Scope, limitations and mechanistic aspects of the photo-induced cross-linking of proteins by water-soluble metal complexes

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Background: Chemical cross-linking is a valuable tool with which to study protein–protein interactions. Recently, a new kind of cross-linking reaction was developed in which the photolysis of associated proteins with visible light in the presence of ammonium persulfate and tris(2,2′-bipyridyl)ruthenium(II) dication or palladium(II) porphyrins results in rapid and efficient covalent coupling (Fancy, D.A. & Kodadek, T. (1999). *Proc. Natl. Acad. Sci. USA* **96**, 6020–6024 and Kim, K., Fancy, D.A. & Kodadek, T. (1999). *J. Am. Chem. Soc.* **121**, 11896–11897). Here, mechanistic and practical aspects of the reaction of importance for its application to biochemical problems are examined.

Results: It is shown that the photo-initiated cross-linking chemistry can be optimized for the analysis of protein–protein interactions in crude cell extracts. A number of commonly used epitope or affinity tags survive the reaction in functional form, allowing the simple visualization of the cross-linked products, or their isolation. It is shown that very little light-independent oxidation of protein residues occurs and that significant perturbation of complexes of interest prior to the brief photolysis period does not occur. Finally, evidence is presented that is consistent with a mechanistic model in which ammonium persulfate functions simply as an electron acceptor, facilitating the generation of the key high valent metal complex from the photoexcited species by electron transfer. In the absence of an electron acceptor, a much lower efficiency reaction is observed that appears to involve products resulting from reaction of the excited state metal complex with molecular oxygen.

Conclusions: These results provide useful practical information for chemists and biochemists who may wish to employ this new cross-linking chemistry for the analysis of protein complexes. They also shed new light on the mechanism of this interesting reaction.

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Keywords: Chemical cross-linking; Protein complex; Protein–protein interaction

Received: **28 April 2000**
Revisions requested: **9 June 2000**
Revisions received: **26 June 2000**
Accepted: **29 June 2000**

Published: **1 August 2000**

Chemistry & Biology 2000, **7**:697–708

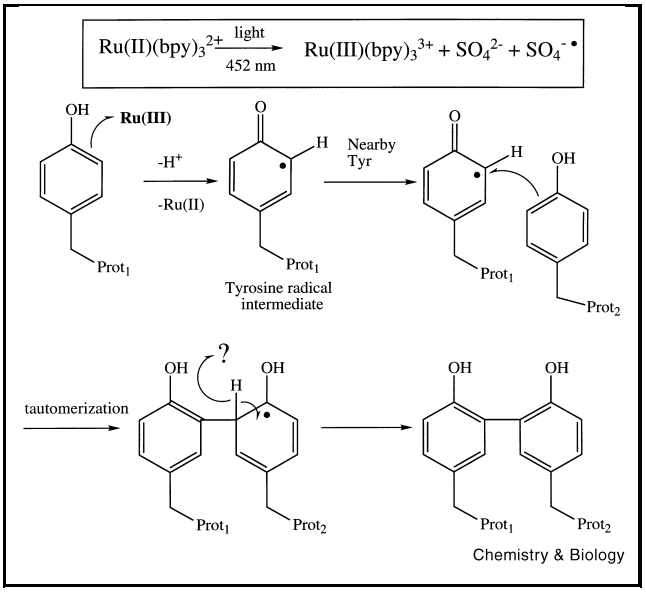
1074-5521/00/$ – see front matter

PII: S1074-5521(00)00020-X

Introduction

Chemical cross-linking is a useful technique to analyze associations between proteins and an enormous number of different chemical cross-linking agents are available commercially. The vast majority of these compounds contain two reactive groups tethered together by a linker arm. In most cases these reactive groups are electrophiles, for example activated carboxylates, α-halo carbonyl compounds or α,β-unsaturated carbonyl compounds, that couple with nucleophilic groups in proteins, such as lysine or cysteine side chains. Cross-linking between associated proteins can occur if each polypeptide has a suitable nucleophilic residue within the distance spanned by the linker arm of a given cross-linker. While this type of chemistry has been of great utility in the biochemical analysis of protein–protein interactions [1–4], it has significant limitations. Because solvent water is itself a reasonable nucleophile, one can only use cross-linkers with electrophilic groups of modest reactivity. This makes cross-linking using bifunctional electrophiles a relatively slow and often inefficient process. The problem of artifactual cross-linking must also be considered when using these reagents. Coupling of one end of a bifunctional cross-linker to a protein side chain can change the physical properties of that protein significantly, for example quenching the charge of a lysine and adding a hydrophobic linker arm to that position. This can lead to protein denaturation and aggregation and the production of artifactual cross-linked products since a reactive group remains on the other end of the cross-linking reagent.

In part to circumvent some of these limitations, we have recently developed a fundamentally new type of protein cross-linking chemistry based on radical coupling reactions. In this method, the proteins of interest are mixed with water-soluble metal complexes such as tris(2,2′-bipyridyl)-ruthenium(II) chloride hexahydrate ($Ru(II)(bpy)_3^{2+}$) [5], or palladium(II) porphyrins [6], and ammonium persulfate



Scheme I.

(APS). The solution is then photolyzed briefly with visible light (> 400 nm), which serves to photooxidize the metal complex. It is believed that the activated metal complex (either a Ru(III) or Pd(II) porphyrin radical cation) extracts an electron from amino acids such as tyrosine or tryptophan, leading to a radical species that can then attack a wide variety of other groups (Scheme I). This eventually leads to covalent cross-linking. In most cases examined to date, cross-linking is rapid and efficient. Irradiation times of 0.5 to 5 s are sufficient to achieve 30–95% yields of cross-linked products in most cases using a 150 W Xe lamp as the light source. Even using a simple hand-held flashlight, comparable yields can be obtained by increasing the photolysis time to 10–30 s. Therefore, it appears that this chemistry will be of utility as a general method to probe the interactions between proteins in vitro.

In this report, we further characterize the photo-initiated protein cross-linking reaction. Mechanistically, the role of ammonium persulfate in the reaction is addressed. The results are consistent with a model in which this compound acts solely as an electron acceptor in the ruthenium-mediated process. In the absence of this cofactor, a much slower cross-linking reaction is observed that appears to result from at least two different pathways involving the reaction of the excited state ruthenium complex with molecular oxygen. The other studies reported here focus on various issues of practical importance for researchers wishing to employ this method. First, it is demonstrated that proteins which are not stably associated are not cross-linked under conditions normally used for biochemical experiments. However, some 'artifactual' cross-linking due to random collisions in solution is observed in concentrated solutions of purified proteins. Second, it is demonstrated that this reaction can be employed to study protein–protein interactions in crude extracts, a significant extension over previously reported experiments, which employed only purified proteins. Third, it is shown that some commonly used epitope and chemical tags survive the reaction in functional form. This is important with regard to carrying out cross-linking experiments in crude extracts since these tags greatly simplify analysis of the results or isolation of the products. Finally, it is shown that little oxidative damage to proteins occurs under the reaction conditions prior to photolysis, though slow oxidation of methionine can be observed with extended incubation times.

