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Aspects of *Rhodobacter sphaeroides* ChrR required for stimuli to promote dissociation of σ^E /ChrR complexes

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Abstract

In the photosynthetic bacterium *Rhodobacter sphaeroides*, a transcriptional response to the reactive oxygen species singlet oxygen (1O_2) is mediated by ChrR, a zinc metalloprotein that binds to and inhibits activity of the alternative sigma factor, σ^E . We provide evidence that 1O_2 promotes dissociation of σ^E from ChrR to activate transcription *in vivo*. To identify what is required for 1O_2 to promote dissociation of σ^E /ChrR complexes, we analyzed the *in vivo* properties of variant ChrR proteins with amino acid changes in conserved residues of the C-terminal cupin-like domain (ChrR-CLD). We found that 1O_2 was unable to promote detectable dissociation of σ^E /ChrR complexes when the ChrR-CLD zinc ligands (His¹⁴¹, His¹⁴³, Glu¹⁴⁷, and His¹⁷⁷) were substituted with alanine, even though individual substitutions caused a 2- to 10-fold decrease in zinc affinity for this domain relative to that of wild-type ChrR ($K_d \sim 4.6 \times 10^{-10}$ M). We conclude that the side chains of these invariant residues play a crucial role in the response to 1O_2 . Additionally, we found that cells containing variant ChrR proteins with single amino acid substitutions at Cys¹⁸⁷ or Cys¹⁸⁹ exhibited σ^E activity similar to those containing wild-type ChrR when exposed to 1O_2 , suggesting that these thiol side chains are not required for 1O_2 to induce σ^E activity *in vivo*. Finally, we found that the same aspects of *R. sphaeroides* ChrR needed for a response to 1O_2 are required for dissociation of σ^E /ChrR in the presence of the organic hydroperoxide, *tert*-butyl hydroperoxide (t-BOOH).

Keywords

Bacterial signal transduction; metalloproteins; Oxidative stress; Reactive oxygen species (ROS); zinc

Introduction

Oxygen (O_2) has several crucial roles in biological systems. O_2 can function as a terminal electron acceptor for bioenergetic pathways like aerobic respiration and serve as a signal to control gene expression^{1–3}. While O_2 is relatively inert, it can be converted to reactive oxygen species by either electron addition (superoxide, hydrogen peroxide, hydroxyl radical) or energy transfer (singlet oxygen) reactions¹. Considerable information is available on the role of metalloproteins in the responses to superoxide, hydrogen peroxide,

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hydroxyl radicals, or organic hydroperoxides ^{4–6}. However, much less is known about how singlet oxygen (¹O₂) damages proteins or cells ^{7–9}. We are interested in determining how biological systems are altered by and respond to ¹O₂.

This reactive oxygen species has broad relevance since several biological processes generate ¹O₂ ¹⁰. Photosynthetic organisms, in particular, produce significant amounts of ¹O₂ as a byproduct of solar energy capture ¹¹. Our studies capitalize on what is known about a transcriptional response to ¹O₂ in the photosynthetic bacterium *Rhodobacter sphaeroides*. In this bacterium, a transcriptional response to ¹O₂ ¹² is controlled by the alternative sigma factor, σ^E , and the zinc metalloprotein ChrR. In the absence of ¹O₂, ChrR forms a 1:1 heterodimeric complex with σ^E , preventing the σ factor from binding core RNA polymerase and initiating transcription of target genes ^{13–14}. It has been proposed that the functions of this sigma factor and its metalloprotein regulator in controlling a transcriptional response to ¹O₂ have been conserved through evolution, as homologues of *R. sphaeroides* ChrR and σ^E , as well as some target genes of σ^E , are distributed across the bacterial phylogeny ¹⁵. Indeed, there is emerging evidence that prokaryotes and eukaryotes activate transcription of genes encoding similar functions to *R. sphaeroides* σ^E target genes after exposure to ¹O₂ ¹⁶.

Molecular insight into how ChrR inhibits σ^E activity was obtained from the 3-dimensional structure of the σ^E /ChrR complex ¹⁷. In the σ^E /ChrR complex, the ChrR N-terminal domain coordinates a zinc atom and makes extensive contacts with regions of σ^E predicted to bind either RNA polymerase or promoter DNA. As predicted by the structure, a truncated ChrR protein comprised of only the N-terminal domain (ChrR85) was able to inhibit σ^E activity *in vivo* and *in vitro*. Consequently, the ChrR N-terminal domain was termed the anti- σ domain (ChrR-ASD) ¹⁷. The ChrR N-terminus contains a zinc atom that is essential for anti- σ factor activity, as substitutions to any of three amino acid residues coordinating this zinc (His⁶, Cys³⁵, or Cys³⁸) render it unable to form a complex with and inhibit the activity of σ^E ¹⁷. The ChrR C-terminal domain structurally resembles the cupins, a diverse protein family containing a conserved β -barrel fold ^{18–19}, so it has been called the ChrR cupin-like domain (ChrR-CLD). The ChrR-CLD can also bind a zinc atom via the side chains of His¹⁴¹, His¹⁴³, Glu¹⁴⁷, and His¹⁷⁷ ¹⁷. The affinity of the two zinc atoms within ChrR is apparently different, since dialysis in the presence of the zinc chelator DTT only removes the metal from the ChrR-CLD ¹⁷. Two additional relevant observations were made when function of σ^E /ChrR complexes lacking the ChrR-CLD was tested *in vivo* ¹⁷. First, loss of the ChrR-CLD does not prevent the ChrR-ASD from inhibiting σ^E activity. Second, loss of the ChrR-CLD blocked ¹O₂ from initiating the σ^E -dependent transcriptional response ¹⁷, suggesting that this domain contains one or more elements necessary for ¹O₂ to promote this response.

This work analyzed how the presence of ¹O₂ triggers this σ^E -dependent transcriptional response. We found that *R. sphaeroides* can initiate the σ^E -dependent transcriptional response to ¹O₂ in the absence of *de novo* protein synthesis, suggesting that this reactive oxygen species somehow promotes dissociation of σ^E /ChrR complexes rather than using newly synthesized σ^E to control the response. Based on this finding and previous observations that the ChrR-CLD is needed for ¹O₂ to stimulate the σ^E -dependent transcriptional response ¹⁷, we probed the ability of this reactive oxygen species to promote dissociation of σ^E /ChrR complexes with single amino acid substitutions at invariant residues within the ChrR-CLD. We found that alanine substitutions at amino acid ligands to zinc in the ChrR-CLD prevented ¹O₂-mediated dissociation of σ^E /ChrR complexes, even though these variant σ^E /ChrR complexes retained the ability to bind zinc. While some amino acid substitutions at conserved cysteine residues in the ChrR-CLD caused a partial defect in inhibiting σ^E activity or exerted an effect on the affinity for zinc by this domain (though located over 12 Å away from the zinc site), they did not prevent cells from activating σ^E expression during exposure to ¹O₂. Our results do not support previous models that zinc

binding by the ChrR-CLD is necessary for $^1\text{O}_2$ to promote dissociation of σ^E /ChrR complexes¹⁷. We also present evidence that the organic hydroperoxide *tert*-butyl hydroperoxide (t-BOOH), but not cadmium, induces σ^E activity in *R. sphaeroides*. Thus, the *R. sphaeroides* σ^E /ChrR system has similarities to and differences from the homologous *Caulobacter crescentus* σ^E /ChrR system²⁰.

Results

$^1\text{O}_2$ promotes dissociation of σ^E /ChrR complexes

We sought to distinguish between two mechanisms by which the presence of $^1\text{O}_2$ could initiate the σ^E -dependent transcriptional response. $^1\text{O}_2$ could initiate the σ^E -dependent transcriptional response by promoting dissociation of σ^E /ChrR complexes. Alternatively, $^1\text{O}_2$ could activate this transcriptional response by preventing newly synthesized ChrR from binding σ^E . To distinguish between these possibilities, the effect of $^1\text{O}_2$ on the σ^E -dependent transcriptional response was analyzed in cells treated with the protein synthesis inhibitor chloramphenicol (Cm). We reasoned that σ^E -dependent transcription would increase in Cm-treated cells if $^1\text{O}_2$ promoted dissociation of existing σ^E /ChrR complexes but not if *de novo* synthesized σ^E was required for this response. Results of control experiments indicated that adding 200 $\mu\text{g/ml}$ Cm to exponentially growing cells for 15 min was 95% efficient at inhibiting protein synthesis relative to untreated cultures (data not shown).

