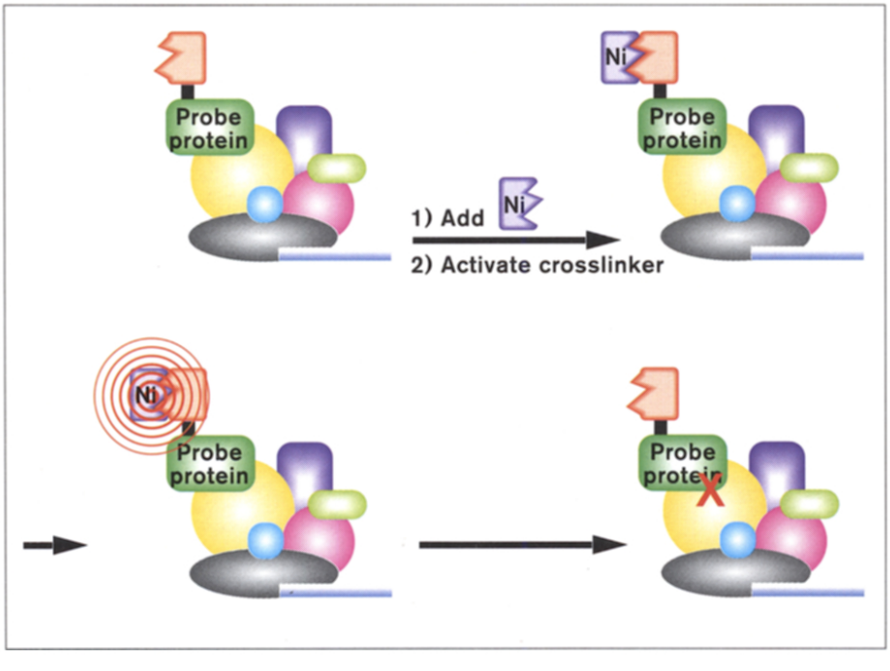
Site-localized crosslinking strategies should be particularly useful for the study of very large protein complexes,

where the complexity of product mixtures and the high level of protein modification generated by traditional reagents are extremely problematic. Unfortunately, this approach has been limited by the need to synthesize affinity-delivered crosslinkers and to develop the appropriate reaction conditions to link them to a single residue on the probe protein. For example, to use this strategy in the analysis of something as complicated as a ribosome or a eukaryotic polymerase holoenzyme [9,10] would require a scheme to reconstitute the complex with a single modified protein. This is usually not feasible.

It would be much more desirable to incorporate into the target protein a small peptide 'receptor' that would bind with high specificity to an activatable crosslinking reagent added exogenously. Ideally, a chimeric protein could be produced *in vivo* from an engineered gene, allowing incorporation of the protein into the complex of interest naturally. Following lysis under conditions that leave the complex intact, a limiting amount of the latent form of the crosslinker would then be delivered specifically to the target protein and activated, resulting in a highly localized reaction that covalently couples the target protein to its binding partners (Fig. 1). This molecular-recognition-based, site-directed crosslinking strategy would require no covalent posttranslational modification of the target protein.

We report here the first example of the use of such a strategy. The design of this system was based on our recent discovery that, when activated with a peracid, a nickel complex of the tripeptide NH_2-Gly-Gly-His-COOH (NH_2-GGH) is an efficient protein-crosslinking agent [11]. Although the detailed mechanism of crosslinking has yet to be elucidated, it is thought to proceed via a high-valent nickel complex, which may extract an electron from an aromatic residue with which it collides. This, in turn, may lead to protein crosslinks by coupling the oxidized aromatic ring to another nearby aromatic sidechain. Whatever the detailed pathway may be, a key observation in the previous study [11] was that no crosslinking was observed in the absence of a metal-binding peptide ligand. It thus seemed reasonable to assume that, if a nickel-binding peptide were incorporated into a target protein, the crosslinking chemistry could be restricted to the immediate vicinity of this tag. We thus explored the use of the six-histidine (His_6) tag, which is commonly used in the purification of proteins through metal ion affinity chromatography, to produce a nickel-binding 'receptor' on a polypeptide. Since the His_6 moiety will bind nickel when placed at any position in a protein, it is a much more flexible metal receptor than NH_2-GGH which must be located at the amino terminus of a protein to function (for previous reports of NH_2-GGH fusion proteins as specific cleavage reagents, see [12–14]). Here, we show that the His_6–Ni(II) chelate can indeed support specific oxidative crosslinking in complex protein mixtures. The reaction is localized and is not the result of a diffusible intermediate. The His_6 tag can be used to purify the protein and associated factors on nickel-saturated NTA agarose beads, and the crosslinking reaction can be carried out on the beads. Alternatively, the

Figure 1



Schematic representation of affinity-directed protein crosslinking. The gene for the 'probe protein' is engineered so the protein product contains an amino terminal His_6 tag (orange). Ni(II) is added, and the His6–Ni(II) complex is activated with a peracid, causing the probe protein to be crosslinked to neighboring proteins.