Results

An electron acceptor, but not ammonium persulfate per se, is required for efficient protein cross-linking by $Ru(II)(bpy)_3^{2+}$

APS is known to act as an electron acceptor in order to generate $Ru(III)(bpy)_3^{3+}$ from photoexcited $Ru(II)(bpy)_3^{2+}$ [7–9] and this is undoubtedly a critical role that the cofactor plays in the protein cross-linking reaction. We had also speculated that the sulfate radical that results from the electron transfer reaction might act as a hydrogen atom abstracting agent [5], since at some point in the cross-link-

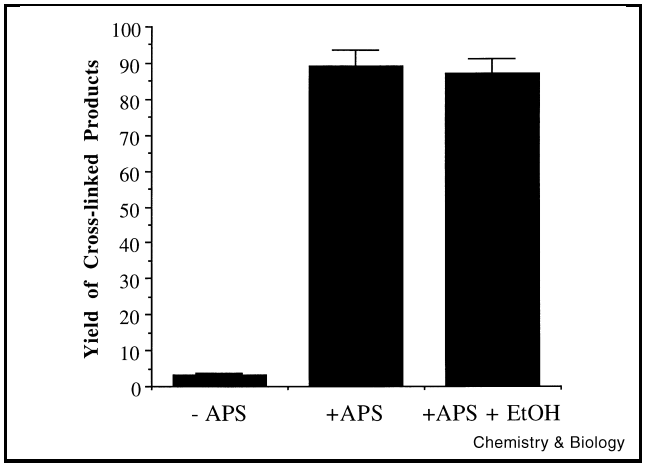


Figure 1. Ammonium persulfate plays an important role in the cross-linking reaction but the sulfate radical is not an obligatory intermediate. A radiolabeled polypeptide containing the Gal4 activation domain bound to the Gal80 repressor was photolyzed for 0.5 s in the presence of $Ru(II)(bpy)_3^{2+}$. APS and ethanol were either present or absent as indicated. The cross-linking reaction is strongly dependent on APS, but ethanol had no effect. This suggests that APS acts as an electron acceptor only and that the resultant sulfate radical is not an obligatory intermediate in the cross-linking reaction. The data shown were obtained by phosphorimager analysis of a SDS–polyacrylamide gel. The quantitation is based on a ratio of the sum of all product bands (see Figure 2) to total labeled protein.

ing process a hydrogen atom must be lost from one of the coupled proteins. In all cases examined to date, much lower yields of cross-linked products have been obtained in the absence of APS ([5] and unpublished results). For example, as shown in Figure 1, a complex comprised of the acidic activation domain of the yeast Gal4 transcription factor [10,11] and the Gal80 repressor [12] cross-linked in excellent yield in the presence of $Ru(II)(bpy)_3^{2+}$ and APS when the sample was photolyzed for 0.5 s. An identical solution, but which lacked APS, provided only a 3% yield of cross-linked products. This could reflect one or both proposed roles of the APS in the cross-linking reaction.

To test the possible requirement of the sulfate radical, the cross-linking reaction was repeated in the presence of ethanol, which is known to quench the sulfate radical efficiently [13]. As shown in Figure 1, the result was indistinguishable within experimental error of that obtained in the absence of ethanol. This result argues that the sulfate radical is not an obligate intermediate in the cross-linking reaction. However, it is possible that the carbon-based radical formed by the reaction of ethanol with the sulfate radical could act as a hydrogen atom abstraction agent. To probe this point, the efficiency of $Ru(II)(bpy)_3^{2+}$-mediated reactions was examined using either APS or cobalt(III) pentamine chloride complex as the cofactor. The Co(III) complex is known to accept an electron from excited state $Ru(II)(bpy)_3^{2+}$ irreversibly ([14] and references therein), producing the Co(II) aqua ion and ammonia, which would not be expected to act as hydrogen atom abstracting agents. As shown in Figure 2A, $Ru(II)(bpy)_3^{2+}$-mediated cross-linking of the Gal4 activation domain and Gal80 was observed when the cobalt complex was employed as the cofactor. A similar result was obtained when glutathione-S-transferase (GST), a homodimer that aggregates into high order species, was employed as the substrate (Figure 2B). These experiments argue that the sulfate radical formed by APS during photolysis is not required for cross-linking mediated by $Ru(II)(bpy)_3^{2+}$, though somewhat greater efficiencies are generally observed using APS (Figure 2 and unpublished results).

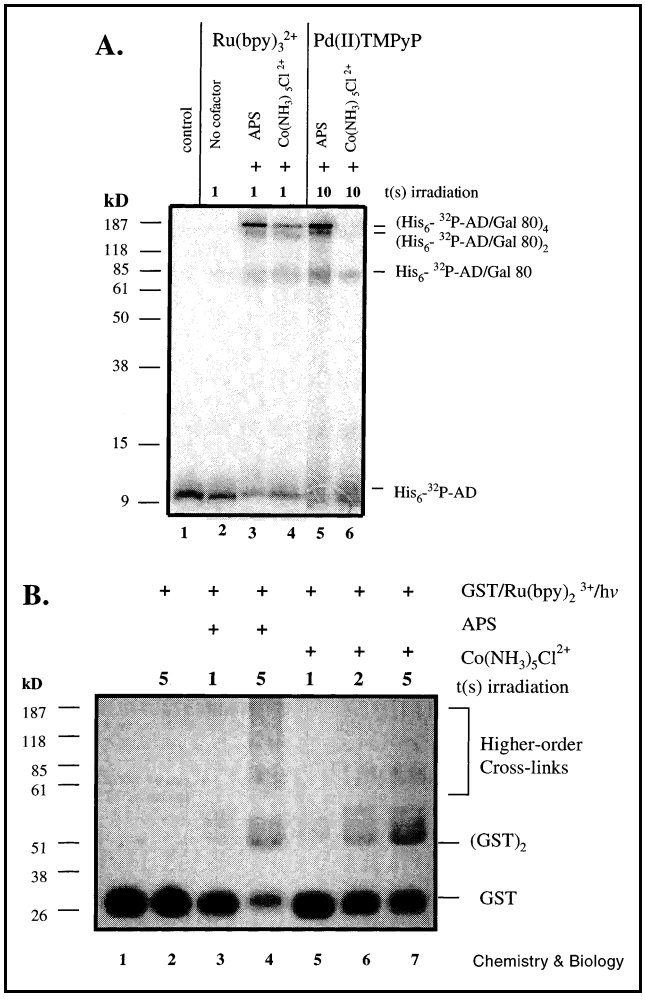


Figure 2. Comparison of APS and $Co(NH_3)_5Cl^{2+}$ as cofactors in the photo-induced cross-linking reaction. **(A)** Cross-linking of a ^{32}P-labeled His_6-Gal4 activation domain fragment and Gal80 repressor. A phosphorimage of the gel is shown, revealing activation domain-containing bands. The reagents included in each reaction are indicated above the gel. **(B)** Cross-linking of GST by $Ru(II)(bpy)_3^{2+}$ and light in the presence of APS or $Co(III)(NH_3)_5Cl^{2+}$. A Coomassie Blue-stained gel is shown.

Pd(II) porphyrins also mediate the photo-initiated cross-linking of proteins in the presence of APS [6], and it seems likely that the mechanism in this case is similar with the exception that the electron is probably lost from the ligand to form a Pd(II) porphyrin radical cation that proceeds to initiate oxidative cross-linking [15,16]. It is therefore surprising that $Co(III)(NH_3)_5Cl^{2+}$ supports photo-initiated cross-linking poorly in the presence of the tetra-(*N*-methylpyridinium) palladium(II) porphyrin (Pd(II)TMPyP) (Figure 2). Only a small amount of product is formed under the same conditions where the presence of APS results in efficient coupling. This is apparently not the result of poorly matched oxidation potentials, since cobalt(III) pentamine chloride has been used previously to support the photooxidation of palladium porphyrins [17]. However, these earlier studies employed different porphyrin ligands, so this remains a possibility. In any case, while we do not understand the mechanistic basis for this result, from a practical standpoint, it is important to point out that if one uses Pd(II)TMPyP as the cross-linking reagent it is important to use APS as the cofactor.

