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Characterization of the HOCl-specific transcription factor HypT and the effects of HOCl on the metabolism of *E. coli*

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PART A

Introduction and Summary

I. Reactive Oxygen Species (ROS)

Reactive oxygen species (ROS) play a crucial role for the innate immune system of host organisms by killing invading pathogens (Winterbourn et al., 2006). Further, ROS are associated with a series of diseases like cancer, chronic inflammation, pulmonary and neurodegenerative diseases. Moreover, ROS accompany the aging of several organisms and cause age-related oxidative stress symptoms (Stadtman, 1992; Dukan and Nyström, 1998).

I-1 Generation and Types of ROS

All aerobically living cells and organisms encounter ROS, which are generated by different mechanisms in biological and physical processes. Most ROS are directly generated by enzymatic processes, e.g. by activated neutrophils and macrophages of the innate immune system in humans and higher eukaryotes (Ha et al., 2005; Winterbourn et al., 2006), or at low rates as by-products of the normal aerobic respiration of prokaryotes and eukaryotes (Miller and Britigan, 1997; Halliwell and Gutteridge, 1999). Other ROS are generated by physical reactions. They can be formed by high levels of radiation in cells, or further exogenous substances like metal ions, ozone, or various forms of smoke (Davies, 2005). There are two different types of ROS, radical and non-radical oxidants. As the name indicates, the basis for ROS generation is molecular oxygen (O_2). Upon addition of electrons to O_2 (e.g. by reduction), several different ROS can be generated such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (·OH) (Fig. 1) (Kiley and Storz, 2004; Imlay, 2013). The main generators of O_2^- and H_2O_2 are flavin cofactors, such as found in NADH dehydrogenase II (Messner and Imlay, 1999), glutathione reductase (Massey et al., 1969), lipoamide dehydrogenase (Grinblat et al., 1991), and glutamate synthase (Geary and Meister, 1977). The flavin cofactors of enzymes can autooxidize, even if not involved in aerobic respiration, and react thereby with O_2 (Messner and Imlay, 1999; Korshunov and Imlay, 2010). This autooxidation occurs when O_2 collides with the dihydroflavin group of the reduced enzyme. This in turn results in the generation of O_2^- and a flavosemiquinone species (Imlay, 2013). Moreover, O_2^- can quickly reduce itself under physiological pH, subsequently forming H_2O_2 (Miller and Britigan, 1997). ·OH can form when H_2O_2 reacts with unincorporated intracellular ferric iron in the Fenton reaction (Imlay et al., 1988). Copper can also serve as metal catalyst. Without such a transition metal catalyst the reaction is of little biological

relevance because the reaction rate is relatively low at physiological pH (Miller and Britigan, 1997). Further reactive species generated by the immune system or physical reactions, respectively, are hypochlorite (HOCl), mono chloramine (NH_2Cl) and nitric oxide (NO·), which is an intermediate of denitrification processes (Zumft, 1997).Thereby, NO· can act itself as an oxidant or it can react with superoxide radicals ($\cdot O_2^-$) from H_2O_2 to generate peroxynitrite ($ONOO^-$) and finally decompose to ·OH (Beckman et al., 1990; Pryor and Squadrito, 1995).

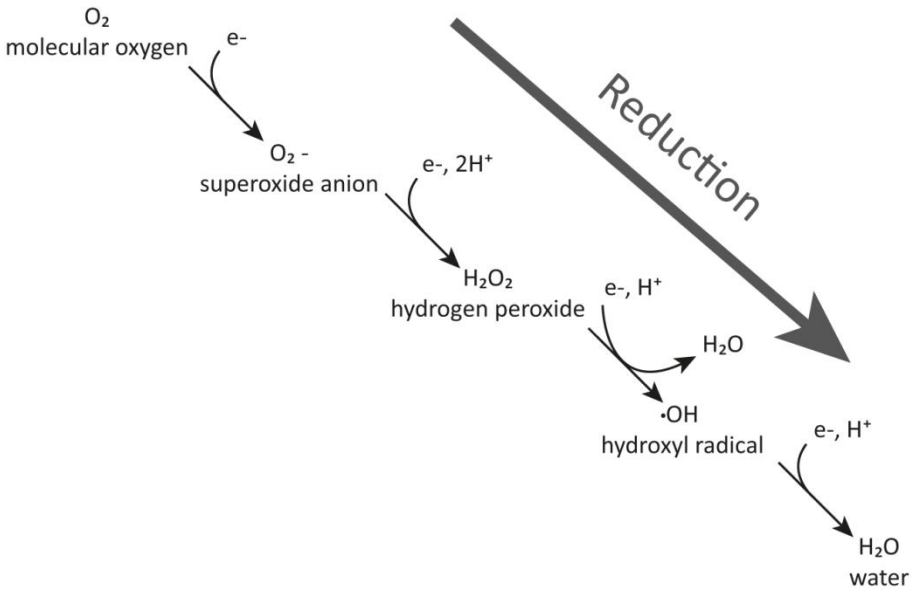

Figure 1 Reduction of molecular oxygen by addition of electrons to generate various forms of reactive oxygen species. Figure adopted from Kiley and Storz, 2004.

I-2 Reactivity of ROS with Biomolecules

ROS, especially the highly reactive radicals, can react with almost all biological macromolecules, including DNA, RNA, carbohydrates, lipids, cholesterol, proteins and antioxidants (Davies et al, 2005). In contrast, non-radical ROS are less reactive but more specific. The peroxides (e.g. H_2O_2) are rather selective, attacking and modifying specific sites of their target molecules.

The reaction rates of ·OH with macromolecules at pH 7 ranges from 8×10^8 M^{-1} s^{-1} for DNA to 4×10^{11} M^{-1} s^{-1} for collagen (Buxton et al., 1988). DNA damage was identified to be mainly driven by ·OH, formed via the Fenton reaction from H_2O_2 in the presence of iron (Imlay et al., 1988; Imlay, 2003). In contrast, H_2O_2 as precursor of ·OH does not directly attack DNA. This conclusion is derived from the observation that bacterial killing, resulting from accumulated DNA damage, is completely abolished when H_2O_2-treated cells are simultaneously treated

with cell-penetrating iron chelators, which inhibit Fenton reaction and concomitant ·OH formation (de Mello Filho and Meneghini, 1985; Imlay et al, 1988). The rate constants for reactions of ·OH with various side-chains of amino acids are relatively similar, ranging from 10^7 to 10^{10} M^{-1} s^{-1} generating further radical intermediates. The rate constant for hydrogen atom abstraction of the α-carbon from protein backbones by ·OH is 10^9 M^{-1} s^{-1} (Buxton et al., 1988). This reaction creates either an imine (Garrison, 1987) or a hydroperoxide (Davies, 1996), subsequently leading to backbone fragmentation.