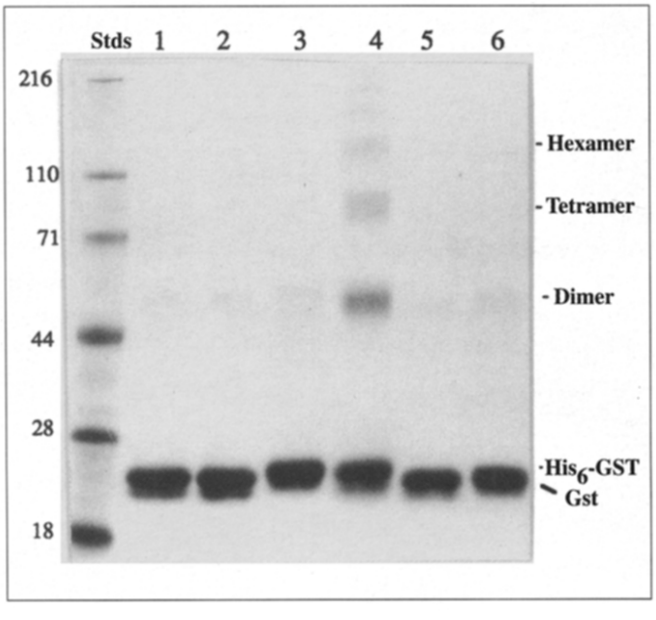
crosslinking reaction can be carried out in solution and the products purified by binding to Ni–NTA agarose. Either method allows the convenient separation of the target protein and its crosslinked products from most other species present, greatly simplifying analysis of the reaction. We anticipate that this protocol will find widespread use in the study of multiprotein complexes.

Results and discussion

Ni (II)–His_6 mediates efficient oxidative crosslinking

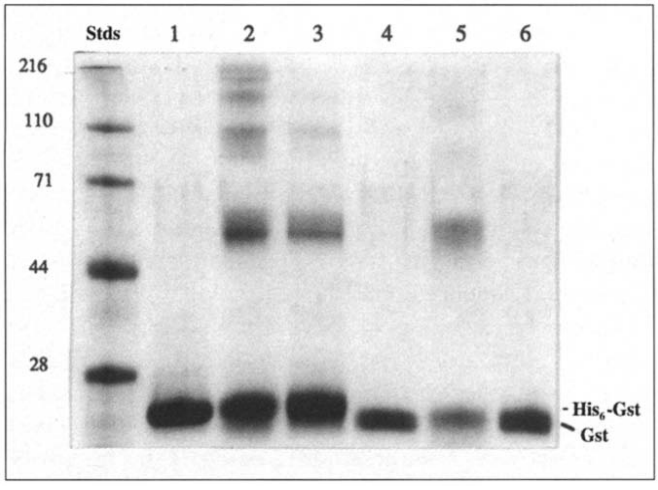
Glutathione S-transferase (GST), which dimerizes in solution, was used as a test case to determine whether the His_6 ligand could support Ni-mediated oxidative crosslinking. Two proteins were purified, one with an amino-terminal His_6 tag (His_6-GST) and one without (GST). In separate reactions, the proteins were exposed to nickel(II) acetate and then treated with the oxidant magnesium monoperoxyphthalic acid (MMPP). Crosslinked products were observed only for His_6-GST (Fig. 2, lane 4). Most of the product had an apparent molecular weight consistent with a covalently crosslinked dimer, although some tetramers and higher-order species were also formed. The crosslinking reaction was completely dependent on the presence of both nickel and the oxidant. Neither nickel acetate (lane 2) nor MMPP (lane 3) alone elicited crosslinking. When nickel acetate and MMPP were added to GST, no crosslinked products were detected (lane 6), although GST dimers were readily detectable when the protein was treated with NH_2-GGH–Ni and MMPP (see Fig. 3, lane 5).

Figure 2



Ni (II)–His_6 mediates oxidative protein crosslinking. Crosslinking occurs only when the protein is His_6-tagged and when MMPP and $Ni(OAc)_2$ are both present. Lane 1: 5 μM His_6-GST; lane 2: 5 μM His_6-GST, and 200 μM $Ni(OAc)_2$; lane 3: 5 μM His_6-GST, and 100 μM MMPP; lane 4: 5 μM His_6-GST, 200 μM $Ni(OAc)_2$, and 100 μM MMPP; lane 5: 5 μM GST; lane 6: 5 μM GST, 200 μM $Ni(OAc)_2$, and 100 μM MMPP.

Figure 3



Dimerization of His_6-GST is not inhibited by exogenous tyrosine, indicating that the reaction between the oxidatively-activated His_6–Ni complex and the protein is an intramolecular process. Lane 1: His_6-GST; lane 2: His_6-GST, $Ni(OAc)_2$, MMPP; lane 3: His_6-GST, $Ni(OAc)_2$, MMPP and tyrosine (0.7 mM); lane 4: GST; lane 5: GST, NH_2-GGH–Ni (II), 100 μM MMPP; lane 6: GST, NH_2-GGH–Ni (II), 100 μM MMPP and 0.7 mM tyrosine.

