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Periodate-Triggered Cross-Linking of DOPA-Containing Peptide−Protein Complexes

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Chemical cross-linking is a potentially powerful method for the analysis of protein−small molecule and protein−protein complexes. To complement existing methodologies, most of which are limited in many ways, we have developed a series of oxidative coupling reactions that are capable of cross-linking many protein−protein and protein−peptide complexes rapidly and in good yield.[1] However, a limitation of this approach is that when one examines a large multiprotein complex or carries out experiments in complex mixtures, a large number of multiply cross-linked species are produced, complicating the separation and analysis of products.[2] For many applications, such as identifying the target of bioactive molecules,[3] it would be desirable to be able to focus the reaction to a single molecule of interest without perturbing any other proteins present. We describe here a useful method of this sort that allows molecules containing an ortho dihydroxyphenyl ring to be linked covalently to associated proteins in a process that is orthogonal to the functional groups present in most proteins. Our hypothesis, based on the known oxidation chemistry of 3,4-dihydroxyphenylalanine (DOPA)-containing proteins common in mollusks,[4] was that this side chain could be oxidized selectively using sodium periodate, an oxidant to which common proteins are inert. This reaction would be expected to produce an ortho quinone intermediate that could be attacked by nearby nucleophiles, resulting in a stable cross-link.

As a model to test this hypothesis we used a complex between the yeast Gal80 transcriptional repressor[5] and a 20-residue peptide that had been selected by phage display to bind Gal80 (NH_2-YDQDMQNNTFDDLFWKEGHR-COOH; K_D = 300 nM).[6a] The binding properties of this peptide to Gal80 and other transcription proteins are of interest because the peptide functions as an artificial activation domain in vivo[6a] and in vitro[6b] when fused to suitable sequence-specific DNA-binding domains. Two Gal80-binding peptide analogues were synthesized by replacing the N-terminal tyrosine of the peptide with either DOPA or 3,4-dimethoxyphenylalanine. All three peptides were extended at the N terminus with two glycines and a biotinylated glutamic acid to allow cross-linked products to be detected by blotting with a NeutrAvidin−horse radish peroxidase (HRP) conjugate. A six-histidine (His_6)-tagged Gal80 protein was incubated with each of the three peptides. Thirty seconds after periodate addition the reaction was quenched using a buffer containing 100 mM DTT. The products were analyzed by denaturing gel electrophoresis and blotting with NeutrAvidin−HRP. Cross-linking occurred only between the DOPA-containing peptide and Gal80p in the presence of periodate (Figure 1, lanes 1 and 2). No reaction was observed when the peptide contained the native tyrosine (lanes 5 and 6) or a dimethoxy-substituted ring (lanes 3 and 4) or when periodate was omitted (lane 7). These results, particularly the lack of reactivity of the dimethoxyphenylalanine-containing molecule, are consistent with an ortho quinone intermediate that then cross-links to nearby residues on Gal80.

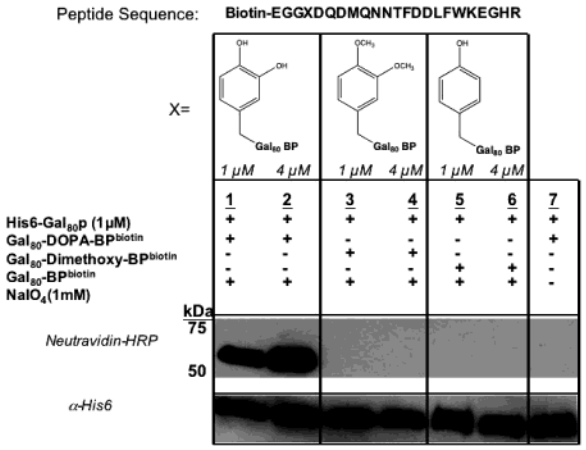

Figure 1. Lanes 1 and 2: Chemical cross-linking reaction between His6-Gal80p and Gal80-DOPA-BP. Lanes 3 and 4: Chemical cross-linking reaction between His6-Gal80p and Gal80-dimethoxy-BP. Lanes 5 and 6: Chemical cross-linking reaction between His6 Gal80p and the Gal80-Y-binding peptide. Lane 7: His6-Gal80p +Gal80-DOPA-BP without the addition of periodate. Probing the blot with anti-His6 antibody showed that equivalent amounts of protein were loaded and that the His_6 tag had not been damaged by the cross-linking reaction.

A problem with many cross-linking reactions is that they can produce products between molecules not tightly associated in solution (for a discussion, see ref 2). To assess this issue in the context of this chemistry, His_6-Gal80 was mixed with a large excess of 10 other proteins, and the experiment was repeated. As shown in Figure 2, lane 6, only a single cross-linked product was obtained. Indeed, the result was almost identical to that of the control experiment lacking the competitor proteins (lane 3), except that the yield was reduced somewhat. This was due to some competition of the peptide−Gal80 complex by the other proteins, which was not surprising since activating peptides typically bind nonspecific proteins promiscuously. Low levels of cross-linked products to some of the other proteins could be observed on overexposed blots (not shown). As a control, the same experiment was done with a biotinylated DOPA-containing peptide (biotin−KG-DOPA-AH-NRLIYMQD) not known to associate with any of the proteins in solution (lane 8). No reaction was observed between the DOPA-containing control peptide and any of the proteins present. We conclude that this reaction reports only stable intermolecular associations.[7]