The destructive and bactericidal effect of O_2^- arises from the oxidation of iron-sulfur clusters (for example [4Fe-4S]), which typically form the catalytically active center of many enzymes (e.g. dihydroxy-acid dehydratase; Flint et al., 1993a). O_2^- has the tendency to be electrostatically attracted to the catalytic iron atom of these [4Fe-4S] clusters. Oxidized clusters are unstable and quickly degraded (Flint et al, 1993b). Nevertheless, the destructive effect of O_2^- seems to play only a subordinated role *in vivo*. The rate of O_2^- formation is 5 µM/s in *Escherichia coli* and therefore relatively high. However, bacteria produce high levels of superoxide dismutase (SOD) keeping the steady-state level of O_2^- at less than 1 molecule per cell (Imlay and Fridovich, 1991).

H_2O_2, as well as HOCl, oxidizes thiol groups of cysteine residues in proteins generating sulfenic acid that is able to form disulfide bonds with other cysteines. Although the rate constant for oxidation of free cysteine is relatively low (k = 2 M^{-1} s^{-1}; Winterbourn and Metodiewa, 1999), the number of potential oxidation products is enormous. Sulfenic acid can be further oxidized to sulfinic acid or even to sulfonic acid (Fig. 2) (Imlay, 2003; Kiley and Storz, 2004). These products can be sources for further modifications to thiosulfinate, thiosulfonate or disulfide trioxide (Jeong et al., 2011).

Another major target of ROS is methionine, which contains a thioether group. The rate constant of the reaction of free methionine with HOCl (3.8 x 10^7 M^{-1} s^{-1}) and homobromous acid (HOBr; 3.6 x 10^6 M^{-1} s^{-1}) are relatively high illustrating the importance as a scavenger of these oxidants (Hawkins et al., 2003; Davies, 2005). The major product of methionine oxidation is methionine sulfoxide (Met-SO). Further oxidation can generate methionine sulfone, although to a much lesser extent (Nielson et al., 1985). Noteworthy, the oxidation of methionine generates two stereoisomers of the sulfoxide group (e.g. S- and R-isomer) and the cellular repair of these modifications is stereospecific (Fig. 3). The ratio of the two isomers depends on the position of the different methionine residues within the protein structure, at least *in vitro* (Sharov and Schoneich, 2000). The generation of methionine sulfoxide can be achieved by several different mechanism and intermediates. The oxidation with ·OH forms a short-living hydroxyl sulfuranyl (R-·S(OH)-R) species (10^9 M^{-1} s^{-1}), which

rapidly undergoes a further reaction to sulfoxide (Hiller et al., 1981; Schoneich et al., 1993). H_2O_2 and other peroxides react with methionine residues to a R-S^+(OH)-R species (6 x 10^{-3} M^{-1} s^1), which is then deprotonated to sulfoxide (Davies, 2005). An analogous reaction occurs with HOCl or HOBr via the formation of an sufenyl chloride R-S^+(Cl/Br)-R species. This unstable intermediate undergoes rapidly hydrolysis (Drozdz et al., 1988; Davies, 2005; Gray et al. 2013b).

Moreover, ROS are also able to oxidize aromatic residues mainly by addition reactions. One example for this is the formation of tyrosine phenoxyl radicals by a wide range of oxidants. This product arises from either direct oxidation of the aromatic ring and subsequent deprotonation at the hydroxyl group triggered by peroxides, or an addition-elimination reaction due to ·OH. The primary fate of this radical is the formation of a cross-linked di-tyrosine. Reactions of aromatic acids and HOCl as well as nitrating species generate chlorotyrosine, chlorinated tryptophan and nitrated products (Davies and Dean, 1997; Hawkins and Davies, 2001b).

I-3 Specific Characteristics of Reactive Chlorine Species (RCS)

Reactive chlorine species (RCS) are a subtype of ROS. The major representatives are HOCl and chloramines. HOCl is an oxidant with one of the highest oxidizing potential and therefore has a strong bactericidal activity. Due to this bactericidal characteristic it is widely used in household bleach as disinfectant (Thomas, 1979; Miller and Britigan, 1997). As a key effector of the innate immune system it is produced in activated neutrophils by the enzyme myeloperoxidase from H_2O_2 and chloride ions (Winterbourn et al., 2006). Its bactericidal activity arises from its reactivity with literally all kinds of cellular components, especially with proteins. The reaction with proteins results in proteome-wide unfolding and protein oxidation (Winter et al., 2008; Hawkins et al., 2003).

HOCl reacts with sulfur-containing compounds 100-fold more rapidly than with other macromolecules. Such compounds are basically cysteine, methionine and GSH (γ-L-glutamyl-L-cysteinyl-glycine). As mentioned in section I-2, HOCl-mediated thiol oxidation of cysteine residues generates an unstable sulfenyl chloride intermediate, which reacts with H_2O to sulfenic acid. Mono-oxidized thiols can be reduced by reductants such as thioredoxin family or can further oxidize to irreversible thiol modifications, such as sulfinic and sulfonic acids. These di- and tri-oxidized species normally lead to protein degradation (Hawkins and Davies, 1998a; Pattison and Davies, 2001; Hawkins et al., 2003). Further, sulfenyl chlorides

are able to react with primary or secondary amines to form irreversible sulfonamide linkages (Fig. 2) (Gray et al., 2013).