We used qRT-PCR to monitor RNA levels from two known σ^E target genes: *rpoE*, the structural gene for σ^E , and RSP1409, annotated to encode a fasciclin-like protein of unknown function. Transcript levels from a control gene *rpoZ* (encoding the Ω subunit of RNA polymerase) that lacks a σ^E promoter and is not a known member of this $^1\text{O}_2$ transcriptional response were measured to normalize transcriptional activity. As expected, *rpoZ* transcript levels did not change significantly in the absence or presence of $^1\text{O}_2$ and/or Cm (data not shown). In the absence of $^1\text{O}_2$, we detected a low steady-state level of *rpoE* and RSP1409 transcripts, as expected^{12–14, 17}. When cells were exposed to $^1\text{O}_2$ in the absence of Cm, we observed an expected increase in abundance of these σ^E -dependent transcripts¹². After normalization to *rpoZ* transcript levels, there was a significant increase in the abundance of both *rpoE* (15-fold) and RSP1409 (32-fold) transcripts after 5 min of $^1\text{O}_2$ exposure; the abundance of these transcripts either remained high or increased further after 15 min of continued $^1\text{O}_2$ exposure (Figure 1). Similarly, in Cm-treated cells, the normalized levels of the *rpoE* and RSP1409 transcripts increased after 5 min of $^1\text{O}_2$ exposure (12-fold and 25-fold, respectively), and remained high throughout this experiment (Figure 1). These results indicate that $^1\text{O}_2$ can initiate the σ^E -dependent transcriptional response when protein synthesis is inhibited by the addition of Cm. Since new protein synthesis is not required for $^1\text{O}_2$ to initiate the σ^E -dependent transcriptional response, we conclude that this reactive oxygen species promotes dissociation of σ^E /ChrR complexes, presumably by somehow inactivating extant ChrR.

Single substitutions at conserved amino acids in the ChrR-CLD do not prevent ChrR from inhibiting σ^E activity

Since previous studies indicated that the ChrR-CLD is required for this transcriptional response to $^1\text{O}_2$ *in vivo*¹⁷, we analyzed function of a series of variant ChrR proteins each containing amino acid substitutions at residues in the ChrR-CLD (comprised of ChrR residues 86–213) that are conserved across 73 predicted homologues¹⁷. One set of these variant ChrR proteins contained alanine substitutions at conserved amino acid residues that function as zinc ligands in the wild type ChrR-CLD (His¹⁴¹, His¹⁴³, Glu¹⁴⁷ or His¹⁷⁷). We also tested the function of ChrR variants with either alanine or serine substitutions at Cys¹⁸⁷

or Cys¹⁸⁹. Combined, these variant ChrR proteins contain single amino acid substitutions in six of the ten conserved residues in the CLD across 73 predicted homologues of this anti- σ factor (the other conserved residues being glycine residues and one proline residue).

We first tested the ability of these variant ChrR proteins to inhibit σ^E activity in an *Escherichia coli* reporter strain that contains a σ^E -dependent promoter fused to *lacZ*^{13–14, 21}. We did not expect single substitutions in the ChrR-CLD to affect this activity since loss of the entire CLD did not prevent the ChrR-ASD from inhibiting σ^E ¹⁷. As predicted, when β -galactosidase activity was measured in this *E. coli* reporter strain we found that each of the ChrR proteins with single alanine substitutions in any of these six residues inhibited σ^E activity (Figure 2A). Thus, single alanine substitutions at these positions do not block the ability of ChrR to inhibit σ^E activity. It was also not surprising that single alanine substitutions at Cys¹⁸⁷ or Cys¹⁸⁹ allowed these variant ChrR proteins to inhibit σ^E activity since proteins containing individual serine substitutions at the same positions were previously shown to function as anti- σ factors²¹. A variant ChrR protein containing alanine at both Cys¹⁸⁷ and Cys¹⁸⁹ was unable to inhibit σ^E as effectively as wild-type ChrR in this tester strain (Figure 2A), a result that was similar to that obtained when a ChrR variant contained serine substitutions at both positions²¹. Thus, it appears that replacement of both Cys¹⁸⁷ and Cys¹⁸⁹ with alanine or serine²¹ blocks the ability of ChrR to inhibit σ^E . One explanation for the behavior of the variant ChrR proteins with amino acid substitutions at both of these cysteine residues is that these proteins are in a conformation that favors complex dissociation rather than complex association. In contrast, variant ChrR proteins containing single alanine or serine substitutions at the cysteine residues in the ChrR-CLD are functional as anti- σ factors in this *E. coli* tester strain (Figure 2A).

In agreement with their behavior in *E. coli*, each of the singly-substituted variant ChrR proteins inhibited σ^E activity in *R. sphaeroides* though the strain expressing ChrR-C187S has somewhat higher σ^E activity than each of the other ChrR variants (Figure 2B, see below).

We recognize that a failure to accumulate σ^E /ChrR complexes containing the variant ChrR proteins could also formally explain the low levels of σ^E -dependent reporter gene activity. To test if the ChrR variants and σ^E were accumulated in *R. sphaeroides*, Western blot analysis was performed using a polyclonal antibody that recognizes both σ^E and ChrR. This analysis showed that σ^E and the variant ChrR proteins were accumulated in these strains (Figure 3). We conclude that each of the variant ChrR proteins containing these single amino acid substitutions in the ChrR-CLD functioned as anti- σ factors in *R. sphaeroides*. Thus, any effect on σ^E activity that we observe when cells expressing these variants are exposed to ¹O₂ can be attributed to the role of the corresponding amino acid in dissociation of σ^E /ChrR complexes.

Amino acid side chains that ligate zinc in the ChrR-CLD are required for ¹O₂ to promote dissociation of σ^E /ChrR complexes

For those variant ChrR proteins that retained their ability to function as anti- σ factors, we tested whether ¹O₂ promoted dissociation of the corresponding σ^E /ChrR complexes. To test the ability of ¹O₂ to stimulate the σ^E -dependent transcriptional response in *R. sphaeroides*, we measured the differential rates of β -galactosidase synthesis from a σ^E -dependent *rpoE* P1::*lacZ* reporter fusion prior to and during exposure to ¹O₂¹². We found that *R. sphaeroides* cells containing a wild-type ChrR protein had a low rate of β -galactosidase synthesis from this reporter gene in the absence of ¹O₂ but, as expected¹², this rate increased (~8 to 11-fold) after ¹O₂ exposure (Table 1).

When we tested the effect of $^1\text{O}_2$ on cells containing variant ChrR proteins with single alanine substitutions at each of the four amino acid residues that function as zinc ligands in the ChrR-CLD (H141A, H143A, E147A and H177A), we saw no significant increase in the rate of β -galactosidase synthesis regardless of whether this reactive oxygen species was generated from methylene blue or the photosynthetic apparatus (Table 1). In the absence of $^1\text{O}_2$, *R. sphaeroides* cells expressing the H143A or H177A variant ChrR proteins reproducibly have a slightly higher basal rate of σ^E activity compared to those containing wild-type ChrR, suggesting there is less binding of these variant proteins to σ^E . Regardless, there is no increase in σ^E activity when cells expressing the H141A, H143A, E147A or H177A variant ChrR proteins are exposed to $^1\text{O}_2$. Thus, we conclude that $^1\text{O}_2$ is unable to inactivate ChrR proteins containing an alanine at any one of the four amino acid residues that ligate zinc in the ChrR-CLD (His¹⁴¹, His¹⁴³, Glu¹⁴⁷ or His¹⁷⁷).