Crosslinking of His_6-GST was repeated under a variety of conditions to determine whether the reaction is compatible with buffer components commonly used in biochemical studies. Crosslinking was effective in the presence of Tris, PBS, or HEPES buffer (data not shown). The latter result is interesting, because HEPES buffer quenches crosslinking mediated by exogenous NH_2-GGH–Ni(II) and MMPP [11]. Presumably, the fact that the oxidatively-activated metal center is formed in close proximity to the protein target allows the His_6–Ni-mediated intramolecular crosslinking reaction to escape quenching. Crosslinking activity is also maintained in the presence of low levels of Tween 20, NP-40, Triton-X-100, or Sarkosyl (data not shown), and common protease inhibitors, such as aprotinin, pepstatin, bestatin, and leupeptin, also had no effect on crosslinking efficiency. Crosslinking was, however, sensitive to dithiothreitol or β-mercaptoethanol, when their concentrations exceeded 50 μM. These reducing agents react with the MMPP oxidant directly, inhibiting its effectiveness.

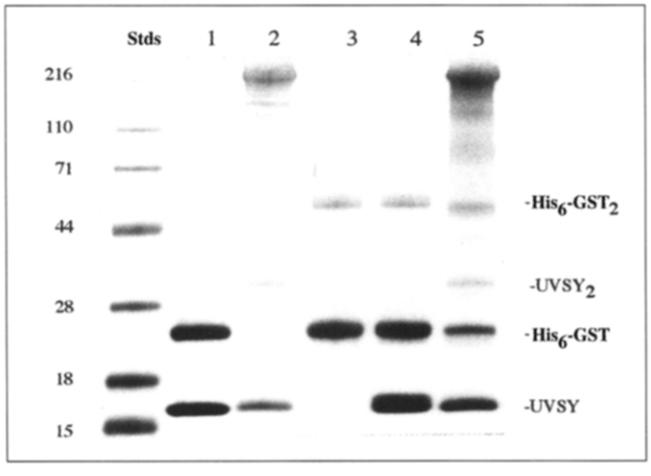
His_6–Ni(II)-mediated crosslinking is localized

An important assumption in the development of this technology is that the oxidatively activated His_6–Ni complex reacts only with groups in close proximity and that the active species is not a diffusible intermediate. To test this idea, we examined whether exogenous aromatic

amino acids could inhibit the reaction (see Fig. 3). NH_2-GGH–Ni(II)-mediated crosslinking of GST (lane 5) is quenched almost completely by tyrosine (>100-fold excess over GST, lane 6), presumably because the amino acid competes with the target protein for the oxidatively activated nickel–peptide complex. An intramolecular reaction should be far less sensitive to inhibition. As predicted, the addition of the same large excess of tyrosine to a reaction containing His_6-GST, nickel acetate and MMPP had little or no effect on the yield of crosslinked dimers compared to a reaction lacking tyrosine (compare lanes 2 and 3). The production of higher-order crosslinked products was, however, diminished greatly, which may reflect the fact that GST is a stable dimer and that the dimers themselves may associate tenuously with one another. This interaction is detected by the crosslinking reaction if tyrosine is not included to quench active intermediates that do not rapidly lead to a crosslink.

Another simple way to test whether the reaction is exclusively intramolecular is to ask whether a His_6-tagged polypeptide is crosslinked exclusively in the presence of a protein that is crosslinked efficiently with the NH_2-GGH–Ni reagent in the presence of MMPP. To examine this issue, His_6-GST was mixed with the uvsY recombination protein of bacteriophage T4 [15]. The latter is known to multimerize in solution and is a good substrate for oxidative crosslinking mediated by NH_2-GGH–Ni/MMPP (Fig. 4, lane 2). When both His_6-GST and the uvsY protein were mixed together and treated with NH_2-GGH–Ni and MMPP, both His_6-GST dimers and the various uvsY crosslinked products were obtained (lane 5). However, when only nickel acetate and MMPP were added to the two proteins (NH_2-GGH was not included), only the His_6-GST dimer was formed (lane 4). No products containing the uvsY protein were observed. Thus, the His_6-supported reaction is an intramolecular process that does not bleed oxidation equivalents into solution.

Figure 4



Crosslinking is specific for proteins containing the His_6 tag. Lane 1: 5 μM His_6-GST and 5 μM uvsY; lane 2: 5 μM uvsY, 100 μM NH_2-GGH–Ni (II), and 100 μM MMPP; lane 3: 5 μM His_6-GST, 200 μM $Ni(OAc)_2$, and 100 μM MMPP; lane 4: 5 μM His_6-GST, 5 μM uvsY, 200 μM $Ni(OAc)_2$, and 100 μM MMPP; lane 5: 5 μM His6-GST, 5 μM uvsY, 100 μM NH_2-GGH-Ni (II), and 100 μM MMPP.