Pathways for protein cross-linking in the absence of an electron acceptor

As shown in Figure 1, a low, but detectable, yield of cross-linked products of the Gal4 activation domain–Gal80 complex is observed in the absence of an added electron ac-

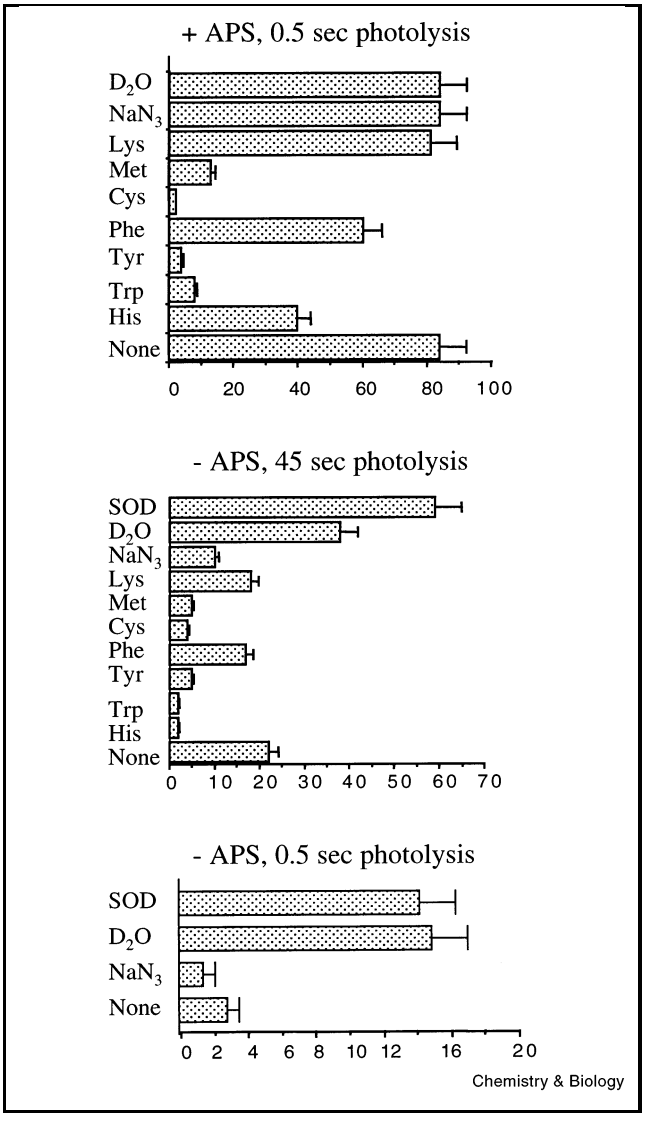


Figure 3. Effect of additives on the Ru(II)$(bpy)_3^{2+}$/light-mediated cross-linking of the Gal4 activation domain and Gal80 protein in the presence or absence of APS. The numbers on the horizontal axes represent percent yield of cross-linked products.

ceptor. This is typical of the results observed with most protein complexes examined to date. This yield can be increased substantially by increasing the photolysis time to 45–60 s. In some cases, the SDS–PAGE mobilities of the cross-linked products formed under these conditions were different than when APS was present and short irradiation times were employed (see Figure 2 in [5]). This suggested that coupling might involve fundamentally different mechanisms under these conditions. In the absence of an efficient electron acceptor like APS, the excited state ruthenium complex can react with molecular oxygen [18,19] to produce singlet oxygen, which is known to oxidize proteins in a number of ways that could result in subsequent cross-linking reactions [20–23]. For example the oxidation of arenes can produce aldehyde products that could form Schiff base adducts with free lysines [22]. Some experiments have been carried out using the Gal4 activation domain–Gal80 complex in which the effect of various additives on the reaction have been examined and these data are consistent with the idea that at least some of the cross-linking in the electron acceptor-independent pathway is due to singlet oxygen-mediated processes.

As shown in Figure 3, the addition of D_2O, which is known to increase the lifetime of $^1O_2$ [24], to a reaction lacking APS resulted in a significant increase in the yield, from about 20 to 40%. This difference was much greater when the photolysis was limited to 0.5 s (3 vs. 15%; Figure 3). On the other hand, sodium azide, which quenches singlet oxygen efficiently [25,26], suppressed the yield of the cross-linking products when APS was absent (Figure 3). Neither D_2O nor sodium azide had any effect on the cross-linking reaction when APS was present (Figure 3).

The effect of adding excess free amino acids to reactions that either do or do not contain APS is also instructive. In the presence of the cofactor, amino acids that are easily oxidized are potent inhibitors of cross-linking (Figure 3), presumably because they compete with the protein substrates for the Ru(III) intermediate. Note that histidine is a less potent inhibitor than for example tyrosine or tryptophan under these conditions, as would be expected from oxidation potentials. However, free histidine is a more efficient inhibitor of cross-linking in the absence of APS than in its presence. Histidine reacts efficiently with singlet oxygen [21].

These different sensitivities of the cross-linking reaction to additives in the presence and absence of APS support the argument that different mechanisms are involved in each case. Furthermore, the stimulation of the 'APS' reaction by D_2O and its inhibition by NaN_3 are consistent with the idea that singlet oxygen is an important intermediate in these reactions. However, it seems unlikely that singlet oxygen-dependent pathways are the only source of cross-linked products in the absence of APS. Even at high NaN_3 concentrations, the 'APS' reaction was suppressed by only 50% (Figure 3) whereas previous studies of other singlet oxygen-mediated processes indicate that they can be squelched almost completely [26]. What might this alternative pathway be?

Hammond and co-workers demonstrated that the excited state ruthenium complex is capable of transferring an electron to molecular oxygen to produce superoxide [18] and Ru(III)$(bpy)_3^{3+}$, the critical intermediate that initiates high efficiency cross-linking. However, given the potentials involved, this electron transfer event is not efficient and in-

deed the equilibrium lies towards reduction of superoxide by $Ru(III)(bpy)_3^{3+}$ [24], but this process could nonetheless be responsible for the small amount of product observed. Only under highly acidic conditions (for example in 9 M H_2SO_4) can Ru(III) be formed in high yields via this mechanism by protonation of the superoxide product [18], thus pulling along an otherwise unfavorable equilibrium. Obviously, such conditions are not relevant to biological studies, but this precedent suggested a different method by which to stimulate O_2-dependent Ru(III) production, which was to consume the superoxide enzymatically. Indeed, as shown in Figure 3, the addition of superoxide dismutase to a cross-linking reaction lacking APS stimulated the yield considerably. Horseradish peroxidase (HRP) had no effect (data not shown), nor did SOD or HRP have an effect on the reaction when APS was present (data not shown). Based on this result, we propose that the O_2-dependent generation of Ru(III) does contribute significantly to the production of cross-linked products in the absence of APS.

Is light-independent protein oxidation by APS a serious side reaction?

For the reasons described above, APS is an important player in the photo-induced cross-linking reaction. However, a concern is that APS might oxidize certain sensitive groups in proteins directly, such as methionine or cysteine sulfurs, prior to irradiation. If such reactions altered the activity of the protein, then artifactual results could be obtained, so it is of interest to determine how extensive such light-independent chemistry might be.

Since the type of oxidation events imagined would be unlikely to alter the mobility of a protein on a denaturing polyacrylamide gel, model reactions were carried out in which each of the 20 common amino acids was exposed to APS. The extent of direct oxidation as a function of time was characterized by NMR spectroscopy. For all of the amino acids except cysteine and methionine, no reaction was observed during a 1 h incubation even at an APS concentration 30 times higher than that used in a standard cross-linking reaction. However, some cysteine oxidation was noticeable after a 10 min incubation and after 1 h approximately 25% of the amino acid had been oxidized. Methionine oxidation was somewhat faster (Table 1).

Table 1
Progress of oxidation of amino acids by APS, or $Co(NH_3)_5Cl^{2+}$.