Loss of one amino acid side chain involved in zinc binding in the ChrR-CLD does not prevent zinc occupancy

If zinc binding to the ChrR-CLD were required for $^1\text{O}_2$ to promote dissociation of σ^E /ChrR complexes, then loss of metal binding by variant ChrR proteins with alanine substitutions in these ligands might explain the inability of this reactive oxygen species to elicit the σ^E -dependent transcriptional response. To test this hypothesis, we purified σ^E /ChrR complexes containing each of these variant ChrR proteins and measured their zinc content by two assays (ICP-MS or PCMB/PAR). We know that the zinc atom in the ChrR-CLD can be removed by dialysis in the presence of DTT¹⁷, so the metal content of the σ^E /ChrR complexes was analyzed both under conditions where ChrR should contain only a single (N-terminal) zinc (purification and subsequent dialysis in buffers containing DTT) as well as under conditions where the ChrR-CLD binds zinc (purification and subsequent dialysis in buffers lacking DTT)¹⁷.

We found that σ^E /ChrR complexes containing either wild type or the H141A, H143A, E147A or H177A ChrR proteins contained the expected ~ 1 molar equivalent of zinc per mole of complex when treated with DTT (Table 2). Thus, we conclude that none of the single amino acid substitutions in zinc ligands of the ChrR-CLD has a strong effect on binding of the essential zinc atom in the ChrR-ASD. This was not surprising because deleting the entire ChrR-CLD has no effect on zinc occupancy of the ChrR-ASD¹⁷. As expected, we found that σ^E /ChrR complexes containing a wild-type ChrR protein contained a second zinc atom if samples were prepared in the absence of DTT (Table 3–2). Surprisingly, we found that σ^E /ChrR complexes containing variant ChrR proteins with single alanine substitutions at the zinc ligands contained a second zinc atom when they were prepared in the absence of DTT (Table 2). Thus, the failure of $^1\text{O}_2$ to promote dissociation of σ^E /ChrR complexes containing single alanine substitutions in the zinc ligands of the ChrR-CLD is not due to the inability of these variant ChrR proteins to bind zinc.

Other proteins that contain four-coordinate zinc binding sites, including the ChrR-ASD, can often bind a metal if one of the protein ligands is removed^{17, 21–23}. However, in these cases loss of one of the four protein ligands can alter the zinc-protein interactions. Consequently, we tested if any of the single amino acid substitutions in the zinc ligands of the ChrR-CLD altered the apparent dissociation constant of this domain for zinc.

To do this, we used a zinc-specific chelator to measure the apparent dissociation constant (K_d) for zinc from σ^E /ChrR complexes containing two moles of zinc under conditions where the zinc in the ChrR-ASD is not removed²⁴. Under these conditions, we measured a K_d for the zinc in the wild-type ChrR-CLD of $\sim 4.6 \times 10^{-10}$ M (Table 3). It has been noted previously that dithiothreitol (DTT), commonly used as a reductant, can act as a metal chelator and has a K_d for transition metals, including zinc, of $\sim 10^{-10}$ M^{25–27}. Thus, the

measured K_d for zinc in the CLD of wild type ChrR explains why this metal is removed from this domain during dialysis against buffers containing DTT. When compared to wild-type ChrR, the measured K_d for zinc in the ChrR-CLD of σ^E /ChrR complexes containing the H141A protein was ~2-fold higher, suggesting that loss of this imidazole side chain did not have a large effect on metal binding affinity (Table 3).

In contrast, σ^E /ChrR complexes containing the H143A, E147A or H177A variant ChrR protein had apparent dissociation constants for zinc that were over 10-fold higher than that of the wild type protein, implying that these substitutions weakened the affinity of this domain for zinc (Table 3). As a control, we assayed zinc occupancy of variant ChrR proteins containing multiple alanine substitutions in the zinc ligands: H141A–H143A–E147A (CLD-HHE) and H141A–H143A–H177A (CLD-HHH). In both the CLD-HHE and CLD-HHH ChrR proteins, we were unable to detect binding of a second zinc atom, presumably because zinc coordination in the ChrR-CLD is dramatically altered by loss of three ligands (Table 2). Therefore, the observed zinc binding to the CLD variants with single amino acid substitutions in individual ligands does not reflect non-specific metal association.

When these observations are considered together, we conclude that multiple substitutions in the zinc site of the ChrR-CLD are needed to abolish metal binding, and that the H141A, H143A, E147A and H177A ChrR proteins are capable of specific, albeit weakened in some cases, zinc coordination. The data also indicate that the inability of 1O_2 to promote dissociation of the corresponding σ^E /ChrR complexes was not correlated with a loss of metal binding, as the H141A variant coordinates zinc with an affinity for zinc only two-fold higher than the wild type protein (Table 3) but cells expressing H141A exhibited no detectable activation of σ^E -dependent transcription in the presence of 1O_2 (Table 1). From studies probing the interaction of 1O_2 with model compounds, this reactive oxygen species can modify imidazole rings^{8, 28}. Thus, it is possible that one or more of the individual alanine substitutions in the ChrR-CLD zinc ligands alter either the number or nature of zinc ligands and lead to a defect in the ability of 1O_2 to promote dissociation of σ^E /ChrR complexes even though these variant ChrR proteins can still bind metal (see Discussion).

The conserved cysteine residues in the ChrR-CLD are not required for 1O_2 to induce σ^E activity

Studies probing the interaction of 1O_2 with model compounds, including individual amino acids, have shown that thiols are also subject to rapid oxidation by this reactive oxygen species^{7–8, 29–32}. Variant ChrR proteins form a complex with σ^E when either of the two conserved cysteines (residues 187 or 189) is changed to alanine or serine (Figure 2). Consequently, we also tested the ability of 1O_2 to promote dissociation of the σ^E /ChrR complexes containing variant ChrR proteins with substitutions at these conserved cysteine residues in the ChrR-CLD.

When we assayed the ability of 1O_2 to stimulate the σ^E -dependent transcriptional response, cells expressing C187A showed a ~2.5 fold increase in σ^E activity during exposure to 1O_2 , which is ~3–6 times lower than that observed (8- to 11-fold increase) when wild-type cells are exposed to 1O_2 (Table 1). Similarly, when cells containing a variant ChrR protein with a serine substitution at Cys¹⁸⁷ were exposed to 1O_2 , there is only a 1- to 4-fold increase in σ^E activity. The decreased fold increase in σ^E activity in cells containing the C187A or C187S variant ChrR proteins reflects a higher rate of β -galactosidase synthesis from this reporter gene in the absence of 1O_2 (Table 1). Thus, the loss of the cysteine side chain in the C187A or C187S variant ChrR proteins appears to alter the ability of these proteins to form a complex with σ^E *in vivo* (see Discussion). Thus, the data suggests that the thiol side chain at Cys¹⁸⁷ does not necessarily play a role in stimulating the σ^E -dependent response *in vivo*.

We found that cells containing ChrR C189A or C189S variant proteins exhibited a greater fold increase in the rate of β -galactosidase synthesis after exposure to $^1\text{O}_2$ compared to cells expressing C187A, C187S or wild type ChrR (Table 1). Cells expressing the C189A variant increase σ^E activity 5- to 7-fold and cells expressing the C189S variant increase activity 5- to 11-fold (compared to the wild type rate increase of 8- to 11-fold). The increase in β -galactosidase synthesis rates observed in cells containing variant ChrR proteins with alanine or serine substitutions at Cys¹⁸⁹ suggest that this side chain has no significant negative effect of the ability of $^1\text{O}_2$ to promote dissociation of this σ^E /ChrR complex. Indeed, the rate of β -galactosidase synthesis observed in cells when exposed to $^1\text{O}_2$ is 3- to 7-fold higher in cells containing the C189A or C189S ChrR variants than that found in strains containing wild type ChrR (Table 1), suggesting that these substitutions render the resulting σ^E /ChrR complexes more susceptible to dissociation when cells are exposed to $^1\text{O}_2$. In summary, evidence suggests that neither cysteine side chain in the ChrR-CLD is essential for $^1\text{O}_2$ to promote dissociation of the corresponding σ^E /ChrR complexes *in vivo*, suggesting that oxidation of these cysteine thiols is not a driving force in the dissociation. This suggestion is supported by our ability to modify two thiols with 5-5'-Dithio-bis(2-nitrobenzoic acid) (DTNB), a sulfhydryl-modifying agent ³³, before and after wild-type σ^E /ChrR complexes are exposed to $^1\text{O}_2$ (data not shown). The side chains of Cys¹⁸⁷ and Cys¹⁸⁹ are the only free thiols in the σ^E /ChrR complex ¹⁷, so it appears that these thiols are not direct targets for modification when pure σ^E /ChrR complexes are exposed to $^1\text{O}_2$ *in vitro*. However, the nature of the side chain at positions 187 and 189 play some role in dissociation of σ^E /ChrR complexes, since single serine substitutions at each residue have a higher rate of β -galactosidase synthesis relative to wild type when cells are exposed to $^1\text{O}_2$.