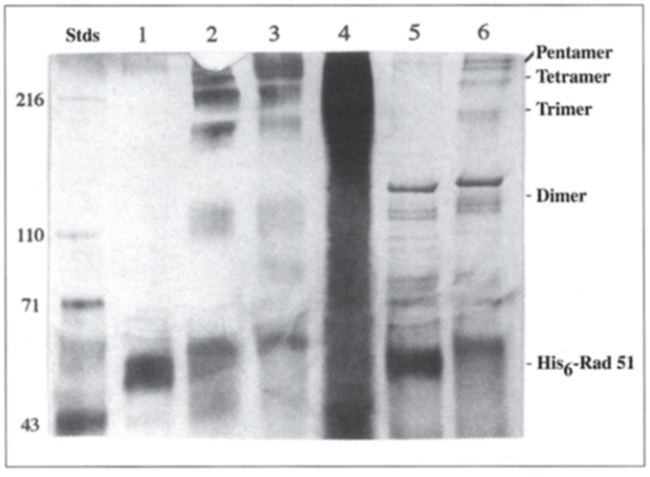
His_6–Ni-mediated crosslinking in a crude lysate

When purified proteins are not available, protein–protein interactions must be studied in complex solutions such as crude whole-cell extracts. Although traditional crosslinking reagents can sometimes be used for such purposes, these experiments are often problematic due to the huge number of products that can be generated by adding an exogenous crosslinker to a solution containing thousands of proteins. The localized nature of the His_6–Ni-mediated reaction, however, should allow selective crosslinking experiments to be conducted under conditions that do not greatly perturb most of the proteins present. Furthermore, it should be possible to use metal-ion affinity chromatography under denaturing conditions to enrich the His_6-tagged protein and its covalently crosslinked products, thus vastly simplifying analysis of the results.

To test these suppositions, we used a His_6-tagged derivative of the *Saccharomyces cerevisiae* Rad51 recombination protein [16], a yeast homolog of the *E. coli* RecA strand transferase, which self-associates and forms polymeric filaments on single-stranded DNA [17]. When treated with nickel acetate and MMPP, purified His_6-Rad51 protein (Fig. 5, lane 1) crosslinks efficiently, forming a ladder of products whose apparent molecular weights are consistent with dimers, trimers, etc. (lane 2), as expected for a filament-forming protein. The mixture was then exposed to nickel-charged chelating sepharose under denaturing conditions (6M guanidinium hydrochloride). After a brief incubation, the beads were pelleted, washed thoroughly, and the bound proteins were eluted by boiling in a loading buffer containing high concentrations of imidazole. The pattern of the bead-bound proteins determined by gel electrophoresis (lane 3) was virtually identical to that observed in lane 2, showing that the metal-ion affinity isolation procedure faithfully reflects the results of the solution crosslinking reaction.

We next applied this protocol to crosslinking in a crude bacterial lysate where His_6-Rad51 was only a minor component (Fig. 5, crude lysate in lane 4). Lanes 5 and 6 show the proteins recovered from this mixture by binding to nickel-agarose beads under denaturing conditions. In lane 6, nickel acetate and MMPP had been added to the lysate, whereas in lane 5 the peracid was omitted. As can be seen at the top of the gel, the His_6-Rad51 protein crosslinked products are readily detectable in lane 6, but are absent in lane 5. Several

Figure 5



Comparison of His_6-mediated crosslinking of a protein in purified form and in a crude lysate. The experiments shown in lanes 1–3 used purified His_6-Rad51 protein (5 μM), whereas those in lanes 4–6 used a crude lysate (120 μg total protein) prepared from *E. coli* cells that express His_6-Rad51 protein. Lane 1: purified His_6-Rad51 protein; lane 2: His_6-Rad51 protein treated with 200 μM $Ni(OAc)_2$, and 100 μM MMPP then loaded directly onto the gel; lane 3: His_6-Rad51 protein treated with 200 μM $Ni(OAc)_2$ and 100 μM MMPP, then bound to Ni–NTA agarose beads under denaturing conditions; lane 4: crude lysate. lane 5: proteins in crude lysate that bind to Ni–NTA agarose beads; lane 6: crude lysate treated with 200 μM $Ni(OAc)_2$ and 100 μM MMPP, then bound to Ni–NTA agarose beads under denaturing conditions.

contaminating bands are also apparent, some of which may obscure the faint dimer product. This result confirms that the His_6 tag is useful for carrying out crosslinking experiments in crude solutions. Products can be enriched by metal-ion affinity chromatography, alleviating the need for monoclonal antibodies to interpret the results of the crosslinking experiment.