Time (min)	Cysteine consumption (±5%)		Methionine consumption (±5%)	
	APS	$Co(NH_3)_5Cl^{2+}$	APS	$Co(NH_3)_5Cl^{2+}$
1	0%	0%	0%	0%
5	0%	0%	5%	0%
10	< 10%	0%	20%	0%
60	25%	0%	50%	0%

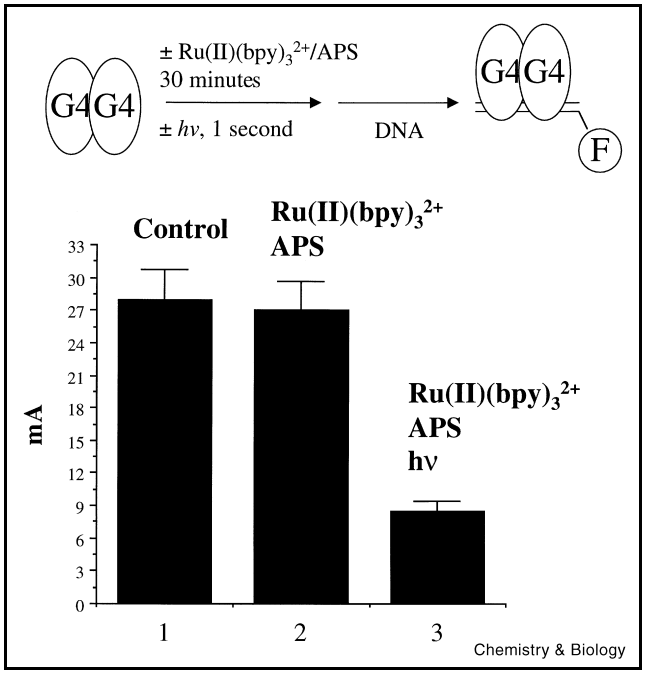


Figure 4. Effect of the cross-linking reagents on the DNA-binding activity of a Gal4 derivative. Purified Gal4(1–93+768–881) was preincubated with $Ru(II)(bpy)_3^{2+}$ and APS or, as a control, in the absence of these compounds, in the dark. One $Ru(II)(bpy)_3^{2+}$/APS-containing sample was photolyzed for 1 s. A fluorescein-labeled oligonucleotide containing the consensus Gal4-binding site was then added and binding was measured by fluorescence polarization.

Given that $Co(III)(NH_3)_5Cl^{2+}$ will also act as an electron acceptor in the ruthenium-mediated reaction, the sensitivity of cysteine and methionine to this reagent was also assessed. As expected, no reaction between these amino acids and the cobalt complex was observed even after long incubation times (Table 1). Therefore, when a particularly sensitive protein is employed for a cross-linking experiment $Co(III)(NH_3)_5Cl^{2+}$ should be the cofactor of choice. If APS is employed, incubation periods should be limited to no more than a few minutes prior to photolysis.

As another test of the level of protein damage that might occur prior to photolysis, the effect of the cross-linking reagents on the activity of the DNA-binding domain (DBD) of the yeast Gal4 protein was assessed. The Gal4 DBD contains a $Zn(\text{-S-Cys})_4$ cluster [27], the integrity of which is critical for sequence-specific DNA-binding. It binds as a dimer [28] to 17 base pair sites that resemble the consensus sequence 5′-CGGAGGACTGTCCTCCG [28,29]. Given the anticipated sensitivity of this metal–thiolate cluster to oxidation, this seemed like a fairly stringent test for light-independent oxidation.

A purified fragment of Gal4p containing the DBD as well

as the C-terminal activation domain (residues 1–93+768–881) was exposed to APS or $Co(III)(NH_3)_5Cl^{2+}$ and $Ru(II)(bpy)_3^{2+}$ for 30 min in the dark. Some of the solutions were then photolyzed for 1 s while others were not. Finally, a 5′-fluoresceinated double-stranded 21 base pair oligonucleotide containing the consensus Gal4-binding site was added to each solution and the extent of binding was monitored by fluorescence anisotropy [30]. The results are shown in Figure 4. The 30 min incubation in the dark in the presence of $Ru(II)(bpy)_3^{2+}$ and APS had no effect on the DNA-binding activity of the Gal4 derivative, as evidenced by the fact that the anisotropy value observed in this sample is identical within experimental error to a control that was not exposed to the cross-linking reagents. However, when the Gal4 derivative was cross-linked by irradiating this sample for 1 s, a significant decrease in the DNA-binding activity of the sample was observed (about 4-fold, see Figure 4). Analysis of the protein by SDS–PAGE revealed that the cross-linking yield in this experiment was approximately 40% (data not shown). We conclude that even for a tetrathiolate-containing zinc finger protein such as this Gal4 derivative, $Ru(II)(bpy)_3^{2+}$ and APS have little or no effect on the activity of the protein in the absence of light. However, photolysis of the sample results in significant loss of activity. This could be the result of the cross-linking reaction, other chemical modifications of the protein by the Ru(III) intermediate and the sulfate radical, or both. Essentially the same results were observed using the cobalt complex in place of APS. In the absence of light, no effect on the DNA-binding activity of Gal4p was observed. However, photolysis resulted in a significant decrease in activity (data not shown).

'Spurious' cross-linking can occur in concentrated solutions of purified proteins but not in more biologically relevant samples

A concern with any new cross-linking reaction is that it faithfully reports only stable protein associations and does not produce artifactual cross-links. These could occur as a result of protein denaturation and aggregation over extended reaction periods, or could result from trapping random collisions of proteins in solution, i.e. transient, nonspecific interactions. Because the $Ru(II)(bpy)_3^{2+}$/APS-mediated reaction is so fast, the former issue was not expected to be a problem. However, since the putative radical intermediates are quite reactive, cross-linking due to transient interactions was a concern. Figure 5 shows the results of $Ru(II)(bpy)_3^{2+}$/APS-mediated cross-linking experiments using four different proteins at relatively high concentrations (20 μM). Gal4-VP16 [31], a different derivative of the Gal4 protein containing the dimeric DBD fused to the heterologous VP16 activation domain [32], was employed as a positive control. Lysozyme, ubiquitin and maltose-binding protein (MBP) have been reported to be monomeric and so should not provide high yields of cross-linked products. However, as shown in Figure 5A, lysozyme, ubiquitin and maltose-binding protein (MBP) did provide cross-linked products, though less efficiently than was the case for Gal4-VP16.

The effect of protein dilution was examined using ubiquitin as a model. As shown in Figure 5B, the cross-linked band disappeared when the protein was diluted. Note that the bacteriophage T4 uvsY protein [33,34], a stable hexamer [35], was cross-linked to high molecular weight products in almost quantitative yield at a concentration where ubiquitin did not cross-link. A similar result was obtained

