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Chemoselective Tryptophan Labeling with Rhodium Carbenoids at Mild pH

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Abstract: Significant improvements have been made to a previously reported tryptophan modification method using rhodium carbenoids in aqueous solution, allowing the reaction to proceed at pH 6−7. This technique is based on the discovery that *N*-(*tert*-butyl)hydroxylamine promotes indole modification with rhodium carbenoids over a broad pH range (2−7). This methodology was demonstrated on peptide and protein substrates, generally yielding 40−60% conversion with excellent tryptophan chemoselectivity. The solvent accessibility of the indole side chains was found to be a key factor in successful carbenoid addition, as demonstrated by conducting the reaction at temperatures high enough to cause thermal denaturation of the protein substrate. Progress toward the expression of proteins bearing solvent accessible tryptophan residues as reactive handles for modification with rhodium carbenoids is also reported.

Introduction

Bioconjugation reactions have provided key tools for the study of proteins for nearly a century. These techniques are based on chemoselective reactions that can target specific amino acid side chains, often with the goals of abrogating a side chain's normal function, altering its pK_a or electrochemical properties, or appending a new chemical probe.[1] Numerous techniques have been developed for the labeling of lysine, cysteine, tyrosine, and acidic residues.[2] However, chemical methods with high specificity for tryptophan residues are, in comparison, lacking. Species such as Koshland's reagent (2-hydroxy-5-nitrobenzyl-bromide)[3] or chlorosulfonium ions[4] have long been used to study and quantify tryptophan residues in proteins, but these methods can display cross reactivity with histidine and tyrosine side chains. Given the important role of tryptophan residues as intrinsic fluorophores and mediators of electron transfer, new methods to modify this residue are of significant value to the chemical biology community.

To attach new functionality to tryptophan residues, we have reported an organometallic reaction involving vinyl metallocarbenoids, featuring aqueous reaction conditions and high indole chemoselectivity (Figure 1).[5] This reaction was facilitated by an unusual additive, hydroxylamine hydrochloride, which in many cases served to enhance the efficiency of the tryptophan modification pathway relative to hydrolysis of the rhodium carbenoid intermediate. Since that time, reports have appeared describing the modification of tryptophan residues using malondialdehyde under acidic conditions[6] as well as a *bis*-cinnamoyl malonyl diazo compound that generates fluorescent derivatives using the same rhodium acetate catalyst.[7] Taken together, these

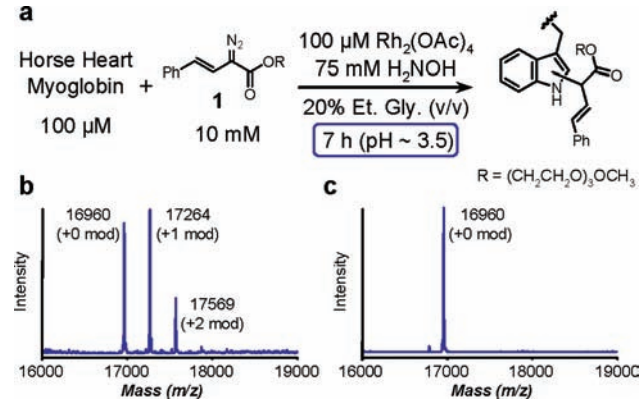

Figure 1. Modification of tryptophan residues with metallocarbenes. (a) Horse heart myoglobin was exposed to **1** and $Rh_2(OAc)_4$ for 7 h. (b) A representative ESI-MS spectrum of modified myoglobin. (c) No modification is observed without $Rh_2(OAc)_4$. All assigned species agree to within 0.1% of the expected mass values.

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strategies expand the set of tools that is available for probing of the roles of tryptophan residues in proteins.

The reaction conditions initially reported by our laboratory proceed at room temperature and are tolerant of several aqueous solvent systems that are compatible with a wide range of protein substrates.[8] Yet, the reaction pH (ca. 1.5−3.5) necessary for efficient protein labeling stands out as a significant drawback, preventing this methodology from finding widespread use for protein targets. As such, we became interested in developing a new reaction protocol that could promote tryptophan modification with rhodium carbenoids at mild pH (ca. 6−8).

Through a thorough investigation of the role of reaction pH and solution additives in the original tryptophan modification system, we have succeeded in developing a new reaction protocol that allows labeling of solvent accessible tryptophan residues at pH 6−7. This report outlines the discovery of these mild pH reaction conditions, as well as the use of this methodology to modify tryptophan-containing substrates. Furthermore, efforts to develop a strategy for the modification of peptidic "tags" containing particularly reactive tryptophan residues are presented. The low frequency of surface accessible tryptophans in native protein sequences would suggest that these tags could be used for site selective labeling in many cases.

Results and Discussion

In our initial report of this tryptophan modification reaction, $Rh_2(OAc)_4$ was used to generate a carbenoid intermediate from vinyl diazo compound **1**, which was synthesized from the ester of styryl acetic acid (Figure 1).[5] The use of hydroxylamine hydrochloride was identified as a key additive for the protein modification reaction, although the mode of action of the salt was not elucidated. The selectivity of the reaction for tryptophan residues was shown by proteolytic digestion of modified horse heart myoglobin followed by MS/MS analysis. Under the reaction conditions, it was noted that myoglobin was denatured and the heme dissociated from the protein due to the low pH (∼3.5).

Effect of pH on Carbenoid Formation, Myoglobin Modification, and Subtilisin Modification. Our efforts to improve the pH range of the tryptophan modification methodology began with an investigation of the effect of pH on three model systems: (*i*) the rhodium catalyzed degradation of **1** in the absence of protein, (*ii*) the modification of horse heart myoglobin, and (*iii*) the modification of subtilisin Carlsberg (Figure 2). Solutions of **1** (10 mM) in 80% water/20% ethylene glycol were incubated in the presence of 100 μM $Rh_2(OAc)_4$ and 75 mM H_2NOH over a pH range of 1.5−9.0. Reactions were analyzed by reversed-phase HPLC, and the extent of diazo compound consumption was measured relative to a standard sample of **1** that was incubated for an identical period of time in the absence of catalyst. ^{1}H NMR analysis of crude reaction mixtures at pH 3.5 revealed that the major product of this reaction was alcohol **3**, resulting from the O−H insertion reaction of carbenoid **2** with H_2O (data not shown). As shown in Figure 2, **1** was fully consumed below pH 5, while at pH ≥ 6 a rapid drop in reactivity was observed. This indicated that carbenoids (**2**) were not being generated at higher pH. Similar pH profiles were generated for the modification of horse heart myoglobin and subtilisin Carlsberg. For these protein substrates, rather than monitoring the extent of diazo compound degradation, the level of protein