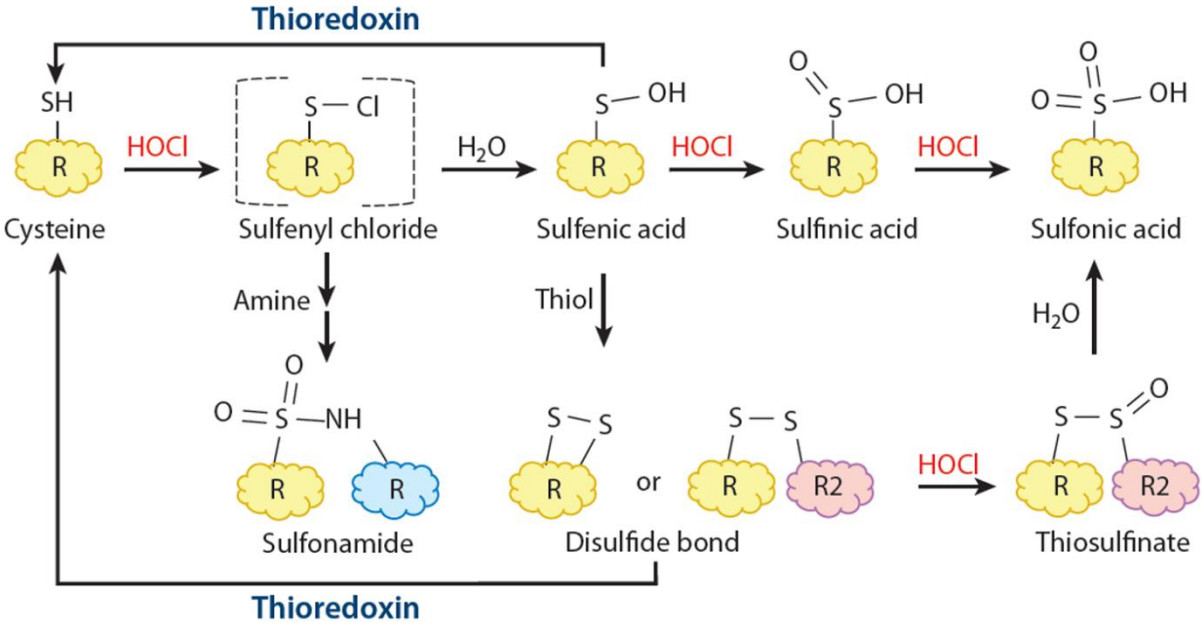

Figure 2 Reaction of HOCl with sulfur-containing biomolecules. Figure taken from Gray et al., 2013b.

Methionine mono-oxidation to Met-SO is reversible by methionine sulfoxide reductases (MsrA, MsrB and fRMsr; Fig. 3). The di-oxidation to methionine sulfone is considered to be irreversible (Peskin et al., 2009). The relative reaction rate for free amino acids with HOCl by competitive reaction with monochlordimedon is Cys > Met > Cystine > His > Ser > Leu (Winterbourn, 1985). However, the order of reactivity changes for the side-chains imbedded within a protein: Met > Cys >> Cystine ~ His ~ α-amino > Trp > Lys >> Tyr ~ Arg > Gln ~ Asn. Connotatively, the second order rate constants for HOCl with the protein backbone depend on the peptide bond environment (Pattison and Davies, 2001).

Besides the S-containing side chains, the primary and secondary amines in proteins are favored targets of HOCl. The amines become chlorinated to chloramines upon reaction with HOCl (Fig. 4). As mentioned above, chloramines show bactericidal activity and are able to chlorinate and oxidize further macromolecules. Thus chloramines can be ascribed to the RCS. Nevertheless, only a few chloramines are stable (e.g. N-chlorotaurine; Nagl et al., 2000), and associated with the innate immune system. The other chloramines rapidly decompose to their respective aldehydes (Hazen et al, 1998). Chloramines can further react

with iron or copper ions generating nitrogen radicals, which are highly reactive by themselves (Hawkins and Davies, 1998b; Hawkins and Davies, 2002; Gray et al., 2013b).

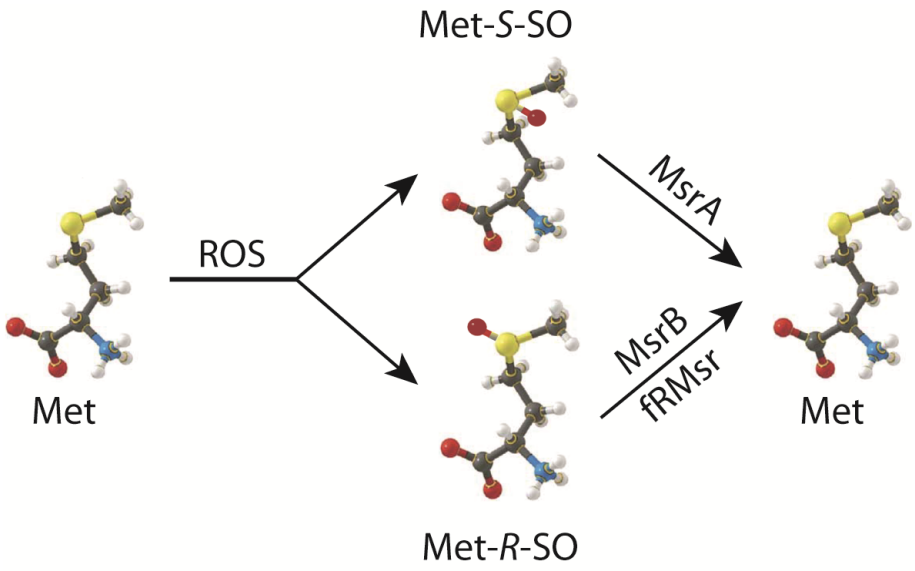

Figure 3 Methionine residues (Met) become oxidized by ROS to the two enantiomers methionine-*S*-sulfoxide (Met-*S*-SO) and methionine-*R*-sulfoxide (Met-*R*-SO). Protein-bound Met-*S*-SO can be reduced by methionine sulfoxide reductase A (MsrA), whereas protein-bound Met-*R*-SO is reduced by methionine sulfoxide reductase B (MsrB). Free Met-*R*-SO can additionally be reduced by fRMsr.