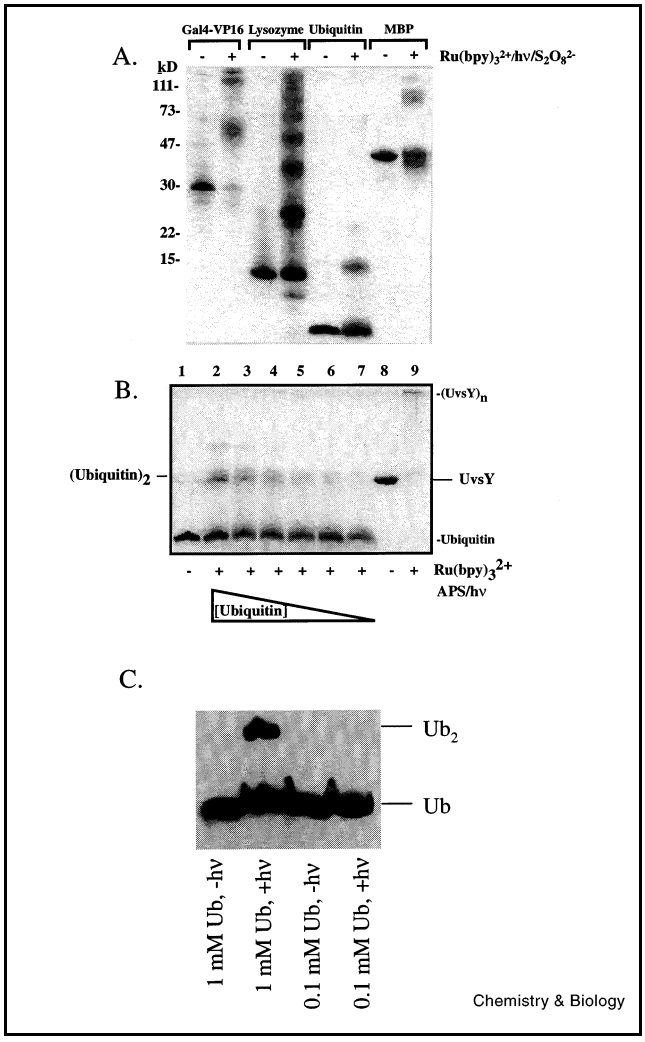


Figure 5. Cross-linking of proteins not stably associated with one another by $Ru(II)(bpy)_3^{2+}$ or Pd(II) porphyrin, APS and light. **(A)** Cross-linking of dimeric Gal4-VP16, and monomeric lysozyme, ubiquitin and maltose-binding protein (MBP). All were present at 20 μM. **(B)** Cross-linking of ubiquitin solutions of various concentrations. Lanes 1, 2: 20 μM ubiquitin. Lanes 3–7: 10 μM, 8 μM, 5 μM, 2 μM, 1 μM, respectively. Lanes 8, 9: 1 μM uvsY protein. The sample represented in lanes 1 and 8 were not photolyzed. **(C)** Cross-linking of ubiquitin with a Pd(II) porphyrin at different protein concentrations.

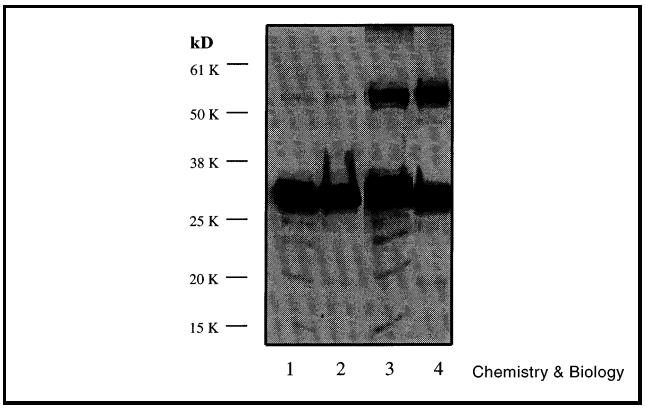


Figure 6. Cross-linking of His_6GST protein in a crude *E. coli* cell extract by $Ru(bpy)_3^{2+}$/APS/hv. A Western blot was used to detect only His_6GST protein (between 28K and 38K markers) and the cross-linked dimer. Lane 1: Crude extract containing His_6GST protein. Lane 2: Purified His_6GST eluted from Ni–NTA agarose beads. Lane 3: Photolysis of the crude extract for 5 s in the presence of $Ru(bpy)_3^{2+}$/APS, followed by direct loading of the sample onto the gel. Lane 4: Photolysis of the crude extract for 5 s in the presence of $Ru(bpy)_3^{2+}$/APS, followed by Ni–NTA agarose chromatography and elution with imidazole.

when Pd(II)TMPyP and APS were employed as the cross-linking reagents. A low level of ubiquitin cross-linking was observed at 1 mM protein, but this product disappeared when the ubiquitin concentration was reduced to 100 μM or less (Figure 5C). In many other experiments, we have never observed spurious cross-linking when the protein of interest is used at a concentration of 5 μM or less (unpublished results). Furthermore, as is demonstrated below, cross-linking of proteins in crude extracts does not result in the production of large numbers of spurious cross-linking products. We conclude that spurious cross-linking is not a problem in solutions normally employed for biochemical experiments, but care should be taken when purified proteins are employed at high concentrations.

$Ru(II)(bpy)_3^{2+}$/APS-mediated cross-linking in extracts

All of the experiments described above and in previous papers from our laboratory have involved cross-linking purified proteins. However, many workers will be interested in carrying out cross-linking reactions with less purified preparations or even in crude extracts.

To address the utility of the $Ru(II)(bpy)_3^{2+}$/APS-mediated photocross-linking reaction in more complex solutions, a cleared extract was prepared from *Escherichia coli* cells that express GST, a homodimer [36,37]. The particular construct employed also included a six histidine tag at the N-terminus of the protein. From a Coomassie Blue-stained gel, it was estimated that the His_6GST protein comprised approximately 10% of the total soluble protein in the sample (data not shown). Western blotting using an anti-GST antibody revealed a single major band (Figure 6, lane 1) which was identified as His6GST by its ability to be purified on a Ni–NTA agarose column (Figure 6, lane 2). When $Ru(II)(bpy)_3^{2+}$ and APS were added to the extract and it was photolyzed for 5 s, Western blotting revealed the formation of a 30% yield of the cross-linked His6GST homodimer (Figure 6, lane 3). This product was not observed when either $Ru(II)(bpy)_3^{2+}$ or APS was omitted from the reaction or if the sample was not photolyzed (data not shown). Thus, relatively efficient cross-linking via this chemistry is possible in crude extracts. It is important to note that there is no evidence on the Western blot of the formation of products other than the homodimer. This demonstrates that spurious cross-linking of His6GST to *E. coli* proteins did not occur to a detectable degree.

Another experiment was done in an extract made from *E. coli* cells that express epitope-tagged Pho4 protein, a dimeric yeast transcription factor [38]. In this case, expression of the protein was not induced, resulting in a low level of Pho4 due to the 'leakiness' of the lac-type promoter under non-inducing conditions. Indeed, the Pho4 protein band was undetectable in a Coomassie-stained gel of the crude extract (data not shown). This situation is a better model for an experiment in which one attempts to use cross-linking to identify partners of a native protein in a crude extract.

As shown in Figure 7, Western blotting using a monoclonal antibody raised against the epitope tag (S10 epitope) can detect the low level of Pho4 in the extract and also reveals the presence of proteolytic degradation products (third lane). Addition of $Ru(II)(bpy)_3^{2+}$ and APS followed by a 1 s photolysis resulted in an approximately 10% yield of a band (fourth lane) with the expected mobility on SDS–

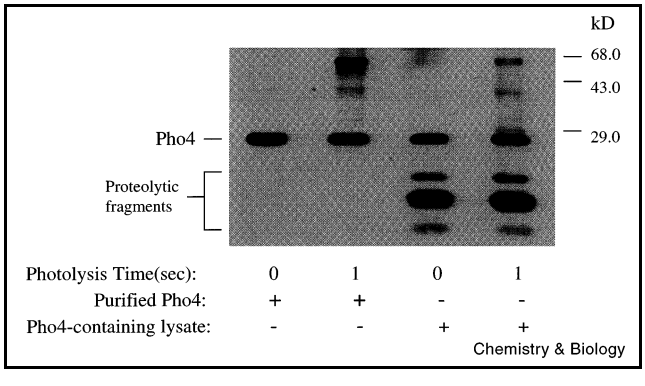


Figure 7. $Ru(bpy)_3^{2+}$/APS/light-induced cross-linking of purified S10-tagged Pho4 protein (first two lanes) or S10-tagged Pho4 protein at low levels in a crude *E. coli* extract (last two lanes). A Western blot obtained using the monoclonal anti-S10 antibody is shown.

PAGE of a covalent Pho4 dimer which co-migrated with the product obtained when purified S10-Pho4 (first lane) was cross-linked (second lane). The results of the experiment using purified S10-Pho4 and the same protein in the extract were very similar except that the yield was higher using the purified protein. This provides another demonstration that spurious cross-linking of the protein of interest to non-interacting proteins does not occur to an appreciable extent.