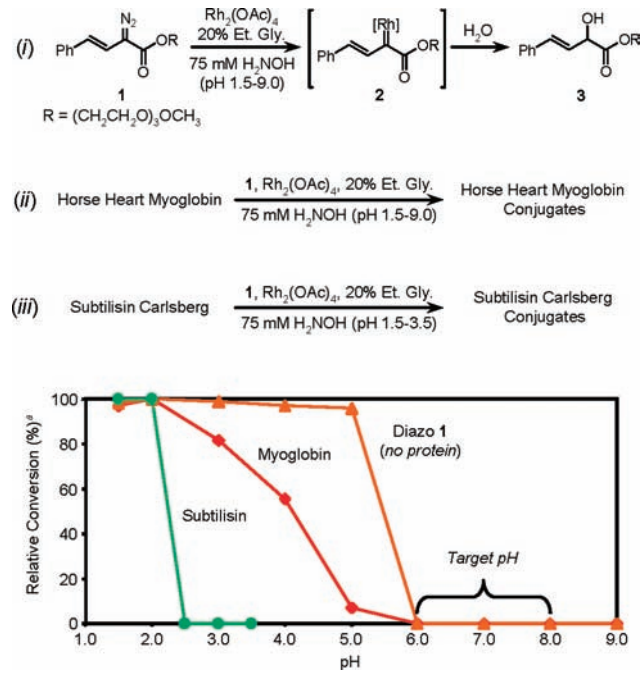

Figure 2. The effect of reaction pH with using H_2NOH as a reaction additive was monitored for (*i*) the catalytic degradation of diazo **1** and for the modification of (*ii*) horse heart myoglobin and (*iii*) subtilisin Carlsberg. In all cases, no reaction was observed at pH ≥ 6. The degradation of **1** was assessed by RP-HPLC. The extent of modification for myoglobin was estimated by ESI-MS, and the values represent the sum total of both singly and doubly modified species. The extent of modification for subtilisin was estimated qualitatively by MALDI-TOF-MS (primarily single modification was observed). *a*The extent of conversion for each data point is expressed as a percentage of the maximum conversion observed for that substrate across the entire pH range. The absolute conversion was estimated to be 90% for myoglobin and 50% for subtilisin Carlsberg.

modification was measured as a function of pH. Analogous to the results obtained for the degradation of **1**, both protein labeling reactions were favored at low pH but failed above a certain pH threshold.

The shape and position of the pH profiles depicted in Figure 2 revealed important factors preventing tryptophan modification in the desired pH range (ca. 6−8). First, with respect to the catalytic degradation of diazo compound **1**, it was clear that the necessary rhodium carbenoid intermediates (**2**) were not being generated above pH 5. This dramatic drop in catalytic activity can be explained by considering the pK_a of the hydroxylamine additive (pK_a = 5.97).[9,10] At pH values below 6, the nitrogen of hydroxylamine is largely protonated, thereby preventing coordination of the nitrogen lone pair to the axial positions of the rhodium dimer. Instead, we propose that these vacant coordination sites are bound by water or possibly the oxygen of protonated hydroxylamine (Figure 3). This scenario is supported by the color of a 1 mM aqueous solution of $Rh_2(OAc)_4$ in 75 mM H_2NOH at pH 3.0. The characteristic blue color and UV−vis spectrum of this solution are consistent with $Rh_2(OAc)_4$ bearing axial water ligands (Figure 3).[11,12] Importantly, the UV−vis spectrum at pH 3.0 is also distinct from $Rh_2(OAc)_4$ bound by chloride,[11,13] indicating that this ion is

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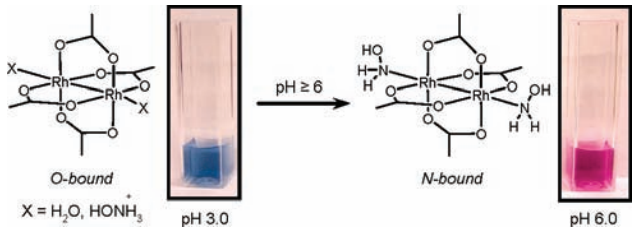

Figure 3. pH-dependent binding of H_2NOH to $Rh_2(OAc)_4$ in aqueous solution. At low pH the color of $Rh_2(OAc)_4$ in solution is consistent with coordination by water or possibly the oxygen of protonated hydroxylamine, while above pH 6.0 the color is consistent with coordination via the nitrogen atom of hydroxylamine. Samples contain 1 mM $Rh_2(OAc)_4$ in 80% water/20% ethylene glycol in the presence of 75 mM H_2NOH at pH 3.0 and 6.0.

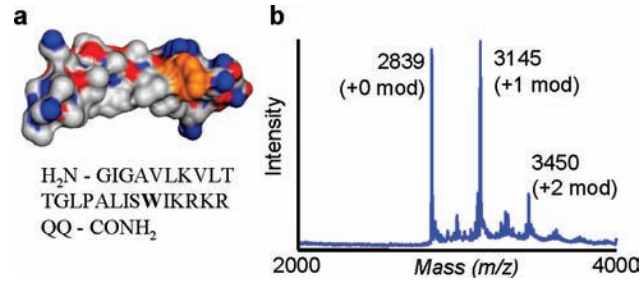

Figure 4. Modification of melittin. (a) Crystal structure of melittin (PDB ID: 2MLT, W in orange) and sequence. (b) Typical MALDI-TOF-MS spectrum for the modification of melittin at pH 6.0 with *t*BuHNOH. Expected masses are *m*/*z* 2846 for unmodified, 3152 for singly modified, and 3458 for doubly modified peptides.

not binding to the catalyst to an appreciable extent (for spectra see Figure S1). Upon increasing the reaction pH to 6.0, the nitrogen of hydroxylamine becomes largely deprotonated, resulting in replacement of the oxygen bound axial ligands with the nitrogen of hydroxylamine. The resulting pink color and UV−vis spectrum is consistent with known complexes of $Rh_2(OAc)_4$ bound to nitrogen-based ligands (Figure 3, S1).[12] This ligand exchange process effectively deactivates the rhodium catalyst and leads to the observed drop in catalytic activity.