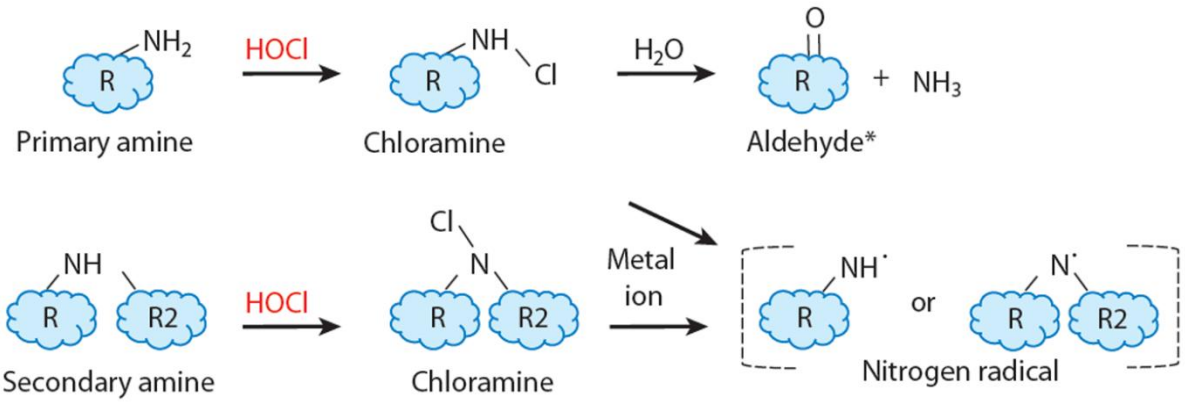

Figure 4 Reaction of HOCl with amines. Figure taken from Gray et al., 2013b.

HOCl-mediated products, generated with relatively slow reaction rates, are 3-chlorotyrosine and 2-oxo-histidine, which have been determined as specific biomarkers for RCS stressed cells, although it has been postulated that 3-chlorotyrosine is not always generated by direct chlorination of the phenol ring (Winterbourn and Kettle, 2000; Pattison et al, 2012). It is more

likely that the formation of a chloramine from a free amine group occurs, and that hemolysis of this N-Cl bond results in a chlorine atom attack on the phenyl ring (Domigan et al, 2005). However, it shows that under certain circumstances direct chlorination of the tyrosine side-chain can occur, if no free amine group is available (Fu et al, 2000, Pattison and Davies, 2001). Tyrosine chlorination and aldehyde formation both lead to irreversible protein unfolding and aggregation (Chapman et al., 2003). Other HOCl-mediated protein dimerization and aggregation mechanisms involve the generation of di-tyrosine, or reactions of carbonyl groups formed from decomposed lysine residues (Hazell et al., 1994).

Further, it has been proposed that HOCl can form intra- and intermolecular sulfonamide S-N crosslinks between thiol groups and arginine and lysine residues (Fu et al., 2002; Hawkins et al., 2003). As mentioned in section I-2, HOCl can also react with DNA and RNA, although with three to seven orders of magnitude slower rates compared to reaction with proteins (Gray et al., 2013b). Lipids are also a target of HOCl forming chlorohydrins by chlorination of double bonds of unsaturated fatty acids (Carr et al., 1997). Moreover, amine containing head groups of lipids (e.g. phosphatidyl ethanolamine) can react to chloramines, nitrogen radicals, and aldehydes. Finally, HOCl-mediated radical formation can generate lipid peroxides, which results in decomposition of unsaturated fatty acids to aldehydes (Niki, 2009; Gray et al., 2013b).

I-4 Antimicrobial Effects of ROS and RCS

ROS and RCS are able to attack all kinds of macromolecules leading to destructive modifications. Thus, several ROS related products contribute to toxicity and cell damaging. Some specific macromolecular modifications are considered as main contributor to the toxic effects of ROS. One of these most sensible points of attack is the DNA and the resulting mutagenesis caused by oxidative stress. Although, H_2O_2 and O_2^- are not able to directly damage DNA, bacteria that are deficient in *sod* as well as catalase/peroxidase (i.e. enzymes that are detoxifying H_2O_2 or O_2^-) show a high mutation rate when treated with H_2O_2 or O_2^-, respectively (Farr et al., 1986; Park et al,. 2005). The underlying reasons are DNA lesions that result from reaction by-products of H_2O_2 or O_2^- with unincorporated iron via the Fenton reaction. As already mentioned above, the Fenton reaction amplifies the damage of oxidative stress by generating additional destructive ·OH. DNA is known as a good iron binder (Rai et al., 2001). Thus, ·OH, as a nonselective although highly reactive oxidant that diffuses not far from its origin of generation, turns DNA to a primary target of Fenton mediated damage. ·OH can oxidize both, ribose and base moieties, respectively, of the DNA (Imlay, 2013). Adenine

is in most cases the initial site of an ·OH attack, but immediate electron movement from the resulting adenine radical to neighboring guanine leads commonly to 8-hydroxyguanine. The 8-hydroxyguanine is highly mutagenic due to its ability to elude the intrinsic DNA repair system when base-pairing with an adenine (Hogg et al., 2005; Imlay, 2013). The reaction of DNA with HOCl results in the formation of chloramines, which can lead to DNA double-strand dissociation and the disruption of hydrogen bond formation (Prutz, 1998; Hawkins et al., 2003). Additionally to the chloramines and their by-products, several stable chlorinated products could be identified e.g. 5-chlorocytosine, 5-chlorouracil, 8-chloroadenine and 8-chloroguanosine that lead to protein inactivation and aggregation (Whiteman et al., 1997; Henderson et al., 1999; Masuda et al., 2001).

Another destructive and bactericidal effect of ROS arises from protein damage. Proteins expose several different sites to oxidative attacks. The different reaction sites for ROS/RCS in proteins have already been discussed in section I-2 and I-3. Most proteins are irreversibly damaged by over-oxidation of sulfur atoms, especially in cysteine and methionine residues, concomitantly resulting in cumulative unfolding and aggregation (Leichert et al., 2008; Rosen et al., 2009) . This was first demonstrated for fibronectin and low density lipoprotein (LDL). Both proteins were treated with increasing concentrations of HOCl resulting in aggregation, whereby the aggregates could not be eliminated under reducing conditions (Vissers and Winterbourn, 1991; O'Connell et al., 1994). Moreover, it was demonstrated that HOCl results in proteome-wide unfolding (Winter et al., 2008). First evidences for amino acid and protein damaging by ROS were shown by Brown and coworker in 1976. They demonstrated that hyperbaric oxygen (one-hundred percent oxygen under high pressure) results in amino acid auxotrophies in *E. coli* (Boehme et al., 1976). Moreover, another study revealed that *E. coli* SOD-deficient mutants lose their ability to grow unless they were supplemented with branched-chain (Leu, Ile, Val), aromatic (Tyr, Trp, Phe) and sulfurous (Cys, Met) amino acids (Carlioz and Touati, 1986; Imlay, 2013). Noteworthy, several proteins can be carbonylated via metal-catalyzed oxidation reactions (Dukan et al., 2000).