The stability of commonly used epitope and affinity tags under cross-linking conditions

When cross-linking experiments are carried out in crude extracts, an issue of major importance is how to analyze the results in these complex mixtures. On an analytical scale, the use of immunological methods (Western blotting) is very convenient. As evidenced by the data shown in Figure 7, the S10 epitope tag survives the cross-linking reaction in functional form and can be used for this purpose. This was somewhat surprising in that the S10 epitope (MASMTGGQQMG) is rich in easily oxidized methionine residues. To examine this issue in more detail, purified S10-tagged Pho4 protein was irradiated in the presence of $Ru(II)(bpy)_3^{2+}$ and APS for 1–15 s. SDS–PAGE followed by Western blotting showed that the epitope is relatively stable under the reaction conditions for up to approximately 5 s but that significant degradation of the tag occurs at longer irradiation times (Figure 8).

Similar experiments were conducted with other tags commonly employed to visualize proteins in complex mixtures. In general, the results were exactly what one would expect based on the ease of oxidation of the residues in the tag. For example, the commonly used hemagglutinin (HA) epitope tag (YPYDVPDYA) is converted rapidly to a form that is not recognized by the cognate antibody (data not shown). This is not surprising given the tyrosine-rich nature of the HA epitope. Fluorescein also proved to be quite sensitive to the reaction conditions (data not shown).

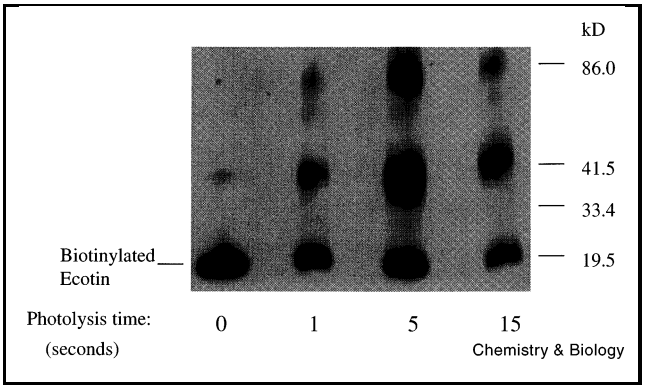


Figure 9. Cross-linking of biotinylated ecotin. Biotinylated ecotin was photolyzed for the times indicated in the presence of $Ru(bpy)_3^{2+}$ and APS. The samples were analyzed by SDS–PAGE and visualized with an HRP–neutravidin conjugate.

Whereas epitope tags are very useful for analytical experiments, other tags are better suited for preparative work where one might wish to employ the tag to physically isolate the cross-linked products for further analysis. For example the six histidine tag is often used for this purpose since this allows proteins to be purified by immobilized metal affinity chromatography under denaturing conditions. Exogenous histidine can inhibit the $Ru(II)(bpy)_3^{2+}$/APS photocross-linking reaction (see Figure 3), suggesting that the imidazole ring can be attacked by the oxidized metal complex. Therefore, it was not obvious that the six histidine tag would survive the cross-linking reaction.

To probe this point, the experiment in which His6GST was cross-linked in an *E. coli* extract (Figure 6) was adapted to assess the state of the metal-binding tag before and after the cross-linking reaction. Rather than immediately boiling the sample and loading it onto the gel (Figure 6, lane 3), the cross-linked extract was applied to Ni-saturated NTA agarose beads. The beads were then pelleted and washed thoroughly. The bead-bound proteins were then analyzed by boiling in denaturing buffer followed by SDS–PAGE and Western blotting using an anti-GST antibody. As shown in Figure 6 (lane 4), the results show that cross-linked products can be isolated by metal ion affinity chromatography. Indeed, comparison of the experiments in which the cross-linked extract was loaded onto the gel directly (Figure 6, lane 3) and in which the intermediate metal-binding step was employed (Figure 6, lane 4) revealed that the ratio of monomeric to dimeric His6GST was almost identical. This argues that there is little degradation of the His6 tag under these conditions. If that were the case, then there should be less dimer relative to the unreacted monomer in the metal-bound fraction than in the sample loaded directly onto the gel. Thus, while a large excess of free histidine can inhibit the cross-linking reaction, this residue does not appear to be oxidized to any great extent in the context of a protein under typical reaction conditions.

Another tag that is useful for the subsequent isolation of products is biotin, which has an extremely high affinity for avidin. Since biotin contains a thioester, it was also unclear if it would survive the reaction in functional form. To test this, biotinylated ecotin, which exists as a mixture of dimers and tetramers, was subjected to $Ru(II)(bpy)_3^{2+}$/APS/light-mediated cross-linking for between 1 and 15 s. The samples were then electrophoresed through a denaturing gel, blotted, and probed with a horseradish peroxidase–neutravidin conjugate. As shown in Figure 9, the biotinylated protein and its cross-linked products were readily detectable even after a 15 s irradiation. This experiment in-

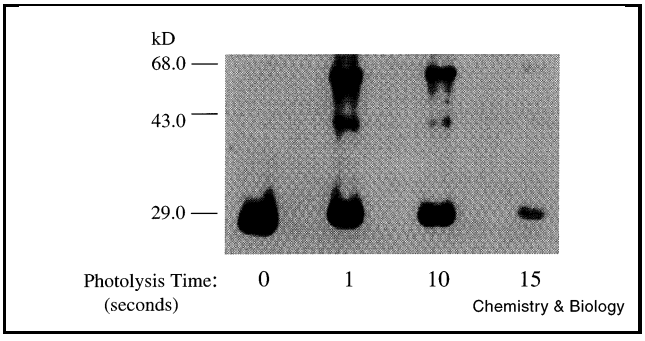


Figure 8. The S10 epitope tag is stable for a short time under the cross-linking conditions. Purified S10-tagged Pho4 protein was photolyzed for the time indicated in the presence of $Ru(bpy)_3^{2+}$ and APS. The samples were analyzed by SDS–PAGE and Western blotting using the anti-S10 antibody.

dicates that using photo-induced cross-linking to search for binding partners of biotinylated probe molecules in crude extracts will be feasible.

Discussion

The visible light-mediated protein cross-linking reaction mediated by $Ru(II)(bpy)_3^{2+}$ or a Pd(II) porphyrin and an electron acceptor has many advantages over traditional cross-linking methods and should be of broad utility in the analysis of protein–protein interactions in vitro. Cross-linking occurs rapidly and in most cases examined to date produces moderate to excellent yields of cross-linked products. Because the reagents are inert until activated with light, this represents a readily 'switchable' system that can be used for studies of dynamic processes over time. Another use of this type of reaction is in affinity cross-linking experiments in which the cross-linker is appended covalently to a probe molecule of interest and must be kept in an inert state until the probe molecule docks with its receptor. Finally, the fact that visible, rather than UV, light is employed to initiate the reaction means that almost all of the light is delivered to the cross-linking reagent, rather than being absorbed by various biological chromophores, an important advantage for experiments conducted in complex mixtures such as cell extracts.

In order to facilitate the routine use of this system, we have investigated various issues of practical importance to biochemists who might wish to employ this technology. It is demonstrated here that the reaction can be used in crude extracts as well as with purified proteins. We have identified some epitope and affinity tags used commonly for the protein visualization in, or purification from, crude extracts that survive the reaction in functional form. These include the S10 epitope tag, the six histidine tag and biotin, though the S10 and biotin tags can be degraded when extended irradiation times are employed, presumably due to oxidation of the sulfur atoms present in these tags. Obviously, the 'safe' time period will vary with the intensity of the light source employed. The values reported here are appropriate for the 150 W Xe lamp we employed in our experiments. This parameter should be determined empirically in any given experiment. Certain other tags do not survive the reaction, including the HA epitope and fluorescein and are therefore incompatible with this technology. In general, it appears that the survivability of any given tag can be predicted based on the ease of oxidation of the functional groups in the tag. Electron-rich aromatic residues seem particularly vulnerable, as one would expect given the proposed mechanism of the reaction. This is an important point to keep in mind when one employs antibodies raised against a native protein of interest to analyze the results of a cross-linking experiment. In these cases, one may not know the epitope or epitopes recognized by the antibody. If that epitope contains sensitive residues, then on a Western blot the tagged protein will appear to be consumed in the cross-linking reaction without the production of observable cross-linked products, when in reality, the epitope has simply been destroyed. Several colleagues using this cross-linking technique have observed this type of result, but were able to obtain useful information by using a different antibody (various personal communications). Polyclonal antibodies are particularly useful in this regard, since it is unlikely that all epitopes recognized by a polyclonal preparation will be destroyed in the reaction.