Amino acid substitutions at Cys¹⁸⁷ or Cys¹⁸⁹ affect zinc binding to the ChrR-CLD

When we analyzed zinc occupancy of σ^E /ChrR complexes with alanine or serine substitutions at either Cys¹⁸⁷ or Cys¹⁸⁹, the metal content of these variants were indistinguishable from the wild-type ChrR protein (Table 2). These results were not surprising given that these cysteine side chains are over 12 Å away from the zinc site in the ChrR-CLD and thus were not expected to have a direct role in zinc coordination ¹⁷. However, we were surprised to find that the apparent affinity for the C-terminal zinc of σ^E /ChrR complexes containing the C187S or C189S proteins was reduced ~10- to 20-fold compared to one containing a wild type ChrR protein (Table 3). Given the distance of these side chains from this metal site, we propose that the reduced affinity of σ^E /ChrR complexes containing the C187S or C189S proteins for zinc in the ChrR-CLD is an indirect effect of the amino acid substitutions. The effects of these substitutions on zinc affinity *in vitro* coupled with the *in vivo* effects observed in cells expressing these variants when exposed to $^1\text{O}_2$ will be incorporated into a working model for how $^1\text{O}_2$ promotes dissociation of σ^E /ChrR complexes (see Discussion).

The ability of other agents to promote dissociation of *R. sphaeroides* σ^E /ChrR complexes

It was recently shown that activity of the *C. crescentus* σ^E homologue is increased by treating cells with $^1\text{O}_2$, the organic hydroperoxide t-BOOH, or cadmium ²⁰. Consequently, we sought to determine whether treatment of *R. sphaeroides* with t-BOOH or cadmium would promote dissociation of σ^E /ChrR complexes.

To do this, we tested if activity of the σ^E -dependent reporter gene was increased when wild type *R. sphaeroides* cells were exposed to either cadmium or t-BOOH. When aerobically grown *R. sphaeroides* cells were treated with 20 μM , 100 μM , or 1 mM CdSO₄ for up to 3 hours, no increase in the rate of β -galactosidase synthesis from this reporter gene was observed (Table 4), unlike what is found in *C. crescentus* where addition of 25 μM exogenous cadmium increased σ^E activity ~2.5-fold ²⁰. In contrast, the addition of 100 μM

t-BOOH increased the rate of β -galactosidase synthesis from this σ^E -dependent reporter gene in either wild type cells (Table 4) or cells containing a wild type copy of ChrR as their only source of this anti- σ factor (Table 5). Thus, t-BOOH but not cadmium is able to promote dissociation of *R. sphaeroides* σ^E /ChrR complexes *in vivo*, suggesting there are differences between the behavior of this system in this bacterium and *C. crescentus* (see Discussion).

To further analyze aspects of ChrR required for t-BOOH to promote dissociation of σ^E /ChrR complexes, we tested the ability of this compound to increase target gene activity in cells containing variant ChrR proteins. We found that the rate of β -galactosidase synthesis cells containing the truncated ChrR85 protein was not increased in the presence of 100 μ M t-BOOH (Table 5). From this, we conclude that the ChrR-CLD was required for t-BOOH to promote dissociation of σ^E /ChrR complexes.

Since the ChrR-CLD is needed for both $^1\text{O}_2$ and t-BOOH to promote dissociation of σ^E /ChrR complexes, we also tested the ability of this organic hydroperoxide to alter activity of the σ^E -dependent target gene in cells containing variant ChrR proteins with single amino acid substitutions in the CLD. We found that the rate of β -galactosidase synthesis in cells containing either the H141A, H143A, E147A or H177A variant ChrR proteins was the comparable before and after addition of 100 μ M t-BOOH. From this we conclude that the histidine or glutamate side chains at these positions in wild type ChrR are required for t-BOOH to promote dissociation of σ^E /ChrR complexes, similar to what was found when cells containing these variant ChrR proteins were exposed to $^1\text{O}_2$ (Table 1). We also found that replacing the cysteine residue at ChrR positions 187 or 189 with either a serine or an alanine produced similar effects on dissociation of σ^E /ChrR complexes in cells containing each of these variant proteins in the presence of t-BOOH (Table 5) as was found with $^1\text{O}_2$ (Table 1). Thus, it appears that there are similar effects of these single amino acid substitutions in the ChrR-CLD in the presence of $^1\text{O}_2$ and t-BOOH (see Discussion).

Discussion

$^1\text{O}_2$ is one of several reactive oxygen species that can destroy biomolecules and kill cells^{7–10, 28–29, 34–38}. Prokaryotes and eukaryotes mount a transcriptional response to $^1\text{O}_2$ ^{12, 15, 36–37, 39–40} but little is known about the mechanisms by which these responses are initiated. Previous work has shown that the transcriptional response to $^1\text{O}_2$ in *R. sphaeroides* is regulated by ChrR, a zinc metalloprotein which forms a complex with the Group IV sigma factor, σ^E , that prevents this sigma factor from binding RNA polymerase *in vitro*^{12–13, 17, 21}. However, it was not known whether $^1\text{O}_2$ activated the σ^E -dependent transcriptional response by promoting disassociation of σ^E /ChrR complexes or by preventing newly-synthesized ChrR from forming this inhibitory complex with σ^E . We found that $^1\text{O}_2$ stimulated the σ^E -dependent transcriptional response when cells were treated with the protein synthesis inhibitor chloramphenicol. Since $^1\text{O}_2$ was able to increase σ^E activity in the absence of *de novo* protein synthesis, we conclude that this reactive oxygen species somehow causes dissociation of σ^E /ChrR complexes to initiate the transcriptional response. Stimulatory signals cause dissociation of complexes between other Group IV sigma factors and their cognate anti- σ factors^{22, 41–49}. Thus, the process by which $^1\text{O}_2$ stimulates the σ^E -dependent transcriptional response is similar to that used for increasing activity of many Group IV sigma factors. In many cases, the inducing signal activates one or more proteases and stimulates turnover of the anti- σ factor^{43–45}. This appears to be true for *R. sphaeroides* σ^E /ChrR as well, since the presence of either $^1\text{O}_2$ or t-BOOH promotes turnover of the anti- σ factor ChrR (Nam, unpublished). However, the mechanism and other proteins needed for these inducers to stimulate dissociation of σ^E /ChrR complexes are unknown.

Previous work demonstrated that ChrR is composed of an N-terminal domain that is sufficient for binding σ^E and a C-terminal cupin-like domain (CLD) that is needed for 1O_2 to promote this transcriptional response *in vivo*¹⁷. To acquire insight into how 1O_2 might stimulate release of σ^E from ChrR, we tested the role of six conserved amino acids (His¹⁴¹, His¹⁴³, Glu¹⁴⁷, His¹⁷⁷, Cys¹⁸⁷ and Cys¹⁸⁹) in the ChrR-CLD for a role in the σ^E -dependent transcriptional response to 1O_2 . While little is known about the reactivity of 1O_2 with proteins, each of these amino acids is conserved in 73 ChrR homologues and studies with amino acids or model compounds predict that cysteine and histidine side chains are potential sites of modification by this reactive oxygen species^{7, 29, 32, 38, 50}.

We found that 1O_2 was unable to promote dissociation of σ^E /ChrR complexes containing variant ChrR proteins with single alanine substitutions at His¹⁴¹, His¹⁴³, Glu¹⁴⁷, or His¹⁷⁷, the amino acid side chains that comprise the 4-coordinate zinc binding site in the wild type ChrR-CLD. It was possible that a defect in metal binding could explain the inability of 1O_2 to promote dissociation of σ^E /ChrR complexes containing alanine substitutions at these residues. When studies were performed on the response of a *Caulobacter crescentus* σ^E /ChrR homologue to 1O_2 and other agents, the authors found that 1O_2 was unable to increase target gene expression in cells containing variant proteins with alanine substitutions at the analogous positions of the CLD²⁰. Based on this data and amino acid similarity to the *R. sphaeroides* ChrR protein, it was proposed that the *C. crescentus* ChrR protein may be able to coordinate zinc at the analogous residues. However, it was recognized that further studies were needed to determine if there is a physiological role for this C-terminal zinc in the ChrR-dependent response in either organism²⁰. Unfortunately, no experiments had been performed in the *C. crescentus* study to monitor zinc binding of mutant ChrR proteins relative to the wild type protein.