Besides, enzymes containing a single metal atom as prosthetic group are inactivated by H_2O_2 or O_2^- via protein-associated Fenton reaction. The iron of these mononuclear enzymes is able to directly bind substrate and stabilizes the resulting oxyanionic intermediate thereby catalyzing several reactions. Examples for such enzymes are epimerases, dehydratases, dehydrogenases, deformylases, and deaminases (Sobata and Imlay, 2011; Anjem and Imlay, 2012; Gu and Imlay, 2013). The most vulnerable metal-containing sites of proteins are iron-sulfur clusters, commonly part of the catalytic center. Thus, the origin for the branched-side chain amino acid auxotrophy mentioned above, induced by O_2^- treatment in SOD-deficient mutants, arises by the destruction of the [4Fe-4S]-cluster of dihydroxyacid

dehydratase (Kuo et al., 1987). Further, enzymes of the tricarboxylic acid cycle such as aconitase A and aconitase B, respectively, and fumurase A contain [4Fe-4S] clusters and explain the inability of SOD mutants to grow prototroph (Imlay, 2013).

II. Bacterial Responses to Oxidative Stress

Bacterial cells continuously face hostile environments. Thus, they have to adapt rapidly to new situations and conditions. Consequently, bacterial cells developed several different stress proteins, on the one hand recognizing the new stress situation and initiating the stress response, and on the other hand proteins acting directly as countermeasures. Therefore, cells express some few global regulators controlling the overall conditions in the cell in a long term perspective, and many other regulators responding immediately to specific stress and inducing the production of repair enzymes or the repression of genes, which would lead to further cell harm. These counteractive proteins act at different points in the cell. Some enzymes can directly act against reactive species by scavenging them. Others repair the damages resulting from the stress. Interestingly, most of the stress responsive elements in bacterial cells are activated by the destructive modifications caused by the stress factors that normally deploy their bactericidal effects.

II-1 Global Bacterial Defense Mechanisms against ROS / RCS

Oxidative stress, no matter in which form it envelops its destructive nature, has to be first detected by stress responsive regulators in order to initiate appropriate countermeasures. Therefore, bacterial cells have developed several transcriptional regulators activated by several or only one specific ROS. One such regulator is SoxR, which belongs to the MerR transcriptional regulator family (Greenberg et al., 1990). In *E. coli* it regulates only one gene, *soxS*. SoxR is basically activated by O_2^- by oxidation of its [2Fe-2S] cluster in the active center, but can also be activated by NO and redox-cycling drugs (Ding and Demple, 1997; Demple et al., 2002; Gu and Imlay, 2011). Concomitantly, the SoxR activation leads to the transcription of *soxS*. SoxS initiates the transcription of several genes encoding SOD, DNA repair enzymes like endonuclease IV, and O_2^- -resistant dehydratases (Storz and Hengge, 2011). A similar transcriptional regulator that is activated by O_2^- is PqrR from *Pseudomonas aeruginosa*, which belongs to the MarR family. PqrR contains, like SoxR, an iron-sulfur cluster in its active center and mediates the oxidative stress response (Rungrassamee et al., 2009).

One of the best characterized redox-sensitive transcriptional regulators is OxyR, which belongs to the LysR-type transcriptional regulator (LTTR) protein family (Kullik et al., 1995).

OxyR is activated specifically upon H_2O_2 stress. Activation is accomplished by the oxidation of two conserved cysteine residues, concomitantly forming a disulfide bond. Thus, OxyR activation induces the expression of several antioxidant genes encoding catalases, AhpC peroxidases, AhpF reductases, the iron sequestering protein Dps, thioredoxin, glutaredoxin, and metal transporters (Imlay, 2008; Storz and Hengge, 2011; Gray et al., 2013b).

H_2O_2 detection in *Bacillus subtilis* is mediated by PerR, which belongs to the Fur family. PerR binds under non-stress conditions a single ferrous iron, thus enabling DNA binding and repression of oxidative stress responsive elements. PerR contains a structural Zn^{2+} and a regulatory Fe^{2+} metal center. Upon H_2O_2 stress the ferrous iron is oxidized in a direct Fenton reaction to ferric iron, concomitantly generating a hydroxyl radical. This ·OH directly oxidizes a metal-coordinating histidyl residue, generating 2-oxo-histidine. Consequently, this reaction results in the dissociation of the ferric iron, irreversibly impeding DNA binding and therefore resulting in expression of stress responsive genes (Bsat et al., 1996; Lee and Helmann, 2006; Imlay, 2008). Noteworthy, PerR responds also to HOCl. It was demonstrated that HOCl additionally oxidizes the Zn^{2+} ion, in contrast to H_2O_2 (Lee and Helmann, 2007). However, it is not completely determined, if HOCl activates PerR by a different mechanism than H_2O_2 (Gray et al., 2013b).

Recently, two HOCl-specific transcriptional regulators in *E. coli* have been identified (Gray et al., 2013b). NemR, which belongs to the TetR repressor family, senses HOCl stress via redox-sensitive cysteine residues. Upon the reversible cysteine oxidation, DNA binding is inhibited and target gene regulation is initiated. Interestingly, NemR is exclusively activated by RCS such as HOCl and taurine chloramine. Neither ROS nor reactive nitrogen species (RNS) result in target gene regulation. NemR regulates electrophile detoxifying enzymes, including *N*-ethylmaleimide reductase and glyoxalase I (Gray et al, 2013a). The other HOCl-specific transcriptional regulator is HypT, formerly known as YjiE. HypT belongs, like OxyR, to the LTTRs. HypT is reversibly activated by methionine oxidation and regulates for example iron acquisition and amino acid biosynthesis (Gebendorfer et al., 2012; Drazic et al., 2013a).

II-2 Bacterial Repair Mechanisms

Several different enzymes are up-regulated by oxidative stress sensing transcriptional regulators, specialized in direct repair of the occurring damages or in the degrading of the reactive species. One of these scavengers is superoxide dismutase. Different isoforms of

SOD are expressed in *E. coli*. Two isoforms, one manganese- and an iron-cofactor type, act in the cytoplasm, whereas a third copper-, zinc-factored SOD is located in the periplasm (Imlay, 2008). SOD scavenges O_2^- by catalyzing the dismutation of O_2^- to O_2 and H_2O_2 (Fig. 5). The catalytic efficiency of SOD is very high and therefore restricts the intracellular steady-state level of O_2^- to approximately 0.1 nM (Imlay and Fridovich, 1991). The exact role of the periplasmic SOD has not been fully elucidated, but there is evidence that it plays a crucial role for bacterial pathogens to withstand oxidative burst of the host's innate immune system (De Groote et al., 1997; Imlay, 2008).