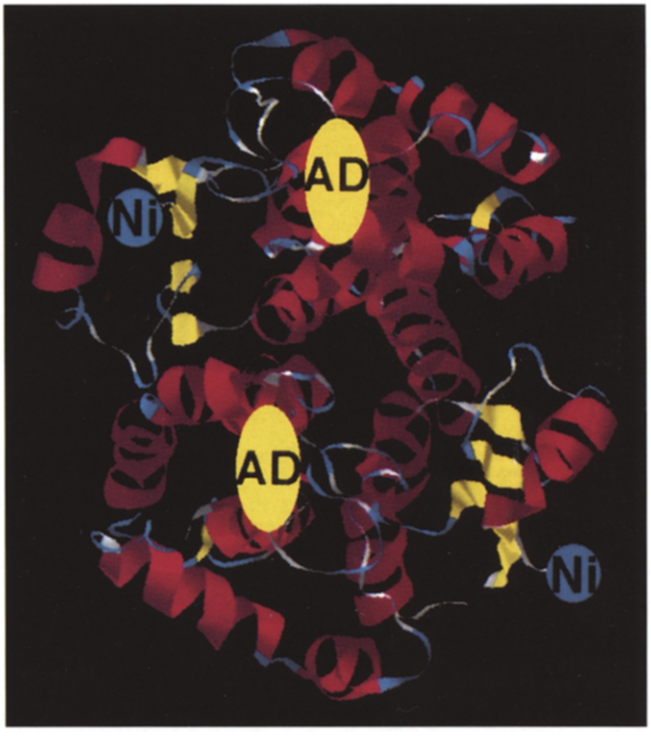
Heterodimer crosslinking

Messenger RNA synthesis in eukaryotic cells involves a very large number of proteins, many of which are included in the polymerase holoenzyme. Gene-specific activators interact directly with one or more proteins in this complex and these contacts somehow stimulate transcription. It is important to identify the direct protein targets of activators. It would also be desirable to understand something about the organization of proteins in the holoenzyme and how this structure might be modified during the transcription cycle (for a review see [18]). The His_6–Ni/MMPP methodology may be ideal for these applications. As a first step towards this goal, we determined whether His_6–Ni-mediated crosslinking could be used to detect binding of the 34-residue activation domain (AD) of the yeast Gal4 transcriptional activator [19] to general transcription factors. In particular, we focused on the TATA-binding protein (TBP), which has been implicated as a target of Gal4 protein [20] and many other activators [21–23].

Two GST fusion proteins, containing the AD of Gal4, were used for these experiments. GST-34 comprises the AD fused to the carboxyl terminus of GST via a linker arm that contains a cleavage site for the highly specific TEV protease [24]. His_6-GST-34 is identical to GST-34 except that it also includes a His_6 tag at the amino terminus of the protein. Although the Gal4 AD and the His_6 moieties are separated by the GST polypeptide, the amino and carboxy termini of GST are relatively close in space (<10 Å). Model building based on the crystal structure of GST [25] suggested that the activated His_6–Ni complex should be able to contact the AD directly (see Fig. 6).

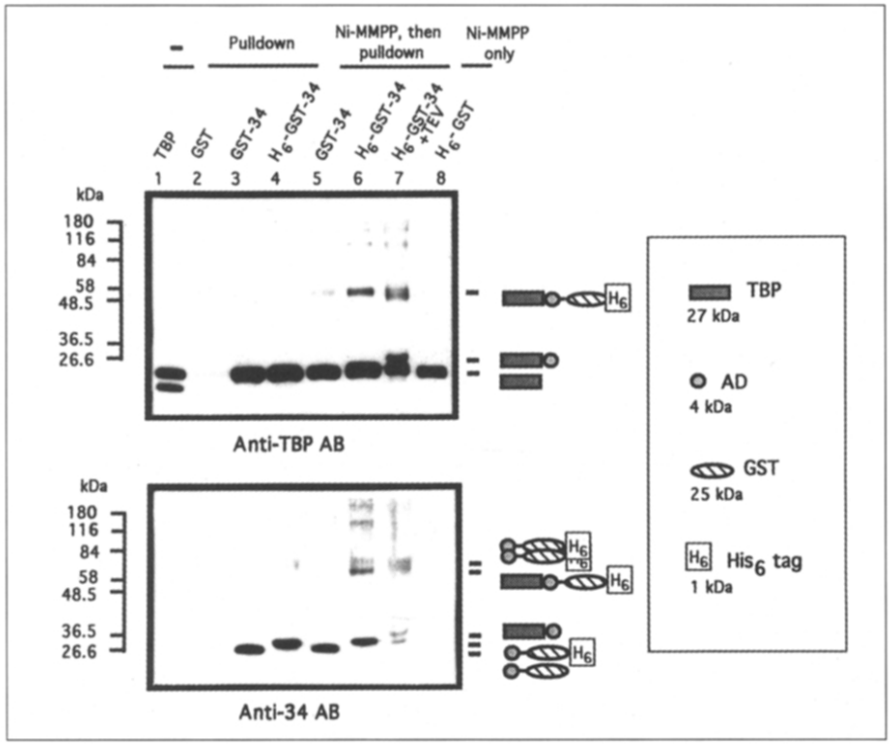
The GST-AD fusions were bound to glutathione agarose resin, and these beads were incubated with purified yeast TBP. Lanes 3 and 4 in the upper panel of Figure 7 (a Western blot using anti-TBP antibodies) show that when pelleted, the bead-bound GST-34 and His_6-GST-34 fusions retained TBP. Binding required the AD, as GST alone did not bind TBP (lane 2). Lanes 5–7 show the products obtained when the same experiment was repeated, except that nickel acetate and MMPP were

Figure 6



Model of the structure of His_6-GST-34, containing the His_6 tag fused to the amino terminus of GST and the 34-residue activation domain (AD) of the yeast Gal4 transcriptional activator fused to the carboxyl terminus. Note the relative proximity of the His_6 tag (shown bound to Ni) and the AD.