In addition to the lack of carbenoid formation at pH ≥ 6, the pH profiles obtained for myoglobin and subtilisin modification indicated that other factors contributed to the failed modification of these substrates at higher pH. For these targets, the pH above which modification fails (pH 2.0 for subtilisin, and pH 4.0 for myoglobin) corresponds to the known pH stability of these proteins, as myoglobin begins to denature below pH 4,[14,15] while, for subtilisin, denaturation occurs below pH 2.[16] The close correlation between the pH stability of these proteins and the pH profiles pictured in Figure 2 suggests that denaturation is necessary for efficient carbenoid addition. This requirement is likely due to the poor solvent accessibility of most tryptophan residues. In the case of myoglobin, the two tryptophan residues (W7 and W14) are buried when the protein is in its native state. This is evident from inspection of the X-ray crystal structure (Figure S2a), as well as the fact that these residues are resistant to derivatization with Koshland's reagent in the absence of denaturant.[17,18] The single tryptophan of subtilisin (W113), though considered to be an example of a solvent exposed tryptophan, maintains close contact with the protein surface, and thus the indole side chain may not extend far enough into solution to allow reaction with the sterically congested carbenoid carbon (Figures S2b, S3).[19,20] The sensitivity of rhodium carbenoids to the steric constraints of incoming nucleophiles has been documented in the literature and is due in large part to the "ligand wall" created by the carboxylate ligands of the rhodium dimer.[21] In addition, recent computational work suggests that the ester of the intermediate carbenoid is twisted 90° out of plane from the rest of the diazo fragment, potentially causing additional steric interactions with incoming substrates.[22] These steric factors, as well as the preference for hydrophobic indole groups to reside on the interior of most proteins, are the likely explanation for why protein denaturation, and a corresponding increase in tryptophan accessibility, is required for successful labeling with rhodium carbenoids.

Identification of *tert*-BuNHOH for Tryptophan Modification at pH 6.0. Recognizing that H_2NOH was ineffective at generating rhodium carbenoids at pH ≥ 6, we set out to identify alternate reaction conditions that could both generate the desired carbenoids and modify tryptophan residues at mild pH. For these studies, we recognized that most protein substrates would be uninformative as the target tryptophan residues would be inaccessible under nondenaturing conditions. To avoid this complication, we elected to use a peptide as a model substrate. Melittin is a 26 amino acid peptide that is a major component of honey bee venom and contains a single tryptophan that is known to be highly solvent accessible (Figure 4a).[23] Using melittin, we screened a number of common buffers, as well as solution additives structurally similar to H_2NOH, in an effort to identify conditions that resulted in melittin modification at pH 6.0 (eq 1, Table 1). From these studies, *t*BuNHOH was found to be highly effective at promoting carbenoid addition, generating over 40% of a singly modified peptide with small amounts of double modification as indicated by MALDI-TOF-MS (Figure 4b). It should be noted that the use of pure water as a reaction solvent proved unsuccessful in these studies, indicating that buffers or solution additives were required for melittin modification. Following this lead, *N*-phenyl and *N*,*N*-dialkyl hydroxylamines were also screened, but these additives led to catalyst decomposition and other unpredictable reaction behaviors.

$$\text{Melittin} \xrightarrow[\text{75 mM buffer (pH 6.0)}]{\text{20 mM } \mathbf{1},\ Rh_2(OAc)_4} \text{Melittin Conjugates} \quad (1)$$

Using *t*BuNHOH, we were able to modify melittin over a pH range that was significantly wider than the successful range with H_2NOH. As depicted in Figure 5, comparable levels of melittin modification were observed from pH 3−6 with *t*BuNHOH, with lower levels at pH > 7. In contrast, reactions containing H_2NOH failed above pH 5, as would be expected by the small molecule experiments described above. Of particular significance were the results at pH 6.0, where

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Table 1. Modification of Melittin in the Presence of Various Solution Additives[a]

Buffer/Additive	+0 mod (%)	+1 mod (%)	+2 mod (%)
—	94	6	0
NaCl	95	5	0
phosphate	90	10	0
borate	92	5	3
TRIS	73	21	6
triethanolamine	96	4	0
HEPES	92	8	0
H_2NOH	96	4	0
*t*BuNHOH	49	43	8
NH_4Cl	91	8	1
Me_3NO	90	9	1
AcNHOH	97	3	0
$H_2NOCH_2CO_2H$	81	17	2
H_2NNH_2	75	22	3

[a] Conditions: 100 μM melittin, 100 μM $Rh_2(OAc)_4$, 20 mM **1**, 2% *t*BuOH (v/v), RT, 24 h. Product ratios estimated by MALDI-TOF-MS. Values represent the average of three independent MALDI-TOF-MS analyses of the same sample.

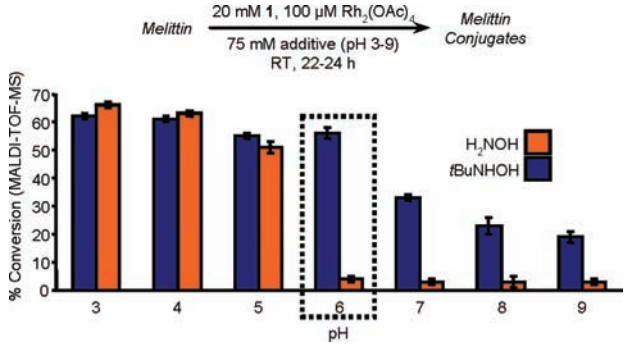

Figure 5. Use of *t*BuNHOH substantially improved the pH range over which successful modification of melittin could be achieved. Note: Percent conversion was estimated by MALDI-TOF-MS (sinapinic acid matrix), and values represent the average of three independent analyses of the same sample (standard deviation indicated by error bars). Conversion values represent the sum total of all modified species.

*t*BuNHOH was found to promote efficient melittin modification, while H_2NOH was completely ineffective.

With regard to the site selectivity of the reaction at pH 6.0, the observation of a doubly modified melittin species suggested the possibility that additional residues besides tryptophan were modified as melittin contains only a single tryptophan residue (W19). In small molecule studies with 3-methylindole, it was observed that multiple additions could be achieved under analogous reaction conditions. LC-ESI-MS analysis revealed that adducts containing as many as three covalent modifications could be obtained (albeit in minute amounts) using a large excess of diazo compound (see Figure S4), although this species has not been observed on peptide and protein substrates. In the case of melittin, ESI-MS/MS analysis of the doubly modified product ultimately indicated that both modifications were indeed confined to W19 (Figure 6). Both modifications were restricted to daughter ions containing W19 and were clearly absent from fragments lacking this residue. For example, a doubly modified y″-ion containing residues 19−26 ($m/z = 1754.0$) was observed, while the y″-ion corresponding to residues 20−26 (lacking W19) possessed zero modifications ($m/z = 955.6$). No fragments were observed that suggested the modification of any other residue. While the modification itself was found to be relatively labile under collisional activation conditions, the use of collision energies of 15 V or less was sufficient to fragment the peptide with minimal loss of the modification.