Indeed, our data indicate that the inability of 1O_2 to promote dissociation of complexes containing these variant *R. sphaeroides* ChrR proteins (H141A, H143A, E147A, or H177A) is not associated with a defect in zinc occupancy in the CLD since all four of these σ^E /ChrR complexes bound zinc when purified (Table 3–2). In addition, the CLD of the H141A ChrR protein binds zinc with an affinity within 2-fold of that of its wild type counterpart and the CLD of the H143A, E147A and H177A ChrR proteins each had an affinity for zinc *in vitro* that was 10- to 20-fold higher relative to wild type (Table 3). Thus, previous predictions that the zinc atom in the ChrR-CLD was somehow linked to the σ^E -dependent transcriptional response¹⁷ seem unlikely since 1O_2 was unable to promote dissociation of σ^E /ChrR complexes containing variant ChrR proteins that have affinities for zinc comparable to a wild type protein. Instead, our data support a model in which zinc binding by the ChrR-CLD is not necessary for 1O_2 to promote dissociation of σ^E /ChrR complexes. However, it is likely that the H141A, H143A, E147A, or H177A substitutions shift the number of protein ligands to the zinc in the ChrR-CLD from 4 to 3, so it is possible that features of the zinc-protein interaction are needed for 1O_2 to promote dissociation of σ^E /ChrR complexes.

This model is not contradicted by the observation that the C187S and C189S ChrR variants, which allow cells to respond to 1O_2 , have an effect on the ChrR-CLD zinc affinity even though these side chains are ~12 Å from this metal. The C187S and C189S ChrR variants exhibit a 20-fold higher affinity for zinc relative to wild type but cells expressing C187S or C189S can still increase σ^E activity when exposed to 1O_2 . The changes in zinc affinity for the C187S and C189S ChrR proteins also indicate that these amino acid substitutions exert an effect on the properties of the zinc binding site in the ChrR-CLD even though these substitutions are not in proximity to the amino acid residues that coordinate this metal. When the metal binding properties and the ability of 1O_2 to promote dissociation of the ChrR H141A, C187S and C189S proteins are considered together, it leads us to propose that

the presence of zinc in the ChrR-CLD is not obligately linked to the ability of this reactive oxygen species to release σ^E .

Combining information from the 3-dimensional structure of the σ^E /ChrR complex¹⁷ with data from this study allows us to refine previous models for how $^1\text{O}_2$ triggers the σ^E -dependent transcriptional response to this reactive oxygen species. We propose that the chemical nature of the His¹⁴¹, His¹⁴³, Glu¹⁴⁷, or His¹⁷⁷ side chains in the ChrR-CLD is required for $^1\text{O}_2$ to promote dissociation of σ^E /ChrR complexes. $^1\text{O}_2$ is known to modify imidazole-containing compounds^{7, 29, 32, 38}, so proper positioning of one or more of these side chains in the ChrR-CLD is likely crucial for $^1\text{O}_2$ to directly or indirectly promote dissociation of σ^E /ChrR complexes. The side chains of His¹⁴¹, His¹⁴³, Glu¹⁴⁷, or His¹⁷⁷ (as well as Cys¹⁸⁹) are each solvent-exposed on one face of the ChrR-CLD in the σ^E /ChrR structure (Figure 4B), so it is possible that modification of one or more of these side chains helps to initiate a conformational change in the ChrR-CLD that promotes dissociation of the σ^E /ChrR complex. Changes in the position of amino acid side chains within the ChrR-CLD brought about by alterations in the zinc coordination state could also explain why the H141A, H143A, E147A, or H177A amino substitutions prevented $^1\text{O}_2$ from promoting dissociation of σ^E /ChrR complexes.

Indeed, significant alterations in the positions of the His¹⁴¹, His¹⁴³, Glu¹⁴⁷ or His¹⁷⁷ side chains, as well as Cys¹⁸⁷, were observed between the 3-dimensional structures of σ^E /ChrR complexes that lacked or contained a C-terminal zinc even though the backbone positions of the ChrR-CLD were similar in these two complexes (maximum rmsd of 0.63 Å² over 85 a-carbons in the ChrR-CLD; Figure 4A)¹⁷. Thus, there is structural evidence for metal-dependent alterations in the positions of His¹⁴¹, His¹⁴³, Glu¹⁴⁷, and His¹⁷⁷ side chains, and even at residues Cys¹⁸⁷ or Cys¹⁸⁹ that are ~12Å away from the C-terminal zinc ligand¹⁷. Cysteine thiols are modified by organic hydroperoxides⁶, so it is possible that changes in the position of the Cys¹⁸⁷ or Cys¹⁸⁹ can alter the ability of these variant σ^E /ChrR complexes to respond to t-BOOH. However, experiments to determine how $^1\text{O}_2$ or t-BOOH promotes dissociation of σ^E /ChrR complexes *in vitro* are needed to test the predictions of this model.

While this paper was being prepared, results of experiments to analyze the response of the *C. crescentus* σ^E /ChrR system to $^1\text{O}_2$ and other stimuli were published²⁰. The *C. crescentus* σ^E /ChrR system can respond to $^1\text{O}_2$, t-BOOH, UV-A radiation, and cadmium, though it was proposed that the mechanism by which cadmium induces σ^E activity is distinct from the other agents²⁰. We found that the *R. sphaeroides* σ^E /ChrR system also responds t-BOOH. However, σ^E activity is not induced when cells are exposed to concentrations of cadmium that activated *C. crescentus* σ^E activity. Thus, our data suggest there are differences in the response of *R. sphaeroides* σ^E /ChrR to cadmium when compared to other agents and between these two bacteria to this compound.

If we further compare the σ^E /ChrR systems of both organisms, we observe that neither *R. sphaeroides* Cys¹⁸⁷ nor *C. crescentus* Cys¹⁸⁶ are necessary for the σ^E -dependent transcriptional response to $^1\text{O}_2$, as cells containing variant ChrR proteins with substitutions at either position induce a σ^E -dependent response to $^1\text{O}_2$ similar to the wild-type σ^E /ChrR systems of each bacterium. While the ChrR-CLD cysteine residues are not important in either organism for promoting complex dissociation when cells encounter $^1\text{O}_2$, in *C. crescentus* the cysteine residues are reported to be important for transient increases in σ^E activity when cadmium is added to cells²⁰. This does not appear to be the case in *R. sphaeroides* since the addition of 1mM cadmium sulfate did not promote dissociation of σ^E /ChrR complexes even in wild type cells.

It has been reported that addition of cadmium can deplete *E. coli* cells for zinc⁵¹ and it is known that zinc limitation can increase *R. sphaeroides* σ^E activity^{12, 52}. However, if the concentrations of cadmium added in this study interfered with zinc homeostasis, and zinc limitation contributed to the ability of cadmium to increase σ^E activity, we would have observed increased σ^E activity in *R. sphaeroides* in cadmium-treated cells.

One obvious difference between the *R. sphaeroides* and *C. crescentus* ChrR proteins is that the latter homolog lacks side chains that coordinate zinc in the *R. sphaeroides* ChrR-ASD. We know that three of four zinc ligands in the *R. sphaeroides* ChrR-ASD (His6, Cys35, and Cys38) are essential for it to form a complex with and inhibit σ^E activity¹⁷. Thus, the absence of zinc in the *C. crescentus* ChrR-ASD may allow the system to respond to alternate stimuli, such as cadmium, relative to *R. sphaeroides* ChrR, and vice versa. Further investigation is warranted to distinguish the mechanistic differences on the ability of $^1\text{O}_2$ and other agents versus cadmium or other yet identified stimuli to promote dissociation of σ^E /ChrR complexes *in vitro* and *in vivo* between these homologous systems.