To evaluate this chemistry in a more demanding setting, we examined the interaction of the Gal80-binding peptide with the 19S regulatory particle of the proteasome,[8] which we have shown to be an important participant in RNA polymerase II transcription.[9] To recruit this complex, the activation domain of the native Gal4 transactivator binds Sug1 and Sug2, two of the 19 proteins in the 19S particle. These binding events are important for efficient

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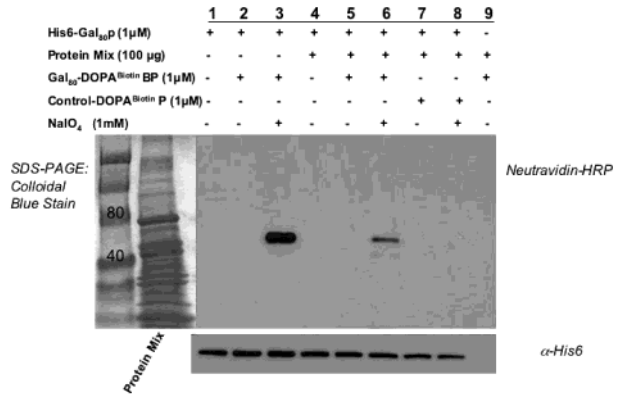

Figure 2. Cross-linking between His6-Gal80p and the Gal80-DOPA-binding peptide is only seen in the presence of periodate (lane 3). The addition of the protein mix shown on a colloidal stained SDS-PAGE gel to the left does not significantly interfere with the cross-linking reaction as shown in lane 6. No cross-linked products were detected when the control-DOPA peptide was subjected to the same reaction (lane 8). The bands in the top NeutrAvidin−HRP blot overlay with the bands in the bottom panel (α-His6), proving that Gal80p is cross-linking to Gal80-DOPA-BP despite the presence of the protein mix.

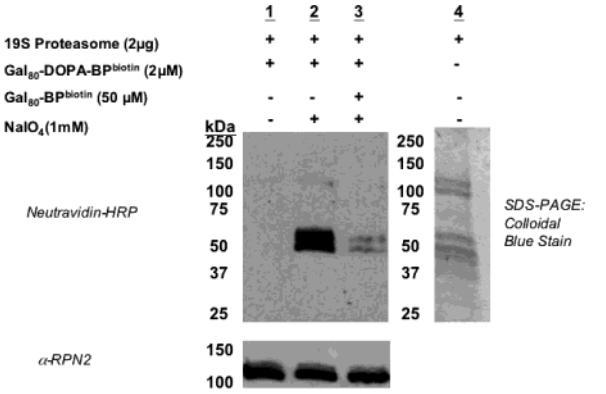

Figure 3. Peptide-19S complex cross-linking. Lane 1: 19S + the Gal80-DOPA-BP in NE buffer without periodate. Lane 2: 19S + the Gal80-DOPA-BP in NE buffer with periodate. Lane 3: 19S + the Gal80-DOPA-BP + excess Gal80-BP+ periodate in NE Buffer. Lane 4: Collodial blue stained SDS-PAGE gel showing the amount of 19S added to each reaction. The bottom α-RPN2 blot serves as a loading control. The blot was stripped and reprobed with anti-Sug1, anti-Sug2, anti-Cim5. These blots are presented in the Supporting Information.

Gal4-mediated transcription.[10] Since the Gal80-binding peptide also functions as an activation domain in vivo,[6] we wondered whether it would exhibit the same binding properties, even though it had not been selected to bind the 19S complex. As shown in Figure 3, the DOPA-containing Gal80-binding peptide cross-linked cleanly to two proteins of about 50 kDa. These were identified as Sug 1 and Sug 2 by Western blotting with highly specific antibodies and overlaying the Western and NeutrAvidin−HRP blots (see Supporting Information). When this experiment was repeated in the presence of a 50-fold excess of the tyrosine-containing Gal80-binding peptide, the DOPA-dependent cross-linked product was reduced drastically (Figure 3, lane 3), showing that the native and DOPA-containing peptides compete for the same sites on the 19S complex. An interesting sidelight to this experiment was the detection of a small amount of a third cross-linked product (lane 2). Western blotting revealed that this species represented cross-linking of the peptide to Rpn2. Since Rpn2 is thought to interact with Sug1 and Sug2,[11] these experiments suggest that the three proteins form a pocket in the 19S complex responsible for binding activation domains.

In conclusion, an operationally simple and highly specific cross-linking chemistry has been developed. Our preliminary studies suggest that the ortho quinone resulting from periodate oxidation of the DOPA residue is an important intermediate in the reaction, but the chemistry of how this species couples to closely associated proteins remains to be determined. Whatever the detailed mechanism, the reaction should be useful for the study of peptide−protein interactions and more generally for small-molecule−protein complexes where the former contains the appropriate oxidizable ring. The chemistry is orthogonal to the 20 common amino acids present in most proteins, and thus the oxidizing power can be focused specifically to the molecule of interest without the production of multimeric species containing protein−protein as well as peptide−protein cross-links.[2] Furthermore, protein-engineering methods developed over the past few years now make it feasible to place such selectively oxidizable residues into specific positions of native proteins,[12] thus making this approach potentially applicable to the study of protein−protein interactions.

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Supporting Information Available: Full experimental procedures and western blot overlays. This material is available free of charge via the Internet at http://pubs.acs.org.

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