The detailed structure of the double addition product remains unknown, largely due to the fact that it is a minor reaction product that is difficult to isolate. Based on our previous small molecule studies, we speculate that N−H insertion and alkylation at the 2-position of the tryptophan indole ring remain the major reaction pathways.[5] Acquisition of both types of modifications on a single tryptophan residue could account for the observed doubly modified melittin product. We are also exploring other reaction possibilities, including the addition of carbenoids to the double bond in the reaction product itself, or oligomerization of the diazo compound prior to tryptophan modification. MS/MS analysis of singly modified melittin was also performed using MALDI-TOF/TOF (Figure S7) and was consistent with modification at W19.

Given the structural similarity between H_2NOH and *t*BuNHOH, it is tempting to hypothesize that a specific and analogous interaction between these compounds and the rhodium catalyst is responsible for the beneficial effects that are observed. Though rare, the use of solution additives and secondary ligands in reactions catalyzed by dirhodium tetracarboxylate dimers have been documented in the literature.[24−26] However, in the case of H_2NOH at pH ~3.5, a specific interaction between hydroxylamine and $Rh_2(OAc)_4$ does not appear to be necessary for the modification of myoglobin with rhodium carbenoids (Figure 7a,b). Rather, the generation of low pH by this HCl salt appears to be the major role for this additive. This is supported by the observation that phosphate buffer at pH 3.5 produced comparable levels of myoglobin modification despite the absence of H_2NOH (Figure 7b).

In contrast, the ability of *t*BuNHOH to promote the modification of melittin at pH 6.0 appears to be unique to this additive (Figure 7c). Phosphate buffer (Figure 7d) or other additives (see Table 1) all yielded significantly lower levels of melittin modification. An important role for *t*BuNHOH appears to be its ability to promote efficient carbenoid formation over a much wider pH range than was attainable using H_2NOH. As depicted in Figure S5, pH profiles measuring the catalytic degradation of **1** in the presence of H_2NOH and *t*BuNHOH showed that while H_2NOH fails above pH 5.0, *t*BuNHOH was able to promote carbenoid formation beyond pH 7.0. Notably, these results suggest that the nitrogen of *t*BuNHOH does not inactivate the rhodium catalyst at higher pH. While the precise mode of action for *t*BuNHOH remains unclear, we speculate that a specific interaction between this additive and $Rh_2(OAc)_4$ is responsible for the substantial increase in catalytic activity. Currently, we propose that the oxygen of *t*BuNHOH binds to $Rh_2(OAc)_4$ rather than the nitrogen, the latter being disfavored by the bulky *tert*-butyl substituent (Figure 8). We have also determined that the pK_a values for *t*BuNHOH and H_2NOH are comparable (Figure S6), ruling out the possibility that significant differences in basicity are responsible for the dramatic differences in reactivity observed for these additives above pH 5.

Modification of Lysozyme under Thermally Denaturing Conditions at pH 6.0. Having demonstrated successful tryptophan

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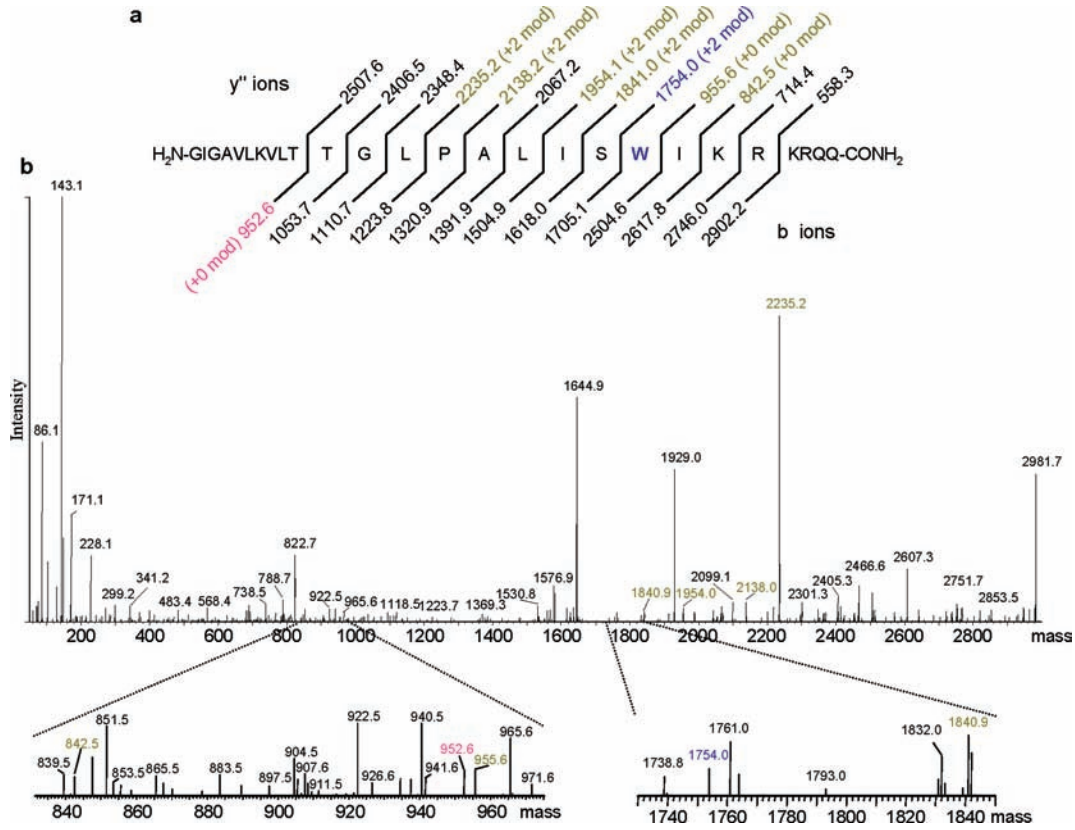

Figure 6. MS/MS analysis of doubly modified species. (a) Melittin sequence and expected y" and b ions assuming double modification of tryptophan. (b) MS/MS spectrum of the $(M+5H)^{5+}$ ion of the mass 3450 Da. Multiply charged ions have been transformed onto a singly charged *x*-axis to simplify spectral interpretation.