In summary, we have shown that $^1\text{O}_2$ stimulates the σ^E -dependent transcriptional response by promoting dissociation of σ^E /ChrR complexes. We have also presented evidence that t-BOOH will increase σ^E activity in *R. sphaeroides*. Finally, we identified amino acid side chains in the ChrR-CLD that play important roles in the process by which $^1\text{O}_2$ or t-BOOH promotes release of σ^E from ChrR. Our data indicate that the ability of $^1\text{O}_2$ to promote dissociation of several variant σ^E /ChrR complexes is not linked to a failure of each anti- σ factor to bind zinc in the C-terminal CLD. We are currently developing systems to test the predictions of our results for how $^1\text{O}_2$ and t-BOOH promote dissociation of σ^E /ChrR complexes. For example, experiments are in progress to test whether these reactive oxygen species chemically modify ChrR, if dissociation of σ^E /ChrR complexes caused by $^1\text{O}_2$ or t-BOOH require additional proteins *in vivo* and *in vitro*, and how these and other amino acid changes in ChrR alter its structure and zinc-protein interactions in the CLD.

Experimental Procedures

Bacterial Strains and Growth Conditions

E. coli strain VH1000 ($\Phi\lambda$ JDN1), containing an *rpoEP1::lacZ* fusion, was used to assay ChrR function in this host^{13–14, 21}. Plasmids were maintained in *E. coli* DH5 α , *E. coli* S17-1 was used as a donor for plasmid conjugation into *R. sphaeroides*, and *E. coli* strains M15(pREP4) and BL21(DE3) were utilized for protein expression. *E. coli* strains were grown at 37°C in Luria-Bertani growth media⁵³ supplemented with 100 $\mu\text{g}/\text{ml}$ ampicillin, 25 $\mu\text{g}/\text{ml}$ kanamycin, and/or 10 $\mu\text{g}/\text{ml}$ tetracycline to maintain plasmids where necessary. *R. sphaeroides* 2.4.1 (wild type) and TF18 ($\Delta rpoEchrR-1::drf$) were grown at 30°C in Sistrom's succinate-based minimal medium A⁵⁴. Media used for growth of *R. sphaeroides* strains containing low-copy *rpoE P1::lacZ* reporter plasmids^{14, 21, 55} were supplemented with 25 $\mu\text{g}/\text{ml}$ kanamycin and 1 $\mu\text{g}/\text{ml}$ tetracycline where necessary. *R. sphaeroides* strains grown by aerobic respiration were bubbled with 69% N_2 , 30% O_2 , and 1% CO_2 in the dark. Photosynthetic cultures were grown by bubbling with 95% N_2 and 5% CO_2 using a light intensity of 10 W/m^2 ¹². $^1\text{O}_2$ was generated for experiments with *R. sphaeroides* as previously described^{12, 17}.

Mutagenesis of *chrR*

Variant ChrR proteins were generated via site-directed mutagenesis using the QuickChange Mutagenesis kit (Stratagene, La Jolla, CA) with *chrR* genes contained on plasmids pJDN48²¹ or pLC37¹⁷. Each mutant *chrR* gene was sequenced to confirm that only the desired change was present before testing ChrR function^{13, 21}. To determine if the variant ChrR

proteins inhibited σ^E activity, β -galactosidase assays were performed in *E. coli* strain VH1000 ($\Phi\lambda$ JDN1). To assay function of variant ChrR proteins in *R. sphaeroides* before or during exposure to $^1\text{O}_2$, each mutant *chrR* gene was cloned into pJDN18, transformed into S17-1 and mated into *R. sphaeroides* strain TF18 containing the low-copy *rpoEP1::lacZ* reporter plasmid, pJDN30^{13, 21}.

β -galactosidase Assays

β -galactosidase assays were performed as previously described. Data is presented in Miller units for experiments using *E. coli*¹⁴ and *R. sphaeroides* when testing the ability of ChrR variants to inhibit σ^E activity, and as the differential rate of β -galactosidase synthesis to assay σ^E activity when *R. sphaeroides* cells were treated with $^1\text{O}_2$ ^{12,17}.

Inhibition of Protein Synthesis

R. sphaeroides cultures were treated with chloramphenicol (Cm) to inhibit protein synthesis. To assay protein synthesis, 10 ml of exponential-phase *R. sphaeroides* cultures ($\sim 2 \times 10^8$ cells/ml) were split, and one flask was treated with 200 $\mu\text{g/ml}$ Cm for 15 min. After 15 min, 50 μCi of EasyTag EXPRE³⁵S³⁵S Protein Labeling Mix (Perkin-Elmer) was added to each culture for 1 min followed by a 2 min exposure to a mixture of 1 M cysteine and 1 M methionine. Ice-cold trichloroacetic acid (5% final concentration) was added, proteins were harvested by centrifugation ($13,000 \times g$ for 10 min), the supernatant was aspirated and the pellet washed twice with ice-cold 80% acetone. After evaporation of residual acetone, the cell pellet was resuspended in 1 ml of TBS-Tween and radioactivity measured via a scintillation counter. The ratio of the ³⁵S incorporated into acid-precipitable material in Cm-treated versus Cm-untreated cells was used to monitor protein synthesis. In three replicate experiments, treatment of *R. sphaeroides* with 200 $\mu\text{g/ml}$ Cm for 15 min was 95% efficient at arresting protein synthesis.

Monitoring transcript levels before and during $^1\text{O}_2$ exposure

RNA was isolated, with slight modification, as previously described⁵⁶. Photosynthetic *R. sphaeroides* 2.4.1 cultures (500 ml) were grown to a density of $\sim 2 \times 10^8$ cells per ml, and 25 ml samples were taken at time points prior to and during exposure to $^1\text{O}_2$. To digest DNA, the samples were treated with RNase-free DNase (Sigma, Woodlands, TX) in 50 mM Tris-Cl (pH 7.5), 50 mM MgCl_2 , and 10 mM DTT for 20 min at room temperature. The RNA was further purified using an RNeasy CleanUp kit (QIAGEN, Valencia, CA). RNA quality was assayed (i.e., A_{260}/A_{280} ratio ~ 1.8 – 2.0) and quantitated spectrophotometrically (NanoDrop Technologies, Wilmington, DE).

cDNA synthesis

For cDNA synthesis, 5 μg of RNA was mixed with 3 μl of random hexamer primers (Invitrogen, Carlsbad, CA) in a final volume of 30 μl . The mixture was incubated at 70°C for 10 min, then at 25°C for 1 hr. Superscript II Reverse Transcriptase and recommended buffers (Invitrogen, Carlsbad, CA) were added, the mixture was incubated for 1 hr at 37°C, followed by 1 hr at 42°C. To digest the RNA after cDNA synthesis, 20 μl of 1N NaOH was added before placing samples at 65°C for 30 min. After 20 μl of 1N HCl was added, the cDNA was purified using a PCR Purification Kit (QIAGEN, Valencia, CA) and resuspended in 30 μl of buffer. The concentrations of cDNA generated were quantitated spectrophotometrically (NanoDrop Technologies, Wilmington, DE).

qRT-PCR

Real-time PCR was used to measure expression from known σ^E target genes (*rpoE* and RSP1409^{12, 15}) and *rpoZ* (encoding the Ω subunit of RNA polymerase) as a σ^E -

independent control gene using a Bio-Rad iCycler (Bio-Rad, Hercules, CA). Primers used were: *rpoZ* forward (5'-TTG AAG ACT GCG TTG ACA AGG TCC-3'), *rpoZ* reverse (5'-GTT CTT GTC ATT GTC GCG GTC GAT-3'), *rpoE* 110F (5'-TGA AGG GCT TCC TGA TGA AAT CCG-3'), *rpoE* 210R (5'-ATC GAA GAG ATG AGC CTT CTG CCA-3'), RSP_1409F (5'-GAT CTG CTG AAG CCC GAG AAC AAG-3'), and RSP_1409R (5'-CTT CCG TCA GAT CCG AGG ACA TCA-3'). Triplicate assays were performed in reactions containing 12.5 μ l of the IQ SYBR Green Super-mix (Bio-Rad, Hercules, CA), 20 ng of cDNA (diluted to 4ng/ μ l), 5 pmol of each primer, and water to 25 μ l. The PCR cycling conditions were: denaturation at 95°C for 5 min, followed by 40 cycles of 30 s at 95°C, 30 s at 60°C, and 30 s at 72°C. To assay amplification products for each target gene, a melting curve was generated by treating for 1 min at 95°C, then incubating at 55°C for 1 min and increasing the temperature 0.5°C every 10 s up to 95°C. Real-time PCR efficiencies were determined by 5-fold serial dilutions of the cDNA template from 20 ng down to 0.16 ng. Data were analyzed by comparative Ct method⁵⁷. To report the relative change in RNA abundance, the value was compared to that prior to the generation of ¹O₂ stress (time equals zero).