When using the reaction to examine interactions between purified proteins, it is important that one employs the proteins at levels where transient, biologically irrelevant associations do not occur, since this very efficient cross-linking chemistry can trap those transient interactions (see Figure 5). This critical concentration for non-specific interactions will vary from protein to protein, but in general it appears that when using solutions containing 20–100 μM or more of a single protein spurious cross-linking is a concern. As demonstrated in Figure 5, a simple titration experiment can easily distinguish between high and low affinity contacts. Gratifyingly, in experiments that best model what we anticipate will be the most popular applications of this chemistry, probing interactions in crude extracts or between purified proteins in less concentrated solutions, there was no evidence of spurious cross-linking.

Finally, we have addressed certain aspects of the role of the APS cofactor in this reaction. The results are consistent with the idea that it functions purely as an electron acceptor, serving to generate a Ru(III) intermediate from photoexcited $Ru(II)(bpy)_3^{2+}$. The fact that ethanol has no effect on the cross-linking reaction and that a Co(III) complex can substitute for APS argues that the sulfate radical that is formed by electron transfer from the excited Ru(II) complex to APS does not play a critical role in the coupling

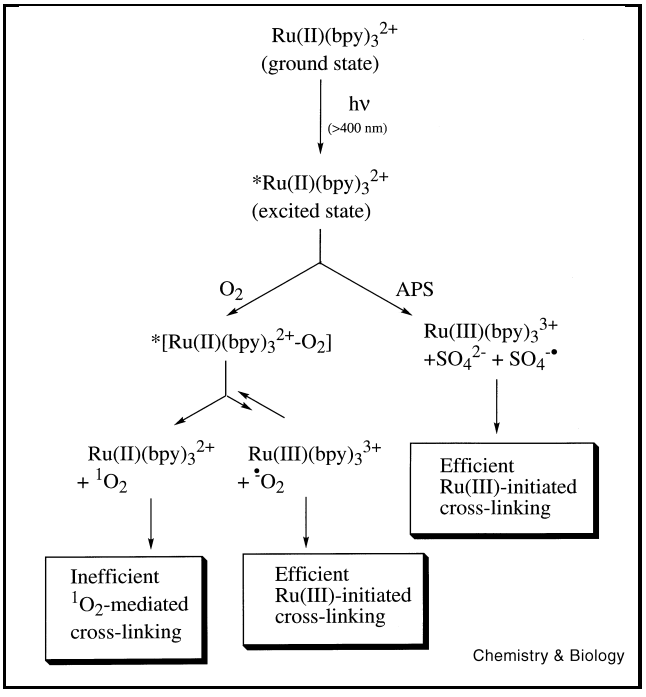


Figure 10. Proposed pathways for the generation of reactive intermediates upon photolysis of $Ru(bpy)_3^{2+}$.

reaction. It remains unclear why the Pd(II) porphyrin-mediated reaction does not proceed when $Co(III)(NH_3)_5Cl^{2+}$ is substituted for APS. It was demonstrated here that in the absence of an electron acceptor, other pathways can lead to less efficient cross-linking. These pathways have not been studied in great detail, since they are less efficient and therefore of lesser interest from a practical point of view. However, the data available suggest that some of the products obtained under these conditions might result from singlet oxygen-mediated chemistry.

Of possible future significance is the observation that superoxide dismutase can stimulate the cross-linking process in the absence of an electron acceptor. Based on literature precedent [18], we have interpreted this result to indicate that electron transfer from the photoexcited Ru(II) complex to molecular oxygen can occur and that destruction of the resultant superoxide product can pull this otherwise inefficient reaction along. If production of Ru(III) via electron transfer from Ru(II) to oxygen could be rendered efficient, it might be of utility in developing photo-initiated cross-linking reactions for use in living cells. $Ru(II)(bpy)_3^{2+}$ and palladium porphyrins are cell-permeable, but APS is not. This will be the subject of future investigations. A summary of our current model for the reactions by which intermediates capable of cross-linking proteins are generated is shown in Figure 10.

Significance

Chemical cross-linking is potentially a powerful alternative to genetic analyses of protein–protein interactions, such as the two-hybrid system. However, the use of chemical approaches to study multi-protein complexes or to search for binding partners of a newly discovered protein has been limited by the relatively slow and inefficient chemistry employed by most classical cross-linkers. The $Ru(II)(bpy)_3^{2+}$- or Pd(II) porphyrin-mediated cross-linking chemistry developed in our laboratory employs fundamentally different chemistry that proceeds rapidly and, in most cases, efficiently and minimizes or obviates many of the drawbacks inherent in traditional cross-linkers (see Introduction). It therefore should be of broad utility as a tool for probing protein–protein interactions in vitro. In this report, we have addressed several important practical issues, such as if the reaction can be used in crude extracts, what tags survive the oxidative chemistry and whether spurious cross-linking of proteins not stably associated with one another is a major problem. These results should facilitate the use of this chemistry in other laboratories. In addition, we have probed certain mechanistic issues revolving around the role of the APS cofactor in the reaction and the pathways that can lead to cross-linking in its absence. These studies have provided interesting basic information and may aid in the design of useful new variants of the basic reaction, for example a system that could be used inside living cells.

Materials and methods

Proteins and reagents

UvsY [34], Gal4-VP16 [31], Gal80p [39] and the radiolabeled carboxyl terminal activation domain of Gal4p (His6-Gal4 AD) [11] were all prepared according to previously published procedures. Gal4 (1–93+768–881) was purified according to the protocol of Reece et al. [40]. Maltose-binding protein (MBP) was purchased from NEB. Ubiquitin, bovine kinase, superoxide dismutase and ammonium persulfate were purchased from Sigma. BSA was purchased from Pierce. Care was taken to remove all reducing agents such as β-mercaptoethanol and dithiothreitol, from the proteins by dialysis from their storage buffers into PBS (15 mM sodium phosphate (pH 7.5), 150 mM NaCl) and 10% glycerol.

Ecotin (kindly provided by Prof. Kathylnn Brown (UT-Southwestern)) was purified by the literature procedure [41] and was biotinylated using the EZ-Link Sulfo-NHS-LC-Biotinylation Kit (Pierce). Sulfo-NHS-LC-Biotin dissolved in water was added in a 12-fold molar excess to a solution containing ecotin protein in PBS buffer. The resulting mixture was incubated on ice for 2 h and then passed over a desalting column.