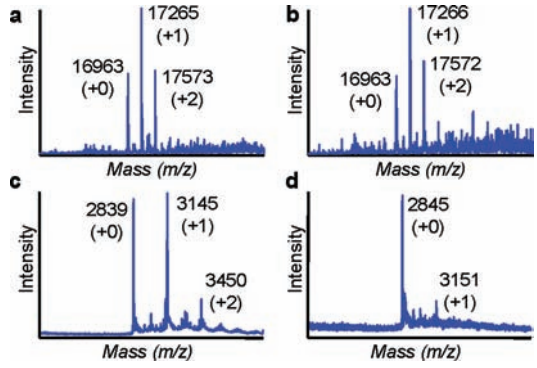

Figure 7. Effect of buffer/additive identity on the modification of myoglobin and melittin. Modification of horse heart myoglobin in the presence of (a) 75 mM $H_2NOH \cdot HCl$ (pH ~3.5) and (b) 75 mM phosphate (pH 3.5). Modification of melittin in the presence of (c) 75 mM *t*BuNHOH (pH 6.0) and (d) 75 mM phosphate (pH 6.0). Note: (a) and (b) are ESI-MS spectra, while (c) and (d) are MALDI-TOF-MS spectra.

modification at pH 6.0 on a peptide model, we sought to apply these conditions to the modification of a protein substrate. As discussed above, this was problematic due to the limited accessibility of most tryptophan residues under nondenaturing conditions. Thus, as an initial demonstration of the utility of *t*BuNHOH, we pursued the modification of a protein at pH 6.0 where denaturation could be induced with temperature rather than extreme pH. To this end, hen egg white lysozyme, which contains six tryptophans, was chosen as a target (Figure S8). Attempts to modify lysozyme in the presence of *t*BuNHOH at pH 6.0 with moderate temperatures were unsuccessful. However, upon heating the reaction to temperatures ≥75 °C, a sudden

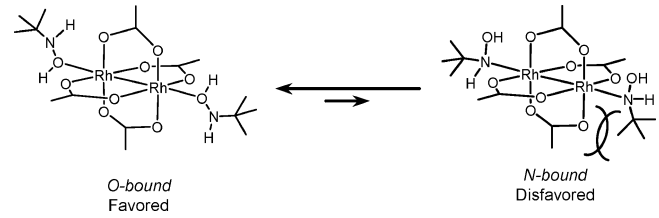

Figure 8. Proposed binding of *t*BuNHOH with $Rh_2(OAc)_4$ at pH 6.0.

surge in reactivity was observed, generating lysozyme conjugates (Figure 9a). Furthermore, the temperature dependence of lysozyme modification closely coincided with the known thermal stability of this protein (Figure 9b). Specifically, lysozyme is known to denature above 74 °C at pH 7, and we only observed modification with rhodium carbenoids at temperatures of 75 °C or higher.[27] Remarkably, control experiments at 85 °C lacking $Rh_2(OAc)_4$ gave no modification, indicating that the diazo compound itself exhibited no background reactivity despite the elevated temperature (Table 2). Moreover, removing *t*BuNHOH or replacing it with H_2NOH or phosphate buffer resulted in significantly lower levels of modification (Table 2).These results demonstrate the usefulness of this reaction as a probe of tryptophan accessibility and could be used to detect conformational changes induced by a variety of factors.

Expression and Modification of Mutant Proteins with Solvent Accessible Tryptophan Residues. While the success achieved with lysozyme at pH 6.0 was encouraging, this

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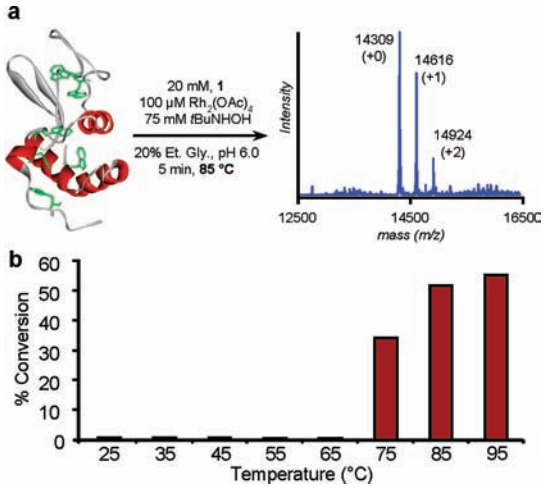

Figure 9. Modification of lysozyme requires thermally denaturing conditions. (a) The six tryptophan side chains are shown in green. A 100 μM solution of lysozyme was modified, and following removal of the small molecules *via* gel filtration, the sample was analyzed by ESI-MS. No modification was observed in the absence of $Rh_2(OAc)_4$ under otherwise identical reaction conditions. (b) Modification of lysozyme using the same conditions as in (a) at various temperatures. Percent conversion is calculated as the combined area of singly and doubly modified peaks as a ratio to the total area for all protein peaks. Conversion was only observed at 75 °C and above.

Table 2. Modification of Lysozyme at 85 °C[a]

Buffer/Additive	+0 mod (%)	+1 mod (%)	+2 mod (%)
*t*BuNHOH	38	53	9
H_2NOH	71	29	trace
none[b]	67	33	0
phosphate	80	20	trace
*t*BuNHOH[c]	100	0	0
*t*BuNHOH[d]	100	0	0

[a] Conditions: 20 mM **1**, 75 mM additive, 100 μM $Rh_2(OAc)_4$, 20% Ethylene Glycol, pH 6.0, 85 °C. [b] pH not adjusted to 6.0. [c] No catalyst added. [d] Reaction run at room temperature.

transformation still required protein denaturation to achieve appreciable levels of modification. Moreover, we realized that tryptophan residues in most protein substrates would be unreactive under nondenaturing conditions due to limited solvent accessibility. However, we hypothesized that solvent accessible tryptophan residues could be introduced by site-directed mutagenesis, followed by selective labeling with rhodium carbenoids. Conceptually, this strategy is very similar to the widely used practice of introducing cysteine residues followed by chemoselective alkylation of the thiol side chain. A similar strategy using tryptophan would therefore represent a much needed complement to this powerful technique, potentially allowing the introduction of two synthetic modifications with exquisite control of site selectivity.