Analysis of purified σ^E /ChrR complexes

σ^E /ChrR complexes containing one or two zinc atoms per complex were prepared as previously described¹⁷. Protein concentration was determined by Bradford assays⁵⁸. To assay metal content of σ^E /ChrR complexes, samples were either analyzed by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS; UW Soil and Plant Analysis Lab, Madison, WI, and Wisconsin State Lab of Hygiene, Madison, WI) or spectrophotometrically via a 4-(2-pyridylazo) resorcinol (PAR) assay²¹ using the organomercurial agent p-chloromercuribenzoic acid (PCMB) to form mercaptide bonds with cysteine residues and ensure the release of all zinc atoms from ChrR denatured by guanidine-HCl (described below).

Zinc Affinity Measurements

Total zinc content ($[\text{ChrR}_{\text{totalZn}}]$) was determined after treating σ^E /ChrR complexes (5 μ M) with guanidine hydrochloride and PCMB (10 mM HEPES-KOH pH 7.4, 150 mM KCl, 40 μ M PCMB, 4.5 M guanidine hydrochloride), incubating with 200 μ M PAR at 70 °C for 20 min, and measuring A₄₉₀ as an indicator of zinc binding to PAR. Zinc occupancy was calculated by comparing the sample A₄₉₀ to ZnSO₄ standards in the same buffer. The apparent dissociation constants for the dialyzable zinc in σ^E /ChrR complexes were estimated by competition with the fluorescent zinc competitor Zinbo-5 (2-(4,5-Dimethoxy-2-hydroxyphenyl)-4-(2-pyridylmethyl)aminomethylbezoxazole)²⁴. To determine the apparent dissociation constants, σ^E /ChrR complexes (2.5 μ M – 5 μ M) were incubated in 50 mM HEPES-KOH pH 7.2, 100 mM KCl, 1 mM β -mercaptoethanol, and Zinbo-5 (2.5 μ M – 5 μ M) for 10 min at 23 °C. Zinc binding by Zinbo-5 (as assessed by increases in A₃₇₆) reached equilibrium within 5 min, while incubations longer than 15 min caused protein aggregation. The extinction coefficient of Zinbo-5 in this buffer was determined by measuring the absorption of 5 μ M Zinbo-5 incubated with increasing concentrations of ZnSO₄ at 376 nm, and calculated values ($\epsilon = 1.70 \times 10^4$ - 2.10×10^4 M⁻¹ cm⁻¹) were consistent with the previously published value ($\epsilon = 1.9 \times 10^4$ M⁻¹ cm⁻¹)²⁴. After the concentration of zinc-bound Zinbo-5 was measured, the concentration of free Zinbo-5 ($[\text{Zinbo-5}_{\text{free}}] = [\text{Zinbo-5}_{\text{total}}] - [\text{Zinbo-5} \cdot \text{Zn}]$) was determined. Assuming that the N-terminal Zn in the ChrR ASD is not released in the presence of Zinbo-5, concentrations of ChrR with 2 Zn ($[\text{ChrR} \cdot \text{Zn}_2] = [\text{ChrR}_{\text{totalZn}}] - [\text{ChrR}_{\text{total}}] - [\text{Zinbo-5} \cdot \text{Zn}]$) or 1 Zn ($[\text{ChrR} \cdot \text{Zn}] = [\text{ChrR}_{\text{total}}] - [\text{ChrR} \cdot \text{Zn}_2]$) were calculated. Based on the published dissociation constant of Zinbo-5 at pH 7.2 with 100 mM ionic strength ($K_D = 1.3 \times 10^{-9}$ M)

²⁴, the binding constant of the dialyzable zinc in ChrR was calculated according to the equation below:

$$K_{dChrRCT.D} = K_{dZinbo-5} \frac{X([ChrR \cdot Zn]X[Zinbo - 5 \cdot Zn])}{([Zinbo - 5_{free}]X[ChrR \cdot Zn_2])}$$

Western Blot Analysis

Proteins from exponential-phase cells ($\sim 2 \times 10^8$ cells/ml) expressing wild type or variant ChrR proteins were collected by TCA precipitation (5% final concentration, see above). Precipitated proteins were resuspended in 100 μ l of 1X LDS loading dye (Invitrogen, Carlsbad, CA) and 20 μ l of each sample was loaded onto a NuPAGE acrylamide gel (Invitrogen, Carlsbad, CA) that was run in 1X MES running gel at 130 volts for ~ 90 min. Proteins were transferred to a charged Invitrolon PVDF membrane (Invitrogen, Carlsbad, CA) using a Hoefer Mighty Small Transfer Tank (Hoefer, Holliston, MA) at 200 mA for 3 hr. The membranes were incubated overnight in 1x TBS, 0.05% NP40, and 6% milk protein before being incubated with rabbit polyclonal antibodies raised against σ^E /ChrR complexes (Cocalico Biologicals, Reamstown, PA). A goat anti-rabbit HRP-conjugated secondary antibody was used for detection using a Pierce ECL Western Blot Substrate (Thermo Fisher Scientific, Rockford, IL).

Treatment of cells with t-BOOH or cadmium

To determine if addition of t-BOOH or cadmium would induce σ^E activity, β -galactosidase assays were performed in exponential phase aerobic cultures of either wild type *R. sphaeroides* 2.4.1 or TF18 ($\Delta rpoEchrR-1::drf$) cells containing a wild type or mutant *chrR* gene on a low copy plasmid (see above). For these experiments, cells were grown by shaking (10 ml in a 125 ml flask) and exposed to either the indicated concentrations of CdSO₄ or 100 μ M t-BOOH. Samples were analyzed for β -galactosidase activity from the low-copy *rpoEP1::lacZ* reporter plasmid, pJDN30^{13, 21} to calculate the differential rate of the β -galactosidase synthesis. Data shown are the averages of three separate cultures.

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Fig. 1. Relative levels of σ^E target gene transcripts (RSP1409 and *rpoE*) before and during $^1\text{O}_2$ stress in the absence (●) or presence (■) of the protein synthesis inhibitor chloramphenicol (Cm) Shown are transcript levels for two known σ^E target genes, RSP1409 (top panel) and *rpoE* (bottom panel), in cells that were or were not treated with Cm before and during exposure to $^1\text{O}_2$ (generated by exposing photosynthetic cells to oxygen in the light). The vertical arrow at zero min indicates the time when cells were exposed to $^1\text{O}_2$. In the Cm-treated culture, Cm was added 15 min prior to exposure to $^1\text{O}_2$. Transcript levels reported were normalized relative to those from a control σ^E -independent gene (*rpoZ*) at each time point.

Fig. 2. Amino acid substitutions in the ChrR-CLD do not block the ability of variant ChrR proteins to inhibit σ^E activity *in vivo*

ChrR proteins with the indicated amino acid substitutions were tested for their ability to inhibit σ^E activity in *E. coli* (Panel A) or *R. sphaeroides* (Panel B). σ^E activity was monitored by measuring β -galactosidase activity (expressed as Miller units) from an *rpoE::lacZ* operon fusion that requires this sigma factor for transcription^{13–14,21}.

Fig. 3. Accumulation of σ^E and ChrR proteins in *R sphaeroides*

Shown is a Western blot analysis of cells containing either a wild type (WT) *rpoEchrR* operon or one that encodes the indicated variant ChrR protein as their only source of these proteins on a low-copy plasmid. The Western blot monitors accumulation of both σ^E (apparent MW ~21kDa) and ChrR (apparent MW ~23kDa; see arrows) since the antibody was raised against purified σ^E /ChrR complexes. Arrows indicate migration of known samples of σ^E and ChrR. Sizes were estimated by running protein molecular weight standards.

