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Heme Degradation by *Staphylococcus aureus* IsdG and IsdI Liberates Formaldehyde Rather Than Carbon Monoxide

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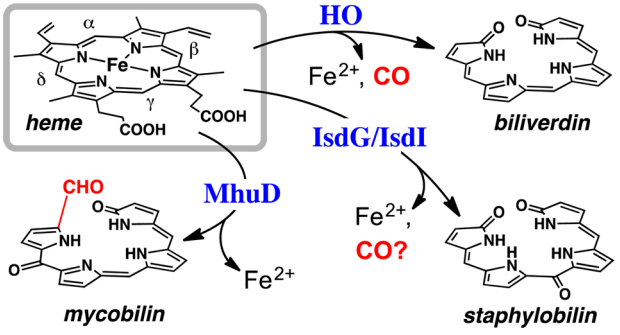
Supporting Information

ABSTRACT: IsdG and IsdI from *Staphylococcus aureus* are novel heme-degrading enzymes containing unusually nonplanar (ruffled) heme. While canonical heme-degrading enzymes, heme oxygenases, catalyze heme degradation coupled with the release of CO, in this study we demonstrate that the primary C1 product of the *S. aureus* enzymes is formaldehyde. This finding clearly reveals that both IsdG and IsdI degrade heme by an unusual mechanism distinct from the well-characterized heme oxygenase mechanism as recently proposed for MhuD from *Mycobacterium tuberculosis*. We conclude that heme ruffling is critical for the drastic mechanistic change for these novel bacterial enzymes.

Heme degradation in pathogenic bacteria is crucial for the acquisition of iron, an essential nutrient required for its survival and infection, from host heme molecules.[1−3] An enzyme family termed heme oxygenase (HO) is known to degrade heme into iron and biliverdin with the release of a *meso* carbon atom as CO (Scheme 1).[4−7] HO catalysis proceeds through three successive mono-oxygenation reactions in which the substrate heme activates oxygen molecules for a series of self-oxidations. Until recently, the HO family was thought to be the only family of heme-degrading enzymes; however, a novel family of heme-degrading enzymes has been identified in *Staphylococcus aureus*.[8] IsdG and its paralogue, IsdI, are heme-degrading enzymes of *S. aureus* composed of a heme uptake system called the Isd system.[8] IsdG-type proteins have distinct structures compared to the HO family enzymes.[9,10] In particular, heme bound to the IsdG-type proteins shows unusual nonplanarity, best described as ruffled. This heme ruffling is expected to significantly modulate the O_2 activation chemistry on the heme molecule, and in fact, a novel tetrapyrrole, staphylobilin, with an additional oxidation at a *meso* carbon is produced (Scheme 1).[10] Nevertheless, the unique reaction mechanism of the IsdG-type enzymes remains unclear.

Scheme 1. Heme Degradation Products of the HO- and IsdG-Type Enzymes[a]



[a]Substituents of the products were omitted for the sake of clarity.

We have recently found that MhuD, an IsdG-type enzyme from *Mycobacterium tuberculosis*, catalyzes heme degradation without generating CO (Scheme 1).[11] The carbon atom at the site of ring cleavage is not released but retained as an aldehyde group, resulting in formation of a novel chromophore termed mycobilin. This unprecedented observation indicates a unique reaction mechanism of MhuD that is distinct from the well-understood mechanism of canonical HO. We have attributed the drastic change in mechanism to the heme ruffling as observed in the *S. aureus* IsdG and IsdI heme-bound structures. The *S. aureus* enzymes, however, have been thought to generate CO despite the lack of any experimental evidence.[10] This assumption is based on the structural similarities of staphylobilin and biliverdin at their ring cleavage sites and removal of the *meso* carbon (Scheme 1). To this end, we have experimentally determined the C1 products of *S. aureus* IsdG and IsdI for the first time.

Single-turnover heme degradation reactions by IsdG and IsdI as well as MhuD and rat HO-1 were conducted using ascorbate as an electron donor. High-performance liquid chromatography (HPLC) and mass spectrometry (MS) analyses confirm that the four enzymes afford their characteristic tetrapyrrole products (Figure S1 of the Supporting Information). The amount of CO produced was determined by trapping CO by a

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H64L variant of myoglobin (Mb) that has an extremely high CO affinity.[12] Figure 1 shows the Soret region difference

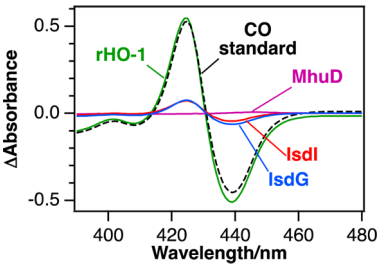

Figure 1. CO quantification as difference spectra of H64L Mb upon formation of its ferrous CO form. Spectral changes in ferrous H64L Mb were calculated from the spectra taken before and after the heme degradation by 5 μM heme complexes of rat HO-1 (green), MhuD (purple), IsdG (blue), and IsdI (red). The black dashed line represents the difference spectrum of 5 μM ferrous H64L Mb upon saturation with CO.

spectra of the ferrous H64L Mb induced by CO generated by single-turnover heme degradation. As reported previously,[11] while the reaction solution of rHO-1 exhibited a significant difference spectrum, essentially no spectral change was observed for MhuD. The CO yields summarized in Table 1

Table 1. Yields of C1 Products of Heme Degradation[a]

enzyme	CO[b]	HCHO[c]	HCOOH[c]
rHO-1	106 ± 4	4 ± 2	1 ± 1
MhuD	1 ± 1	4 ± 1	5 ± 1
IsdG	14 ± 2	82 ± 1	16 ± 1
IsdI	14 ± 1	81 ± 5	15 ± 1

[a]Yields are given in percent. [b]n = 2. [c]n = 3.

show almost stoichiometric formation of CO in the HO-1 reaction and its absence in the MhuD reaction. IsdG and IsdI exhibited the CO-induced difference spectra, but their low intensities indicate low yields of CO [~14% (Figure 1 and Table 1)]. This finding clearly demonstrates that CO is not the major C1 product of the *S. aureus* enzymes.