For these studies, human FKBP was identified as a suitable protein substrate. Wild type FKBP contains a single tryptophan residue (W59) located at the base of the rapamycin binding pocket, and we hypothesized that this amino acid would be unavailable for modification under nondenaturing conditions (Figure 10a).[23,28,29] Indeed, wild type FKBP (containing an additional *C*-terminal threonine residue) showed only trace levels

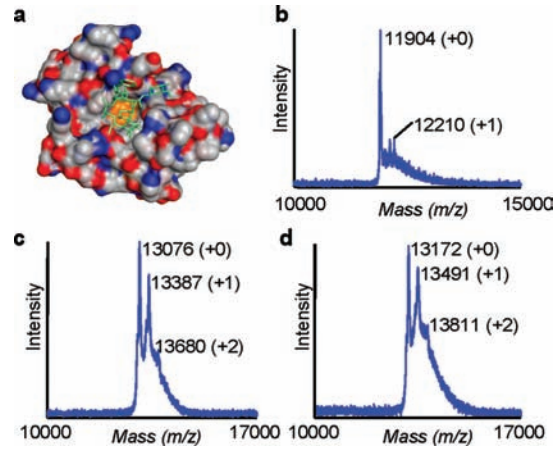

Figure 10. Addition of tryptophan containing "tags" to allow protein modification. (a) Crystal structure of wild type FKBP with rapamycin bound (PDB ID: 1A7X). FKBP contains a single, buried tryptophan residue (in orange) located at the base of the binding pocket. (b) Wild type FKBP (~10 μM) showed only trace levels of modification (expect *m/z* of 11920 for unmodified protein), while (c) lin-FKBP fusion (~90 μM) (expect *m/z* of 13031 for unmodified protein) and (d) mel-FKBP (~30 μM) (expect *m/z* of 13131 for unmodified protein) were modified when treated with 20 mM **1** and 100 μM $Rh_2(OAc)_4$ in 75 mM *t*BuNHOH at pH 6.0 (2% *t*BuOH v/v). The reaction was run for 15 h at RT and analyzed by MALDI-TOF-MS. The expected mass increase for each modification is 306. In both c and d, the total level of modification was estimated to be in excess of 40%.

of modification when treated with **1** and $Rh_2(OAc)_4$ at pH 6.0 in the presence of *t*BuNHOH (Figure 10b).

Having established that wild type FKBP was effectively unreactive toward rhodium carbenoids at pH 6.0, two tryptophan fusion proteins were prepared. To pursue the idea of "reactive tags," we chose two peptide sequences for attachment to the *C*-terminus of FKBP. Given our success with melittin, we chose to incorporate residues 15−23 of this substrate (GALISWIKRKG) into one mutant FKBP construct (designated mel-FKBP). A second fusion protein (designated lin-FKBP) contained a peptide tag (IQKQGQGQWG) that was generated using an online database designed to predict extended, flexible peptide linkers for the construction of fusion proteins.[30] Both FKBP mutants were expressed in *E. coli* as *C*-terminal intein fusions containing a chitin binding domain for affinity purification (Figure S9). Gratifyingly, when exposed to **1** and $Rh_2(OAc)_4$ in the presence of *t*BuNHOH at pH 6.0, both lin-FKBP and mel-FKBP exhibited over 40% modification, as estimated by MALDI-MS analysis (Figure 10c,d). The observed levels of modification for both proteins were far in excess of those observed for wild type FKBP, demonstrating that the reactivity of FKBP to rhodium carbenoids could be induced with the addition of a small peptidic tag.

Conclusion

We have made significant progress in both understanding and improving the tryptophan modification methodology originally reported. We have discovered that the novel solution additive *t*BuNHOH was able to promote the formation of rhodium carbenoids under mild aqueous conditions, and we have applied this chemistry to the selective modification of tryptophan residues in peptides and proteins at pH 6.0. In addition, we have demonstrated that the solvent accessibility of tryptophan residues

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plays a key role in determining the outcome of labeling experiments with rhodium carbenoids. This latter point was manifested in labeling profiles that closely matched the pH and thermal stability of protein substrates and suggests that rhodium carbenoids could serve as probes to detect protein conformational changes (albeit on a relatively slow time scale). Current efforts are directed toward using this information to improve the overall reaction conversion, as well as circumventing the multiple additions of rhodium carbenoids to single indole side chains.

We have also made progress toward developing a general protein labeling strategy based on tryptophan mutagenesis followed by chemoselective modification with rhodium carbenoids. FKBP fusions with tryptophan-containing tags were expressed and shown to be significantly more reactive than the wild type protein. This strategy is conceptually similar to the common practice of introducing cysteine residues followed by chemoselective alkylation of the thiol side chain. Our results suggest that tryptophan could provide a useful complement to the cysteine-based approach as more highly functionalized diazocompounds become available. This would be advantageous for the labeling of proteins that already possess active site cysteine residues or multiple disulfide bonds. Tryptophan modification chemistry could also be used in conjunction with cysteine labeling for the introduction of two different chromophores for FRET studies. Efforts are underway to explore these types of applications and to extend the tryptophan labeling method to additional protein substrates.

Materials and Methods

General Procedures, Materials, and Instrumentation. Unless otherwise noted, all chemicals were obtained from commercial sources and used without further purification. Dichloromethane (CH_2Cl_2) was distilled under a nitrogen atmosphere from calcium hydride. Water (ddH_2O) used in biological procedures or as a reaction solvent was deionized using a NANOpure purification system (Barnstead, USA). Myoglobin (M1882) from horse heart, subtilisin Carlsberg (P5380) from *Bacillus* sp., lysozyme (L6876) from hen egg white, and melittin (M2272) from honey bee venom were purchased from Sigma and used without further purification. $Rh_2(OAc)_4$ was purchased from Strem, and protected amino acids were obtained from Novabiochem (San Diego, CA, USA). Diazo compound **1** was prepared following the procedure outlined previously.[5]

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was performed on a Voyager-DE system (PerSeptive Biosystems, USA). All samples were cocrystallized using a sinapinic acid solution (10 mg/mL in 1:1 MeCN/ddH_2O with 0.1% TFA). Electrospray LC/MS analysis was performed using an API 150EX system (Applied Biosystems, USA) equipped with a Turbospray source and an Agilent 1100 series LC pump. Protein and peptide chromatography was performed using a Phenomenex Jupiter 300 5 μC5 300Å reversed-phase column (2.0 mm × 150 mm) with a MeCN/ddH_2O gradient mobile phase containing 0.1% formic acid (250 μL/min). Protein mass reconstruction was performed on the charge ladder with Analyst software (version 1.3.1, Applied Biosystems). MS/MS analysis for modified melittin was performed on a Waters (Beverly, MA) Q-TOF Premier quadrupole time-of-flight mass spectrometer with an electrospray ionization source. MS/MS analysis for singly modified melittin was performed on a 4700 Proteomics Analyzer (Applied Biosystems, USA) MALDI-TOF/TOF mass spectrometer. The sample was cocrystallized using a CHCA solution (10 mg/mL in 1:1 MeCN/ddH_2O with 0.1% TFA and 2%w/v ammonium citrate). Unless noted, prior to MS analysis, biological samples were desalted and/or separated from small molecule contaminants using μC18 ZipTip pipet tips (Millipore, USA) or NAP-5 gel filtration columns (Amersham Biosciences, USA).