A

B

Fig. 4. Position of conserved amino acid residues in ChrR-CLD

(A) Stereo view of three overlaid structures of the ChrR-CLD. In green is the structure of the ChrR-CLD that contains zinc, in peach and purple are two structures of the ChrR-CLD solved in the absence of zinc. Conserved amino acid residues investigated in this study are labeled and shown as stick figures, with backbone carbons color coded according to above scheme. Side chain atoms are colored as follows: nitrogen, blue; oxygen, red; and sulfur, orange. Zinc is forest-green. (B) Space-fill model of the ChrR-CLD showing solvent-accessibility of the conserved residues. In green is the structure of the ChrR-CLD that contains zinc. Side chain atoms are colored as described above. This view shows the positions and accessibility of the side chains of His¹⁴¹, His¹⁴³, Glu¹⁴⁷, His¹⁷⁷, and Cys¹⁸⁹,

as well as the zinc atom (colored in grey). Structure forms from crystals were accessed from the Protein Data Bank under ID codes 2Q1Z and 2Z2S.

Ability of ¹O₂ to promote dissociation of σ^E-ChrR complexes in cells containing amino acid substitutions in the ChrR-CLD *in vivo*¹.

Table 1

ChrR Protein	Photosynthetic to Aerobic Shift			Aerobic Cultures Treated with Methylene Blue		
	Before ¹ O ₂	During ¹ O ₂	Fold Change	Before ¹ O ₂	During ¹ O ₂	Fold Change
WT	12 ± 1	76 ± 16	6.3	8 ± 2	81 ± 8	10.0
None	1529 ± 389	1208 ± 30	0.8	1285 ± 106	789 ± 174	0.6
H141A	16 ± 2	23 ± 6	1.4	25 ± 7	20 ± 3	0.8
H143A	28 ± 14	21 ± 5	0.8	25 ± 6	19 ± 5	0.8
E147A	14 ± 1	15 ± 4	1.0	19 ± 7	12 ± 3	0.6
H177A	35 ± 1	24 ± 8	0.7	39 ± 5	25 ± 10	0.6
C187A	38 ± 14	97 ± 9	2.5	38 ± 6	98 ± 1	2.5
C189A	27 ± 7	134 ± 41	5.0	34 ± 5	223 ± 9	6.6
C187S	109 ± 19	112 ± 10	1.0	72 ± 19	279 ± 64	3.9
C189S	39 ± 13	212 ± 15	5.4	47 ± 15	561 ± 67	11.9

¹ Shown are the differential rates of β-galactosidase synthesis measured from an *rpoE:lacZ* operon fusion that requires σ^E in cells containing the indicated ChrR protein as their sole source of this anti-σ factor before or during exposure to ¹O₂. The column labeled fold change reports the ratio of these rates during and before exposure to ¹O₂. The left half of the table shows data obtained when ¹O₂ was generated by exposing photosynthetically grown cells to 30% oxygen in the presence of light (10W/m²). The right half of the table shows data obtained when ¹O₂ was generated by adding light and methylene blue (1μM) to aerobically grown cells (30% oxygen).

Table 2Zinc content of σ^E -ChrR complexes containing wild-type or variant ChrR proteins¹

ChrR Protein	+ DTT	– DTT
WT	1.0 ± 0.4 (1)	1.9 ± 0.2 (2)
H141A	1.0 ± 0.2 (1)	2.1 ± 0.4 (2)
H143A	1.2 ± 0.3 (1)	1.6 ± 0.2 (2)
E147A	1.0 ± 0.4 (1)	2.1 ± 0.5 (2)
H177A	1.0 ± 0.3 (1)	1.8 ± 0.7 (2)
C187A	0.9 ± 0.2 (1)	1.8 ± 0.2 (2)
C189A	1.3 ± 0.4 (1)	1.8 ± 0.3 (2)
C187S	0.8 ± 0.3 (1)	1.9 ± 0.3 (2)
C189S	0.6 ± 0.1 (1)	1.9 ± 0.1 (2)
CLD-HHE	0.9 ± 0.2 (1)	0.9 ± 0.3 (1)
CLD-HHH	1.0 ± 0.2 (1)	1.1 ± 0.2 (1)

¹ Ratio of the zinc per σ^E /ChrR complex (expressed as moles of metal per mole of σ^E /ChrR complex) after samples containing the indicated ChrR protein were dialyzed against buffer with or without dithiothreitol (DTT). The numbers in parentheses indicate the ratio of zinc per σ^E /ChrR complex to the nearest whole number. CLD-HHE: ChrR variant with alanine substitutions at His¹⁴¹, His¹⁴³, and Glu¹⁴⁷. CLD-HHH: ChrR variant with alanine substitutions at His¹⁴¹, His¹⁴³, and His¹⁷⁷.

Table 3

Apparent dissociation constants (K_d) for the dialyzable zinc in σ^E /ChrR complexes containing wild type or variant ChrR proteins¹

ChrR Protein	K_d (M)	Relative Affinity
WT	4.6×10^{-10}	1.00
H141A	9.7×10^{-10}	0.48
H143A	5.6×10^{-9}	0.08
E147A	1.6×10^{-8}	0.03
H177A	5.1×10^{-9}	0.09
C187A	3.9×10^{-10}	1.16
C189A	3.9×10^{-10}	1.16
C187S	5.1×10^{-9}	0.09
C189S	1.1×10^{-9}	0.04

¹ Apparent K_d values were calculated based on the dissociation constant of Zinbo-5 ($K_d = 1.3 \times 10^{-9}$ M), as described in materials and methods.

Values in “Relative Affinity” column were calculated by dividing K_d value of σ^E /ChrR complexes containing wild type ChrR to those measured for those containing the indicated variant ChrR protein. Experiments were conducted in three separate trials with separate protein preparations. Results shown are representative data from one experimental trial. In each trial, similar trends in relative affinity for zinc were observed between samples.

Table 4Effects of t-BOOH or cadmium on dissociation of σ^E -ChrR complexes *in vivo*^I

<u>Cadmium (μM)</u>	<u>Before Cadmium</u>	<u>After Cadmium</u>	<u>Fold Change</u>
0	4.5 $\pm$ 1.1	4.8 $\pm$ 1.8	1.1
20	4.0 $\pm$ 0.5	4.6 $\pm$ 1.2	1.2
100	4.1 $\pm$ 0.9	4.7 $\pm$ 1.3	1.2
1000	4.7 $\pm$ 0.2	4.3 $\pm$ 1.1	0.9

<u>t-BOOH (μM)</u>	<u>Before t-BOOH</u>	<u>After t-BOOH</u>	<u>Fold Change</u>
100	4.5 $\pm$ 0.5	17.6 $\pm$ 0.9	3.9

^I Shown are the differential rates of β -galactosidase synthesis measured from an *rpoE::lacZ* operon fusion that requires σ^E in wild type *R. sphaeroides* 2.4.1 cells before or after addition of the indicated concentration of cadmium sulfate or t-BOOH. The column labeled fold change reports the ratio of these rates before and after cadmium sulfate addition.

Table 5

Ability of t-BOOH to promote dissociation of σ^E -ChrR complexes containing wild type or variant ChrR proteins *in vivo*¹.

ChrR Protein	Before t-BOOH	After t-BOOH	Fold Change
WT	19 ± 3	173 ± 64	9.1
ChrR85	34 ± 6	45 ± 7	1.3
H141A	25 ± 2	44 ± 9	1.7
H143A	31 ± 4	38 ± 2	1.2
E147A	20 ± 4	25 ± 6	1.3
H177A	40 ± 8	50 ± 9	1.3
C187A	44 ± 6	82 ± 14	1.9
C189A	27 ± 8	64 ± 12	2.4
C187S	109 ± 26	256 ± 75	2.3
C189S	60 ± 9	262 ± 83	4.4

¹ Shown are the differential rates of β -galactosidase synthesis measured from an *rpoE::lacZ* operon fusion that requires σ^E in cells containing the indicated ChrR protein as their sole source of this anti- σ factor before or after addition of 100 μ M t-BOOH. The column labeled fold change reports the ratio of these rates before and after addition of 100 μ M t-BOOH.