Because staphylobilin actually lacks one *meso* carbon atom, IsdG and IsdI should afford alternative C1 products. The aldehyde group retained in mycobilin compared to staphylobilin (Scheme 1) suggests formation of formaldehyde (HCHO) and/or its oxidized form, formic acid (HCOOH), by the *S. aureus* enzymes. Formaldehyde in the reaction solution was converted by an acetylacetone method to 3,5-diacetyl-1,4-dihydrolutidine,[13] which was quantitated by HPLC analysis (Figure 2A and Table 1). While rHO-1 and MhuD afford a negligible amount of formaldehyde, the prominent signals observed for IsdG and IsdI reveal high yields of formaldehyde in their reactions (~80%). Quantitation of formic acid was based on its enzymatic oxidation.[14] Formic acid dehydrogenase consumes formic acid to generate an equimolar amount of NADH. HPLC quantitation of NADH (Figure 2B and Table 1) indicates negligible (rHO-1 and MhuD) or very low (IsdG and IsdI) yields of formic acid. These observations firmly identify formaldehyde as the primary C1 product of both IsdG and IsdI.

This study shows that *S. aureus* IsdG and IsdI degrade heme by an unusual mechanism in which one *meso* carbon atom of the porphyrin ring is liberated primarily as formaldehyde

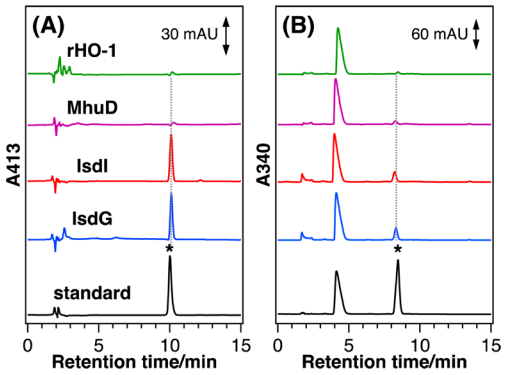

Figure 2. HPLC chromatograms for quantitation of C1 products. (A) Formaldehyde as 3,5-diacetyl-1,4-dihydrolutidine and (B) formic acid as NADH produced by rat HO-1 (green), MhuD (purple), IsdG (blue), and IsdI (red). Product signals are marked with asterisks. Concentrations of enzymes and standards were 20 μM (formaldehyde) and 50 μM (formic acid).

(Table 1). While canonical HO releases the *meso* carbon as CO,[6,7] only small amounts of CO are produced by the *S. aureus* enzymes. The absence of CO production is first reported for heme degradation by MhuD, which retains the *meso* carbon at the cleavage site as the aldehyde group (Scheme 1).[11] The similar aldehyde formation by IsdG and IsdI is suggestive of their mechanistic similarity to MhuD catalysis. The low CO yields indicate that the three IsdG-type enzyme reactions do not involve verdoheme, a key intermediate of ring cleavage by HO (Scheme S1 of the Supporting Information). Among three successive oxygenations in canonical HO catalysis, CO is generated at the second step, *meso*-hydroxyheme to verdoheme.[6,7] Hydroxyheme is highly reactive with O_2, releasing CO, because of its radical character,[6,15] and this rapid autoxidation does not require the assistance by the HO enzyme. Thus, the unique mechanisms of the three IsdG-type enzymes appear to be induced by drastic changes in the intrinsic reactivity of hydroxyheme (or its equivalent). Because the most remarkable structural feature common to hemes in MhuD, IsdG, and IsdI is their severe nonplanarity,[9−11] we propose that heme ruffling is critical to suppressing CO production and promoting aldehyde formation. The ruffling can modulate reactivity of hydroxyheme through the large steric distortion and/or by changing its electronic configuration.[16] The major difference between the MhuD reaction and the IsdG and IsdI reaction is the fate of the aldehyde group, retention in the tetrapyrrole by MhuD or release as formaldehyde by the *S. aureus* enzymes (Scheme S1 of the Supporting Information). While deformylation is known to be mediated by a peroxo−heme complex, such a nucleophilic deformylation normally liberates formic acid,[17] which is a minor product of IsdG and IsdI (Table 1).

Great attention should also be paid to the biological significance of the unusual products of the IsdG-type enzymes. Considering the common absence of CO from the *M. tuberculosis* and *S. aureus* enzymes, the most probable scenario is that microbes employing the IsdG-type enzymes do not favor endogenous production of this toxic gas. Exogenous CO is shown to activate the dormancy regulon of *M. tuberculosis* at biologically relevant concentrations[18] and to have a significant bactericidal effect on *S. aureus* at higher concentrations.[19] Moreover, the unique linear tetrapyrroles and formaldehyde may also have some biological functions.

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In conclusion, this study shows the unusual formaldehyde formation in heme degradation by *S. aureus* IsdG and IsdI probably due to heme ruffling. It has been postulated that heme ruffling in the IsdG-type enzymes enhances its reactivity while controlling the regioselectivity.[10,16] Our results suggest that heme ruffling opens a new path for its degradation.

■ ASSOCIATED CONTENT

Supporting Information

Experimental procedures, Figure S1, and Scheme S1. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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