High performance liquid chromatography (HPLC) was performed on an Agilent 1100 Series HPLC System (Agilent Technologies, USA). Analytical chromatography for small molecule studies was achieved using an Agilent ZORBAX Eclipse XDB-C8 reversed-phase column (4.6 mm × 150 mm) with an MeCN/ddH_2O gradient mobile phase containing 0.1% TFA (1 mL/min). Sample analysis for all HPLC experiments was achieved with an inline diode array detector.

For protein analysis, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was accomplished on a Mini-Protean apparatus (Bio-Rad, USA), following the general protocol of Laemmli.[31] Commercially available markers (Bio-Rad, USA) were applied to at least one lane of each gel for calculation of apparent molecular weights. Visualization of protein bands was accomplished by staining with Coomassie Brilliant Blue R-250 (Bio-Rad, USA). Gel imaging was performed on an EpiChem3 Darkroom system (UVP, USA).

UV−vis spectroscopic measurements were conducted on a Tidas-II benchtop spectrophotometer (J & M, Germany) or a CARY 50 Conc UV−visible spectrophotometer (Varian, USA).

Centrifugations were conducted with either an Allegra 64R Tabletop Centrifuge (Beckman Coulter Inc., USA) or a Sorvall RC5C Plus high-speed centrifuge (Sorvall, USA).

pH Profiles for Catalytic Degradation of Diazo Compound 1, Modification of Horse Heart Myoglobin, and Modification of Subtilisin Carlsberg. Buffered solutions containing 115 mM $H_2NOH\cdot HCl$ were prepared in 84.6% ddH_2O/15.4% ethylene glycol and adjusted to the pH values depicted in Figure 2.

For the small molecule studies outlined in Figure 2, 390 μL of the appropriate hydroxylamine buffer (pH 1.5−9.0) were combined with 60.0 μL of a suspension of vinyl diazo compound **1** in ethylene glycol (33.4 mg/mL, 6.00 μmol), 15.0 μL of an aqueous solution of $Rh_2(OAc)_4$ (1.8 mg/mL, 61 nmol), and 135 μL of ddH_2O in a 1.7 mL microcentrifuge tube. The reactions were incubated on a laboratory rotisserie for 23 h at room temperature and then diluted with 66 μL of MeCN. The diluted samples were then analyzed by reversed-phase HPLC (monitoring at 280 nm). The extent of diazo compound degradation was determined by comparing the observed peak area for **1** to a standard sample of **1** that had been incubated for an identical period of time in pH 3.0 hydroxylamine buffer in the absence of $Rh_2(OAc)_4$.

For the horse heart myoglobin labeling experiments outlined in Figure 2, 390 μL of the appropriate hydroxylamine buffer (pH 1.5−9.0) were combined with 60.0 μL of a suspension of vinyl diazo compound **1** in ethylene glycol (33.4 mg/mL, 6.00 μmol), 15.0 μL of an aqueous solution of $Rh_2(OAc)_4$ (1.8 mg/mL, 61 nmol), and 150 μL of an aqueous solution of horse heart myoglobin (6.8 mg/mL, 60 nmol) in a 1.7 mL microcentrifuge tube. The reactions were incubated on a laboratory rotisserie for 7 h at room temperature. 500 μL of the crude reaction solution were then passed through a gel filtration column (NAP-5) pre-equilibrated with three 3 mL portions of 10 mM NaCl (pH 3.0). The extent of myoglobin modification was then estimated by ESI-MS analysis.

For the subtilisin Carlsberg labeling experiments outlined in Figure 2, 390 μL of the appropriate hydroxylamine buffer (pH 1.5−3.5) were combined with 60.0 μL of a suspension of vinyl diazo compound **1** in ethylene glycol (33.4 mg/mL, 6.00 μmol), 15.0 μL of an aqueous solution of $Rh_2(OAc)_4$ (1.8 mg/mL, 61 nmol), and 150 μL of an aqueous solution of subtilisin Carlsberg (10.9 mg/mL, 60 nmol) in a 1.7 mL microcentrifuge tube. The reactions were incubated on a laboratory rotisserie for 6 h at room temperature. 500 μL of the crude reaction solution were then passed through a gel filtration column (NAP-5) pre-equilibrated with three 3 mL portions of ddH_2O. The extent of subtilisin modification was then estimated by MALDI-TOF-MS analysis (sinapinic acid matrix).

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It should be noted that these estimates were qualitative. The highest levels of subtilisin modification were observed at pH 1.5−2.0, and no subtilisin modification was observed at pH 2.5−3.5.

Buffer/Additive Screen for Modification of Melittin. 100 mM aqueous solutions were prepared for each of the buffer salts and solution additives listed in Table 1. The pH of these solutions was then adjusted to 6.0. To a 0.6 mL microcentrifuge tube were added 45.0 μL of the appropriate buffer, 15.0 μL of an aqueous solution of melittin (1.1 mg/mL, 6.0 nmol), 1.5 μL of an aqueous solution of $Rh_2(OAc)_4$ (1.8 mg/mL, 6.1 nmol), and 1.2 μL of a *t*BuOH solution of **1** (334 mg/mL, 1.2 μmol). The reactions were incubated on a laboratory vortexer for 24 h at room temperature. Reactions employing phosphate, borate, HEPES, TRIS, or triethanolamine buffers were then analyzed directly by MALDI-TOF-MS (sinapinic acid matrix). All remaining reactions were diluted with 10 μL of MeCN prior to MALDI-TOF-MS analysis. Note: All reactions were analyzed in triplicate, and the reported product ratios are based on average peak areas.

pH Profile for Modification of Melittin with *t*BuNHOH and H_2NOH. Aqueous solutions containing 100 mM $H_2NOH\cdot HCl$ or 100 mM *t*BuNHOH·HCl were prepared and adjusted to the pH values depicted in Figure 5. To a 0.6 mL microcentrifuge tube were then added 45.0 μL of the appropriate buffer, 15.0 μL of an aqueous solution of melittin (1.1 mg/mL, 6.0 nmol), 1.5 μL of an aqueous solution of $Rh_2(OAc)_4$ (1.8 mg/mL, 6.1 nmol), and 1.2 μL of a *t*BuOH solution of **1** (334 mg/mL, 1.2 μmol). The reactions were incubated on a laboratory vortexer for 22 h (H_2NOH profile) or 24 h (*t*BuNHOH profile) at room temperature. Reactions were then analyzed directly by MALDI-TOF-MS (sinapinic acid matrix). Note: All reactions were analyzed in triplicate, and the reported product ratios are based on average peak areas.