Figure 7



His_6-mediated crosslinking of the Gal4-protein activation domain and the TATA-binding protein (TBP). Purified TBP was mixed with GST, or a GST chimera, bound to glutathione agarose beads. The chimeras contained an amino-terminal His_6 tag and/or the 34-residue Gal4 activation domain. In lanes 2–7, the beads were pelleted, washed thoroughly and bound proteins then analyzed by electrophoresis and Western blotting. In lane 8, a fraction of the entire solution, not simply the bead-bound fraction, was loaded. Nickel acetate and MMPP were added to the experiments shown in lanes 5–8, but not to those shown in lanes 2–4. The upper panel shows a Western blot using antibodies raised against TBP. The lower panel shows a Western blot using antibodies raised against the Gal4 AD. Lane 1 is a TBP standard.

added to effect crosslinking prior to pelleting the beads. Treatment of the His_6-GST-34/TBP complex with these reagents (lane 6) resulted in the production of a species that was recognized by both the anti-TBP (upper panel) and anti-34 (lower panel) antibodies and was thus identified as a crosslinked His_6-GST-34-TBP heterodimer.

To confirm this assignment, the solution was treated with the TEV protease, which cleaves the AD from His_6-GST, prior to electrophoresis. This yielded a crosslinked species consisting of TBP and the 34-amino-acid activation domain, which migrated more slowly than TBP itself and was recognized by both antibodies (lane 7). Crosslinking between the Gal4 AD and TBP was greatly reduced when the GST-34 fusion protein lacked a His_6 tag (lane 5). The small amount of residual product may be due to weak nickel-binding sites in one or both of the proteins. His_6-GST, lacking a Gal4 AD, did not crosslink to TBP (lane 8).

These experiments demonstrate that the His_6–Ni/peracid-mediated crosslinking procedure can detect interactions between a transcriptional AD and a potential transcription-factor target. They also demonstrate that heterodimerization can be observed between a His_6-tagged and an unmodified protein. We emphasize that these results do not necessarily implicate TBP as the biologically relevant target of the Gal4 AD, but suggest that if Gal4 protein–TBP contacts do exist in the context of native transcription complexes, then this chemistry should allow their detection. Experiments using large transcription complexes are in progress.

Unmodified proteins near the His_6 tag can be crosslinked

It was important to determine whether proteins close in three-dimensional space to the His_6–Ni complex, but which are not themselves tagged with a metal-binding peptide, can be crosslinked. In other words, for a complex of the architecture His_6-$Protein_A$–$Protein_B$–$Protein_C$ where $Protein_B$ is in close proximity to the His_6 tag, can $Protein_B$–$Protein_C$ products be produced, or do all products necessarily include His_6-$Protein_A$? If the mechanistic model in which the oxidized His_6–Ni complex collides with some aromatic residue to initiate the reaction is correct, then the $Protein_B$–$Protein_C$ complex should be produced. If the reaction is restricted completely to the His_6-tagged protein, however, the $Protein_B$–$Protein_C$ species will not be observed.

To answer this question, we used a preformed complex between TBP and His_6-GST-VP16. His_6-GST-VP16 is a fusion protein analogous to His_6-GST-34, except that the 78 residue activation domain of the herpes simplex virus VP16 transactivator [26] was substituted for that of Gal4. Since TBP binds to the VP16 activation domain [23], and the His_6 tag and the VP16 AD are in close proximity in this complex, it is reasonable to assume that the oxidized His_6–Ni complex could react with TBP directly, as well as the VP16 activation domain. Since TBP is a native dimer

when not bound to DNA [27,28], such a direct reaction should produce crosslinked TBP homodimers. If only the His_6-tagged fusion protein is reactive, however, TBP dimers will not be formed. This experiment was not easily conducted using the His_6-GST-34 protein since covalently crosslinked His_6-GST-34-TBP and TBP_2 comigrate in a polyacrylamide gel.

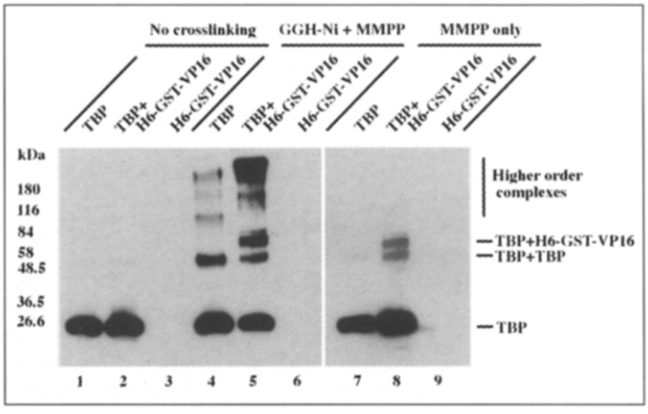
TBP dimers were indeed produced in the context of a preformed His_6-GST-VP16/TBP complex (Fig. 8). A Western blot using antibody directed against TBP is shown in Figure 8. Control experiments using NH_2-GGH–Ni and MMPP as the crosslinking reagent produced TBP dimers (and higher order species) when TBP was alone in solution and when it was mixed with His_6-GST-VP16 (lanes 4 and 5). When the His_6-GST-VP16–TBP complex was adsorbed onto nickel-saturated chelating agarose beads, washed, then treated with MMPP, two products were formed (lane 8). The more slowly migrating species corresponds to the expected His_6-GST-VP16–TBP heterodimer, whereas the band below it corresponds to the TBP homodimer. Note that the TBP dimer is not formed when His_6-GST-VP16 is not present (lane 7).