H_2O_2 is degraded by peroxidases and catalases generating O_2 and H_2O (Fig. 5). Catalase is probably one of the fastest enzymes with catalytic rate constants from 54,000 to 833,000 s^{-1} (Chelikani et al., 2004). The major peroxiredoxin system in *E. coli* is the NADH-dependent AhpCF two-component system. H_2O_2 directly oxidizes a cysteine residue of AhpC forming a sulfenic acid. This sulfenic acid condenses with another cysteine to a disulfide bond, which in return is regenerated by the NADH-reducible flavoprotein AhpF (Imlay, 2008). Ahp activity is like the SOD activity relatively high, which keeps the steady-state H_2O_2 concentration in the cell to a limit of 20 nM. If upon oxidative burst Ahp is saturated and the H_2O_2 concentration exceeds 0.1 µM, OxyR becomes activated and initiates additional countermeasures (Seaver and Imlay, 2001).

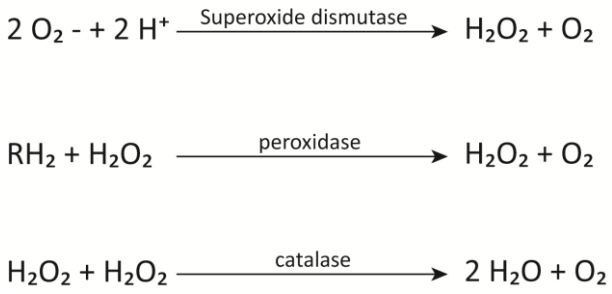

Figure 5 Catalyzed ROS detoxification reactions. O_2^- detoxification is catalyzed by superoxide dismutase. H_2O_2 detoxification is catalyzed by peroxidase and catalase.

Not all ROS can be intercepted in time and so they damage various biological macromolecules. Therefore, bacteria developed several repair enzymes. One of the bactericidal damage is the oxidation of iron-sulfur clusters. Unfortunately, exact repair mechanisms for iron-sulfur cluster repair could not be revealed until now. Several studies indicate roles of different enzymes (e.g. YtfE, YggX, and YgfZ) in this process that are in part regulated by SoxR, and identified catalytic important residues. Nevertheless, the biochemical mechanism remains unclear (Imlay, 2008; Husnain et al., 2012). Moreover, bacterial cells up-

regulate their *de novo* iron-sulfur cluster assembly systems, Isc and Suf, under oxidative stress. Thereby, it seems that the Suf machinery is more a back-up system for the Isc machinery. The Suf machinery is more efficient under H_2O_2 stress than the Isc system, explaining the fact that Suf is induced by OxyR (Outten et al., 2004). It was demonstrated that H_2O_2 inhibits Isc activity, concomitantly the more oxidation resistant Suf system has to support the Isc system (Tokumoto et al., 2004).

Besides the repair of iron-sulfur clusters, the control of unincorporated iron levels is essential for acting against oxidative stress. As mentioned before, ferrous iron and H_2O_2 can generate additional ·OH by the Fenton chemistry, enhancing the destructive effects of the oxidative stress. In most bacteria iron homeostasis is regulated by the transcriptional regulator Fur. The Fur protein is activated by binding ferrous iron, concomitantly inducing the expression of iron-import genes (Neilands, 1993). Bacteria with a *fur* deletion are more susceptible towards exogenous H_2O_2 and show higher rates of mutagenesis (Keyer and Imlay, 1996; Imlay, 2008). The regulation of the *fur* gene is under the control of OxyR and SoxS during oxidative stress (Zheng et al., 1999). Moreover, bacterial cells can directly scavenge excessive unincorporated iron using the iron storage proteins ferritin and Dps. Ferritin is strongly induced by the OxyR and PerR systems (Imlay, 2008).

The inactivation of proteins occurs constantly by the oxidation of cysteine and methionine residues. Bacteria express a whole bunch of reductases to reverse these modifications. The two enantiomers of methionine sulfoxide are reduced by methionine sulfoxide reductases MsrA, MsrB and fRMsr (Ezraty et al., 2005). Importantly, only methionine sulfoxides, not methionine sulfones can be reduced by this system. The primary reductases for intracellular disulfide bonds are thioredoxin and glutaredoxin (Ritz and Beckwith, 2001). Further specific reductases have been identified in *E. coli*, including arsenate reductases and the periplasmic disulfide reducing system DsbC (Imlay, 2008).

Another important bacterial defense mechanism is the DNA damage response. The major DNA repair systems comprise the base-excision repair system and the *rec* recombination system. The base-excision repair system relies on DNA glycosylases that recognize damaged and modified DNA bases. In *E. coli* these glycosylases are MutM and MutY (Michaels et al., 1992). Moreover, endonucleases recognize specific double helix disruptions caused by oxidation of pyrimidine residues (Imlay, 2008). The complete DNA repair mechanism is activated by cleavage of the dimeric LexA repressor (Butala et al., 2009). Therefore, RecA binds to LexA and DNA, forming a filamentous structure, concomitantly forcing LexA into a self-cleavage competent conformational state (Luo et al., 2001; vanLoock et al., 2003). Noteworthy, in *E. coli* the production of DNA repair proteins can also be induced

in a LexA/RecA-independent manner (Hong et al., 2009). This alternative pathway is under the control of IraD and the RpoS regulon (Merrikh et al., 2009; Storz and Hengge, 2011)