MS/MS Sequencing of Modified Melittin. Melittin was modified as described above with *t*BuHNOH as the additive at pH 6.0. The crude reaction mixture was extracted with ethyl acetate (3 × 0.4 mL) to remove excess small molecules. The resulting solution was diluted by half and then analyzed by LC-MS/MS. The $(M+5H)^{5+}$ ion of the doubly modified peptide was mass selected and subjected to collisional activation. The use of collision energies less than or equal to 15 V resulted in immonium and y- and b-type fragment ions with minimal modification losses. MS/MS spectra were deconvoluted using the MaxEnt-3 algorithm of MassLynx (version 4.1, Waters).

High Temperature Modification of Hen Egg White Lysozyme. To a 0.6 mL microcentrifuge tube were added 330 μL of a 136 mM aqueous solution of *t*BuNHOH·HCl (pH 6.0) or control buffer/additive and 150 μL of an aqueous solution of hen egg white lysozyme (5.7 mg/mL, 60 nmol). These solutions were then heated for 10 min at the desired reaction temperature (25−95 °C). A 15.0 μL portion of an aqueous solution of $Rh_2(OAc)_4$ (1.8 mg/mL, 61 nmol) and 120 μL of a suspension of vinyl diazo compound **1** in ethylene glycol (33.4 mg/mL, 12.0 μmol) were then added, and the reactions were incubated at the desired temperature for the following amounts of time: 17 h (25−45 °C), 1.5 h (55 °C), 30 min (65 °C), and 5 min (75−95 °C). The disappearance of the characteristic orange color of **1** was used to determine the reaction end point. 500 μL of each crude reaction solution was then passed through a gel filtration column (NAP-5) pre-equilibrated with three 3 mL portions of ddH_2O. The purified protein samples were then analyzed by ESI-MS.

Construction of FKBP-lin-pTXB1 and FKBP-mel-pTXB1 Plasmids. The template plasmid for these constructs, consisting of the human FKBP gene with an additional *C*-terminal threonine residue ligated into pTXB1 (New England Biolabs, USA) as a *C*-terminal intein/chitin binding domain fusion, was a generous gift from Dante Romanini. The human FKBP gene was amplified out of the FKBP-pTXB1 plasmid described above. The following primers were used to install an *N*-terminal Nde I restriction site and a *C*-terminal Sap I restriction site:

(*FKBP-lin-pTXB1*):5′-CCAGAGCATATGGGAGTGCAGGTG-GAAACCAT-3′

5′-GGTGGTTGCTCTTCCGCAGCCCCACTGGCCCTGGCCC-TGTTTCTGGATGGTTTCCAGTTT-TAGAAGCTC-3′

(*FKBP-mel-pTXB1*):5′-CCAGAGCATATGGGAGTGCAGGTG-GAAACCAT-3′

5′-GGTGGTTGCTCTTCCGCAGCCTTTGCGTTTGATCCAGG-AGATCAGAGCGCCGGTTTCCAG-TTTTAGAAGCTC-3′

The gel purified PCR products were then digested with Nde I and Sap I and ligated into the pTXB1 vector. The identities of the ligation products were confirmed by sequencing.

Expression and Purification of FKBP Mutants. Mutant FKBP plasmids were transformed into *E. coli* strain ER2566 (New England Biolabs, USA) via electroporation with a Micro-Pulser (BioRad, USA) and then plated onto LB agarose plates (ampicillin 100 μg/mL). Colonies were selected and grown in 1 L of sterile LB containing ampicillin (100 μg/mL) at 37 °C until an optical density of 0.5−0.8 was observed at 550 nm. Protein expression was then induced by the addition of isopropyl-β-D-thiogalactopyranoside (0.1 mM final concentration). The cultures were then incubated for 6−7 h at 25 °C. The cells were then spun down at 8500 rcf for 15 min at 4 °C, and the supernatant was discarded. The remaining cell pellet was resuspended in ∼30 mL of 20 mM HEPES containing 500 mM NaCl and 0.1 mM EDTA at pH 8.0 and stored at −20 °C overnight. The cell suspension was then thawed, and the cells were further lysed by sonication with a Branson Digital Sonifier (VWR Scientific, USA) for 2 min with a blunt end tip. Cellular debris was removed by centrifugation at 8500 rcf for 15 min at 4 °C. Mutant FKBP was purified from the supernatant by chitin affinity chromatography. An affinity column was prepared by loading a 25 mL fritted polypropylene tube (Econo-Pac columns, Biorad, USA) with 5−7 mL of Chitin Beads (New England Biolabs, USA). The crude cell lysate was then passed through the column using gravity flow. The resin bound protein was then washed with 10 column volumes of 20 mM HEPES containing 500 mM NaCl and 0.1 mM EDTA at pH 8.0 (wash buffer). The column was then rapidly flushed with three column volumes of wash buffer containing 30 mM sodium 2-mercaptoethanesulfonate (MESNA). The ends of the column were then sealed, and the resin was incubated at 4 °C for at least 17 h. Purified mutant FKBP was eluted using three column volumes of wash buffer. After confirming the purity of the eluted protein by SDS-PAGE, the material was concentrated to ∼1 mL using Amicon Ultra 15 and 4 mL centrifugal filter devices (5000 MWCO). The concentrated material was then passed through a gel filtration column (NAP-10 or NAP-5) pre-equilibrated with 3 column volumes of ddH_2O. Expression yields were calculated by absorbance at 280 nm using extinction coefficients estimated by the technique of Gill and von Hippel.[32] The following yields were obtained (per liter of culture): mel-FKBP 0.7 mg, lin-FKBP 0.1 mg.

Modification of FKBP Mutants. Mutant FKBP proteins were modified in the presence of 75 mM *t*BuNHOH at pH 6.0 using **1** and $Rh_2(OAc)_4$ following protocols analogous to those employed in the modification of melittin. Any minor differences in reaction conditions (reaction times, concentration of **1**, etc.) are indicated in the main text. All reactions were analyzed directly by MALDI-TOF-MS (sinapinic acid matrix).

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

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