This result shows that the oxidized His_6–Ni complex can attack other proteins that are nearby in space and initiate a crosslinking reaction that does not include the His_6-tagged protein in the product. As demonstrated by the uvsY experiment, however, the reaction is not so promiscuous that any protein in solution is crosslinked. The currently available data are consistent with the idea that the 'crosslinking sphere' in these reactions is limited to the proteins that can physically contact the oxidized His_6–Ni complex, though we cannot rule out the possibility that the reactive intermediates created by reaction with a target protein can 'diffuse' further, perhaps by hole hopping between proteins. If this occurs, however, one would expect that the frequency of the crosslinking reaction would drop off with distance. A better understanding of the range of this crosslinking reaction will require more work with structurally well characterized, multi-protein complexes, containing a single His_6-tagged polypeptide. The crosslinking of unmodified proteins is a bonus for the study of large complexes, since production of a product containing a particular protein implicates that factor as being close in three-dimensional space to the His_6 tag. If only products of the His_6-tagged protein are of interest, other products can be removed by binding to nickel beads under denaturing conditions.

Significance

The delivery of chemical reagents to specific sites in macromolecular complexes is a promising method for the analysis of the architecture of these species. We have used a very simple molecular recognition event, metal–ligand complexation, to deliver a novel nickel-based crosslinking reagent to a particular protein in complex solutions. His_6-tagged proteins can be crosslinked to their binding partners without perturbing unmodified proteins, except those close in space to the metal complex, due to the fact that the active intermediates are not highly diffusible species. The His_6 tag survives the crosslinking reaction and can be used to enrich the tagged protein and any covalently crosslinked products by metal-ion affinity chromatography under denaturing conditions, thus greatly simplifying analysis of the results. We anticipate that this technology will be very useful in the study of protein–protein interactions in large multiprotein complexes.

Figure 8



TBP–TBP dimers are formed as a result of His-mediated crosslinking in a $(His_6$-GST-VP16/TBP$)_2$ complex. Crosslinking experiments using His_6-GST-VP16 or TBP or both proteins were carried out as described in Materials and methods. Products were detected by Western blotting using an antibody raised against TBP. Protein components of each reaction and the type of reaction conducted are indicated above the blot. In lanes 7–9, the nickel atoms of the chelating sepharose resin mediated the crosslinking reaction.

Materials and methods

Proteins and reagents

The His_6-tagged Rad51 protein was purified by a protocol developed in our laboratory that will be described elsewhere (H. Jiang, Y. Xie, K. Stemke-Hale, P. Houston, & T.K., unpublished data). The T4 uvsY protein [15], yeast TATA-binding protein [29] GST and the fusion proteins His_6-GST-34, His_6-GST-VP16 and GST-34 [20] were purified according to published procedures from *E. coli* strains carrying the appropriate expression vectors. Prior to their use in crosslinking reactions, the proteins were dialyzed from their storage buffers into 20 mM sodium phosphate (pH 7.6) and 300 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. GGH was purchased from Sigma. Glutathione-Sepharose 4B and Chelating Sepharose Fast Flow resins were purchased from Pharmacia. MMPP and $Ni(OAc)_2$ were purchased from Aldrich.

His_6–Ni-mediated crosslinking reactions

Crosslinking reactions were carried out in a total volume of 20 μl. Final concentrations were 20 mM phosphate (pH 7.6) (unless otherwise noted), 300 mM NaCl, and 5 μM target protein. A complex was formed

between nickel and the His_6-tagged protein by adding unbuffered $Ni(OAc)_2$ in H_2O to the solution to a final concentration of 200 μM and incubating for at least 5 min on ice. The reactions were initiated by the addition of MMPP to 100 μM and were incubated at room temperature for 1 min. The reaction was then quenched by the addition of 7 μl of 4x loading buffer (0.2 M Tris, 8 % SDS, 2.88 M β-mercaptoethanol, 40 % glycerol, 0.4 % bromophenol blue and 0.4 % xylene cyanol) (unless otherwise indicated in the figure legends). The samples were heated to 100 °C for 5 min and then separated by electrophoresis through a 8 % tricine-SDS polyacrylamide gel [30]. The proteins were visualized by staining with Coomassie brilliant blue unless otherwise indicated.