II-3 ROS / RCS Induced Changes in Bacterial Metabolism

Oxidative stress has global effects on bacterial cells including altered protein and transcriptional activity. Consequently, oxidative stress should have a huge impact on the metabolism of the bacterial cell. Concomitantly, the cell should direct its metabolic fluxes in a way that counteract the stress-induced metabolic changes. Several studies exist that investigate the effects of oxidative stress (e.g. H_2O_2- and paraquat-induced stress) by metabolic flux analysis (Shi et al., 2013). Unfortunately, such analyses are missing in the case of RCS stress. All these studies revealed in principle the same changes in the metabolism upon oxidative stress, especially in the central carbon metabolism. Glycolysis and the tricarboxylic acid cycle are down-regulated, whereas glucose flux is re-directed to the NADPH-generating pentose phosphate pathway. Thus, the NADPH pool increases to use it in reductive detoxifying reactions (Rui et al., 2010; Jozefczuk et al., 2010; Valdivia-Gonzàlez et al., 2012; Shen et al., 2013; Gray et al., 2013b). Moreover, it was shown that the *trans*-hydrogenase pathway does not play a direct role in the defense of *E. coli* to oxidative stress (Shen et al., 2012). Most of these changes are generated by post-translational modifications of the appropriate enzymes. Several enzymes involved in glycolysis or tricarboxylic acid cycle contain conserved cysteine residues that are crucial for their enzymatic activity. Thus, oxidative stress results in oxidation of these cysteine residues, concomitantly inactivating them. Such enzymes are glyceraldehyde-3'-phosphate dehydrogenase, phosphoglycerate kinase, aconitase, malate synthase and malate dehydrogenase (Leichert et al, 2008; Gray et al., 2013b).

Some of the mentioned enzymes play also a role in HOCl stress. *E.* coli mutants lacking aconitase or malate dehydrogenase show a higher susceptibility towards HOCl stress (Leichert et al., 2008). Transcriptome analysis revealed in *E. coli* that the production of enzymes, involved in electron transport, oxidative-phosphorylation and carbon-utilization, are altered upon HOCl stress (Gebendorfer et al., 2012). Moreover, it seems that to some extent the catabolite repression system plays a role in H_2O_2- and HOCl resistance. Stress induced decrease of cellular cAMP levels results in inactivation of the cAMP receptor protein (CRP). Subsequently, down-regulation of CRP leads to the de-repression of RpoS and therefore initiating the general stress response in *E. coli* (Barth et al., 2009).

III. Characteristics of LysR Type Transcriptional Regulators (LTTRs)

LTTRs are a large family of transcriptional regulators. They are ubiquitous and highly conserved among bacteria, strictly speaking the LTTRs are the most abundant type of prokaryotic transcriptional regulators. Nevertheless, the number of LTTRs among the different species is highly variable. The *E. coli* K 12 strain MG1655 contains 47 members, whereas *P. aeruginosa* PA01 has 121 different LTTRs (Tatusov et al., 2001). In contrast, *Helicobacter pylori* lacks completely any LTTRs (Knapp and Hu, 2010). Moreover, functional orthologues have been identified in archaea and eukaryotes (Maddocks and Oyston, 2008). In many cases, the gene encoding the LTTRs is located upstream of its divergently transcribed target gene clusters (Tropel and van der Meer, 2004). The LTTRs regulate a wide range of different genes, involved in all kind of metabolic and regulatory processes and represent therefore one of the most important protein families in prokaryotes.

III-1 Function, Structure and Oligomerization of LTTRs

LTTRs are involved in the regulation of various genes, including virulence factors, metabolism, nitrogen source utilization, amino acid biosynthesis and catabolism, detoxification of the cell, quorum sensing, stress response and motility (Schell, 1993; Maddocks and Oyston, 2008). The eponym for this protein family is the *lysA* transcriptional activator LysR. The *lysA* gene encodes the enzyme diaminopimelate decarboxylase, which catalyzes the decarboxylation of diaminopimelate to generate the amino acid lysine (Stragier et al., 1983). LTTRs can function as activators as well as repressors of single and operonic genes. They often auto-regulate negatively and positively, respectively. These feedback loops are initiated by the recognition of co-inducers, basically products or intermediates of the given metabolic pathway that are regulated by the LTTRs. One such LTTR that is negatively auto-regulated, is ArgP. It controls the protein ArgK, which is important for the operation of the arginine transport system. In the presence of L-arginine ArgP inhibits its own synthesis (Celis, 1999). Other LTTRs does not exhibit auto-regulation like the HOCl-specific transcription factor HypT (Gebendorfer et al., 2012).

LTTRs are structurally and functionally conserved. Most LTTRs consist of about 300 amino acids and comprise a N-terminal helix-turn-helix (HTH) motif, which confer the DNA binding ability, and a C-terminal co-inducer binding domain, although not every LTTR binds a co-factor. The HTH motif is present in 95% of all prokaryotic LTTRs, thus the other 5% of the

DNA binding motifs are helix-loop-helix, zinc-finger or *β*-sheet-anti-parallel domains (Aravind et al., 2005; Maddocks and Oyston, 2008). There exists a further variation that is deviated from the HTH motif, the so called winged-helix-turn-helix (wHTH) motif. The difference between the HTH and the wHTH motifs is besides the common three-helical bundles, a single anti-parallel *β* sheet region. In contrast to most HTH-containing transcriptional regulators, which differentiate structurally between activators and repressors (e.g. transcriptional activators have a C-terminal HTH motif, whereas transcriptional repressors have a N-terminal located HTH-motif), the HTH motifs of LTTRs are always located at the N-terminus (Schell, 1993; Pérez-Rueda and Collado-Vides, 2001). In many LTTRs the residues 20-80 are involved in DNA binding and form also the most conserved region, whereas the C-terminus shows little conservation. As already mentioned this region of the regulators is basically involved in co-inducer binding (Stec et al, 2006). Nevertheless, there is evidence that an approximately 80 amino acid region in the C-terminus plays a crucial role in DNA binding, especially upon co-factor binding, which results in conformational changes and altered DNA-protein interactions (Huang and Schell, 1991; Ezezika et al., 2007; Monferrer et al., 2010).