A GST-S10-Pho4p (S10 epitope: MASMTGGQQMG) expressing plasmid was kindly provided by Liping Sun (UT-Southwestern). The DNA encoding the activation domain of Pho4p was amplified from this plasmid by PCR using the following primers: 5′-GAT GCC ATG GCT AGC ATG ACT GGT G-3′ and 5′-GCA TGG ATC CCG TTC TGC TGT AGG TGA CGG ATG TAC C-3′. The resulting product was digested with *Bam*HI and *Nco*I and then cloned into the pQE-60 plasmid (Qiagen) also digested with *Bam*HI and *Nco*I. The resulting plasmid was then transformed into the DH5α strain. The purified protein was obtained by first growing one DH5α-transformant in a 10 ml culture of Luria broth containing ampicillin (75 mg/l) overnight at 37°C and then transferring this saturated culture to 1 l of Luria broth containing ampicillin. This

culture was grown to an OD_{600} of 0.6. Expression of the desired protein was induced by adding IPTG (isopropyl-1-thio-β-D-galactopyranoside) to a final concentration of 1 mM. The culture was then grown for an additional 2 h at 37°C. The cells were harvested by centrifuging the samples at 4000 rpm for 15 min, resuspended in 1×PBS buffer (20 mM sodium phosphate, 150 mM NaCl, pH 7.5) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and then lysed by sonication and centrifuged at 15 000 rpm for 30 min. The resulting supernatant was then loaded onto a Ni–NTA column (Qiagen) pre-equilibrated with 1×PBS buffer. The column was washed with PBS buffer containing 600 mM NaCl and then the Pho4 protein was obtained by eluting with PBS buffer containing 300 mM imidazole. The purified protein was then dialyzed into 1×PBS buffer for subsequent cross-linking experiments.

The Pho4 protein-containing extract used for cross-linking was prepared by growing one DH5α-transformant in a 10 ml culture of Luria broth containing ampicillin (75 mg/l) to saturation overnight at 37°C. The following day the cells were pelleted by centrifugation at 4000 rpm for 15 min and then resuspended in PBS buffer containing 1 mM PMSF. The cells were sonicated and pelleted by centrifugation at 14 000 rpm for 15 min. The concentration of the resulting lysate was determined by the Bradford assay to be approximately 7 mg/ml.

$Ru(bpy)_3Cl_2$ was purchased from Aldrich, and was stored as a 30 mM solution in milli-Q H_2O (Millipore) at room temperature in the dark. Protein concentrations were determined using the Bradford reagent (Bio-Rad) and bovine serum albumin (BSA) as a standard. All other concentrations were determined by dry weight.

Cross-linking reactions

Unless indicated otherwise, cross-linking reactions were carried out in a total volume of 20 μl in a buffer comprised of 15 mM sodium phosphate (pH 7.5), 150 mM NaCl, 0.125 mM $Ru(bpy)_3Cl_2$ and 2.5 mM $(NH_4)_2S_2O_8$ (ammonium persulfate (APS)). When palladium porphyrin was employed, its concentration was also 0.125 mM and when the cobalt complex was substituted for APS, its concentration was 2.5 mM. Protein concentrations varied from 20 μM to 0.5 μM. Immediately following the addition of APS, solutions (in a 1.7 ml Eppendorf tube) were mounted parallel to, and in the beam of light at a distance of 50 cm from a 150 W xenon arc lamp (Oriel Inc.). Light was filtered first through 10 cm of distilled water and then through a 380–2500 nm cut on filter (Oriel #49470). Exposure time was controlled by shining light through timed shutters of a Praktica single lens reflex camera with the lens and back cover removed from the camera body. Exposure times in most cases were 0.5 s unless otherwise indicated. Immediately following irradiation samples were quenched with 7 μl 4×gel loading buffer (0.2 M Tris, 8% SDS, 2.88 M β-mercaptoethanol, 40% glycerol, 0.4% xylene cyanol, 0.4% bromophenol blue) and heated to 95°C for 5 min and then separated by electrophoresis through a 10% Tricine SDS–polyacrylamide gel. Proteins were visualized by staining with Coomassie brilliant blue, by phosphorimage via STORM (Molecular dynamics) or by Western blotting with the appropriate antibodies.

For the cross-linking of biotinylated ecotin, 7 μM of the substrate in a 20 μl reaction volume was cross-linked for varying time intervals. The survival of the biotin tag was then assayed by electrophoresing 1 μl of each reaction through a denaturing polyacrylamide gel and then transferring the proteins to a nitrocellulose membrane and probing with an HRP conjugate of the neutravidin antibody (Pierce). The appropriate bands were visualized with the ECL detection system.

For the cross-linking reactions using the S10-tagged Pho4 protein, 2 μM of purified Pho4 protein was used in each 20 μl reaction and 7 μl of lysate (7 mg total protein/ml). Reaction mixtures were separated by SDS–PAGE, transferred to a nitrocellulose membrane, and then probed with a HRP conjugate of the S10 antibody (Novagen).

Effect of amino acids on $Ru(bpy)_3^{2+}$/APS-mediated cross-linking

The general procedure was followed with the exception that final protein concentrations were 0.5 μM for Gal80p and approximately 0.5 μM for His_6-Gal4 AD in 15 μl of PBS. Additionally, radiolabeled Gal4 AD was incubated with Gal80p for 30 min on ice containing either 7.5 mM histidine, tryptophan, tyrosine, phenylalanine, methionine, cysteine, or lysine. After photolysis and separation of protein fragments by electrophoresis, the gels were dried onto 3 mm paper (Waters) and radioactive bands were visualized and cross-linked products quantitated using a phosphorimager.

Effect of ethanol on $Ru(bpy)_3^{2+}$/APS-mediated cross-linking

The same conditions as those above were employed except that ethanol was present at a final concentration of 125 mM.

Oxidation of amino acids

50 μl of an APS solution in D_2O (1 M) was added into the amino acid/D_2O solution (10 mM). This brought the final APS concentration in the NMR tube to 50 mM. After mixing well, a ^{1}H-NMR spectrum of the solution was obtained every 5 min over a 1 h period. The determination of oxidation of each amino acid was characterized by the formation of a new set of peaks for the corresponding oxidized amino acid.

Gal4–DNA binding experiments

The construction, expression and purification of the Gal4 protein derivative (containing the DNA-binding and activation domains, residues 1–93+768–881) used in this experiment will be reported elsewhere. The concentration of the protein was 30 nM. The fluorescently labeled oligonucleotide was made by annealing fluoresceinated (5′-GAC GGA GGA CTG TCC TCC GAG-3′) and its complementary strand. Its concentration was 10 nM. All polarization readings were taken on a Beacon 2000 instrument (PanVera).

Cross-linking of His_6GST in a bacterial extract

pGEX-His_6GST was transformed into the BL21 strain of *E. coli*. The cells were grown in 1 l of LB medium containing ampicillin (100 μg/ml) at 37°C, and induced with IPTG. Cells were harvested by centrifugation (10 min, 4000 rpm), and the pellet was washed with 1×PBS (15 mM sodium phosphate (pH 7.5), 150 mM NaCl) 2 times. Then, cells were suspended in 10 ml of fresh PBS, and lysed by sonication. The resultant suspension was centrifuged for 40 min at 14 000 rpm. The supernatant was saved for the cross-linking reaction. The total protein concentration of the solution (2.2 mg/ml) was measured by the Bradford method using BSA as the standard. A denaturing protein gel stained with Coomassie Blue showed that His_6GST protein was less than 10% relative to other proteins in the lysate.

60 μl of lysate was transferred into a colorless Eppendorf tube positioned parallel to the beam of a 150 W Xe arc lamp light at a distance of 50 cm. $Ru(bpy)_3^{2+}$ (170 μM) was mixed with the extract. APS (340 μM) was added just before irradiation. Quenching of the cross-linking reaction was accomplished by adding 1 μl of 1 mM β-mercaptoethanol. 30 μl of Ni–NTA agarose bead suspension solution, previously equilibrated with 6 M urea, was added into the reaction mixture and incubated for 30 min at room temperature. The suspension was centrifuged, the beads removed and bound proteins were eluted with 500 mM imidazole solution. Western blots were used to visualize His_6GST proteins using mouse anti-GST antibody.

Acknowledgements

This work was supported by a grant from the National Institutes of Helath (GM-58175) and the Welch Foundation (I-1299). We thank Prof. Kathlynn Brown and Mr. Liping Sun (UT-Southwestern Medical Center) for providing proteins and plasmids.

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