NH_2-GGH–Ni-mediated crosslinking reactions

Crosslinking reactions were carried out in a total volume of 20 μl. Final concentrations were the same as stated previously for the His_6-supported process except that the NH_2-GGH–Ni(II) concentration was 100 μM. The NH_2-GGH–Ni(II) complex was formed by mixing a 1:1 molar ratio of $Ni(OAc)_2$ and NH_2-GGH in unbuffered H_2O. It is important to preform the complex since unliganded nickel precipitates in phosphate buffers. Following a 5 min equilibration, the solution was diluted to the desired concentration with the phosphate buffer. The reactions were initiated by the addition of MMPP to 100 μM and incubated at room temperature for 1 min. The reactions were then quenched and analyzed as stated previously.

Effect of other buffers on the crosslinking reaction

The same conditions as those described above were used, except that either 20 mM Hepes (pH 7.6) or 50 mM TrisCl (pH 7.6) were used. The reactions were carried out at room temperature, quenched with 7 μl of loading buffer and then examined by electrophoresis as described earlier.

Effect of detergents and surfactants on the crosslinking reaction

The same conditions as those described above were used, except that either Triton X-100, NP40, Tween 20, or Sarkosyl (0.1 % in each case) was present in solution prior to addition of MMPP.

Effect of aromatic amino acids on the crosslinking reaction

The same conditions as those described above were used, except that tyrosine or tryptophan were included in solution to a final concentration of 0.7 mM prior to addition of MMPP.

Isolation of crosslinked products by denaturing metal-affinity chromatography

Crosslinking reactions containing a His_6-tagged protein were carried out as described above except that the reaction was quenched with 75 μl denaturing wash buffer (6M guanidinium hydrochloride GHCl, 20 mM TrisCl (pH 7.9), 500 mM NaCl, and 20 mM imidazole). Then 10 μl of a slurry of nickel-charged chelating sepharose Fast Flow in denaturing wash buffer was added and the contents were gently mixed at 4 °C overnight. The resin was then pelleted by centrifugation at 3000 rpm in an Eppendorf centrifuge for 30 s. The supernatant was discarded. 500 μl of denaturing wash buffer was then added and the mixture was agitated at 4 °C for 1 h. After centrifugation, the resin was washed twice with 500 μl urea wash buffer (8.0 M urea, 20 mM TrisHCl (pH 7.9), 500 mM NaCl, and 20 mM imidazole), and twice with Tris wash buffer (20 mM TrisHCl (pH 7.9), 500 mM NaCl, and 0.5 % NP40). The resin-bound proteins were eluted with 10 μl of elution buffer (20 mM TrisCl (pH 7.9), 500 mM NaCl and 1.0 M imidazole). Protein sample buffer was then added and, after heating to 100 °C for 5 min, the eluted proteins were examined electrophoretically as stated previously and visualized by silver staining using the Bio-Rad kit.

Crosslinking of His_6-GST-34 to yeast TBP

10 μg of His_6-GST-34, His_6-GST, GST-34, or GST were bound to 10 μl of glutathione sepharose beads (Pharmacia). The beads were then incubated with 0.7 μg yeast TBP in 100 μl PBS plus 0.1 % Triton X-100 for 2 h on a tiltboard at 4 °C. 25 μl of 60 mM of nickel acetate was added and the solution allowed to equilibrate for 10 min. 25 μl of 60 mM MMPP was then added and the solution incubated for a further 6 min. The His_6-GST-containing reaction was quenched with 50 μl of 4x sample buffer. The other reactions were diluted with 1 ml PBS, pelleted, and the beads washed three more times with 1 ml PBS. The beads containing adsorbed His_6-GST-34 were resuspended in 40 μl PBS, 1 mM DTT, 0.1 mM EDTA, 0.1 % Triton X-100 and split into two aliquots. To one aliquot, 2 μl of TEV protease (Gibco/BRL) was added. Both aliquots were incubated for 70 mins at room temperature on a tiltboard. All reactions were made 1x in sample-loading buffer, separated by electrophoresis through a polyacrylamide gel as described above and blotted to PVDF membranes. Western blots were done using mouse anti-TBP serum at 1:15 000 dilution and rabbit anti-Gal4 activation domain serum at 1:10 000 dilution as primary antibodies. Blots were developed by the chemoluminescence method using the Renaissance kit (Dupont) and secondary horseradish-peroxidase conjugated antibodies (Bio-Rad).

Crosslinking of His_6-GST-VP16 to TBP

6 μg of His_6-GST-VP16 bound to 15 μl of Ni-saturated NTA agarose beads (Qiagen) were incubated with 3 μg of TBP in 450 μl PBS buffer supplemented with 0.1 % Triton X-100. After incubation for 75 min at 4 °C on a tiltboard, the beads were washed extensively with PBS to remove unbound TBP. The beads were then separated into three portions. One was treated with SDS-containing sample buffer without crosslinking to simply release any bound proteins. The second was incubated with 5 μl GGH–Ni (6×10^{-4} M) and 5 μl of MMPP (6×10^{-4} M) for 1 min at room temperature, then quenched as above. The final aliquot was treated with the same amount of MMPP as well as 1.5 μl tyrosine (10 mM) for 7 min, then quenched as above.

In reactions containing only TBP or His_6-GST-VP16, the same conditions were used except that in the case of TBP (600 ng) the beads were not washed, since TBP has no His_6 tag and does not bind directly to them. Western blots were done as described above using an anti-TBP antibody.

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