An important characteristic of LTTRs for their activation is their oligomeric state. Most LTTRs form tetramers in their active state consisting of two dimers. This was first shown by the crystal structure of CbnR, a LTTR that is involved in degradation of chlorocatechols (Muraoka et al., 2003). Another LTTR is OxyR, which is sensing H_2O_2. Thus, H_2O_2 activates OxyR by the formation of a transient disulfide bond between the cysteines 199 and 208. This disulfide bond formation results in conformational rearrangements. OxyR exists as a dimer of dimers, thus it forms a tetrameric structure (Kullik et al., 1995; Choi et al., 2001). Besides, there exist several LTTRs that form more unusual higher oligomeric structures. The LTTR CrgA from *Neisseria meningitides*, which is up-regulated during bacterial-host cell contact, forms stable octameric rings. Crystal structure and electromobility shift assays revealed that CrgA in CrgA-DNA complexes exist as octamer. Nevertheless, mass spectrometry (MS) analysis also indicates that DNA can be bound by two octameric rings, forming together a hexadecameric array (Sainsbury et al., 2009). Moreover, HypT in *E. coli* forms dodecameric ring-like structures in the inactive, non-binding state. Upon activation and DNA presence, respectively, HypT dissociates into several different oligomers. The active, DNA-binding oligomeric state is most probably a dimer of dimers and therefore a tetramer (Gebendorfer et al., 2012; Drazic et al., 2013a). Interestingly, there are some indications that regulatory specificity of LTTRs depends on the formation of hetero-oligomers with non-cognate partner. But, the physiological relevance and the physical basis for these interactions have to be further investigated (Knapp and Hu, 2010).

III-2 Activation of LTTRs and Effects on DNA Binding

As discussed above, the activation and DNA binding ability of LTTRs depend on the presence and absence of co-factors, the oligomeric state and in some cases on potential post-translational modifications. LTTRs bind at multiple DNA binding sites, upstream of their regulated target genes. The LTTRs distinguish between a regulatory binding site (RBS) and an activation binding site (ABS). The RBS lies normally - 35 to + 20 bp of the target gene, whereas the ABS is located at – 40 to – 20 bp referred to the target gene (Lochowska et al., 2001). Albeit, binding sites at – 218 bp as well as + 350 bp have been identified (Maddocks and Oyston, 2008). Thus, LTTRs bind to large regions of the DNA promoter (e.g. 50 to 60 bp) (Muraoka et al., 2003). Normally, a dimer binds in the absence of a co-inducer only to the RBS, whereas upon co-inducer binding a second dimer is able to occupy the ABS, concomitantly resulting in tetramerization (Tropel and van der Meer, 2004). In the case of OxyR, the reduced dimer has contact with two adjacent major grooves, whereas the oxidized OxyR interacts with four major grooves of the DNA. Upon oxidation OxyR repositions its DNA contacts and thereby changes its target promoters. Noteworthy, OxyR functions as activator and repressor, respectively (Toledano et al., 1994).

DNA bending plays an important role in LTTR regulation. Several LTTRs can bend the DNA between 50° and 100°. This bending is dependent on the binding of a co-inducer. Basically, the binding of a co-factor results in the relaxation of the DNA from 9° to 50°. The bending of DNA determines the area bound by the LTTR and seems to be crucial for transcriptional activation or repression (Fig. 6). This mechanism provides additional regulatory possibilities and further controls the interaction between LTTR and RNA polymerase (van Keulen et al., 1998; Akakura and Winans, 2002a,b).

Interestingly, the oligomeric state, the amino acid sequence and the DNA bending seems to be arbitrary in respect to the determination of the LTTR function as activator or repressor. The prediction of this determination has still to be revealed (Maddocks and Oyston, 2008).

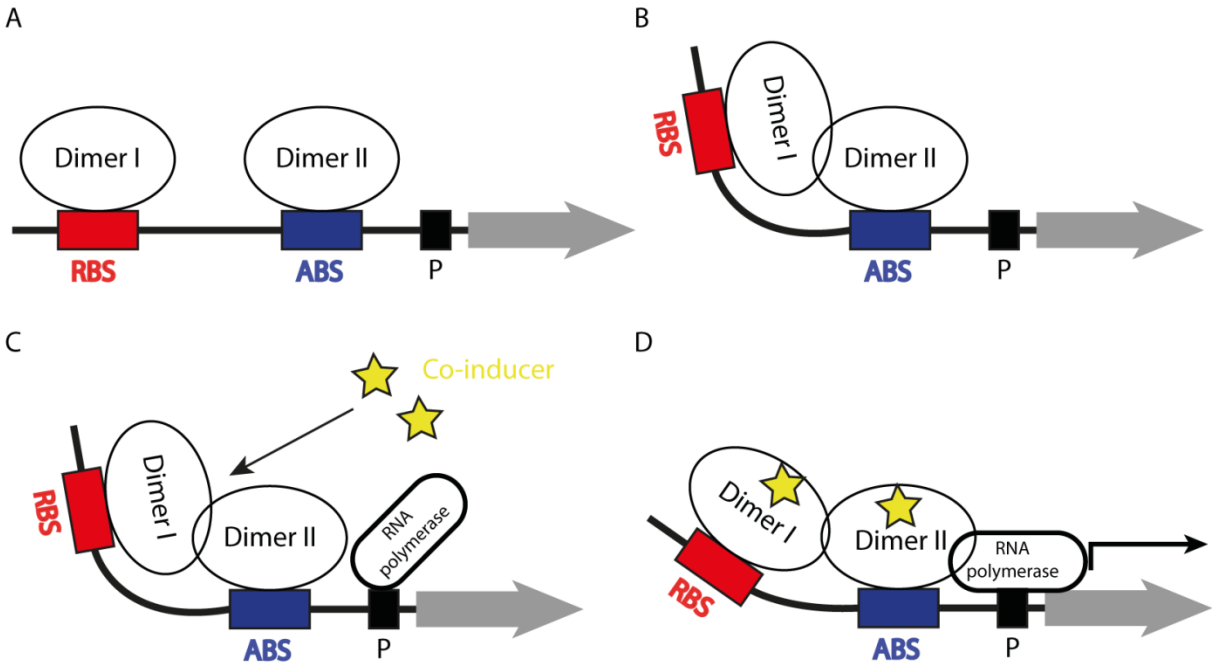

Figure 6 Scheme of interaction between DNA and LTTR. A) LTTR dimer I binds to the regulatory binding site (RBS) and dimer II binds to the activation binding site (ABS) of the DNA. B) Protein-protein interaction between the two LTTR dimers to a tetramer results in DNA bending. C) RNA polymerase binds to the promoter region without transcriptional activity in the absence of LTTR co-inducers. D) Co-inducer binding leads to conformational changes in the LTTR structure resulting in DNA bend relaxation. Concomitantly, the LTTR tetramer interacts with the RNA polymerase at the promoter of the target gene, leading to transcriptional activation. Figure was adopted and altered from Maddocks and Oyston, 2008.

III-3 LTTRs Involved in ROS / RCS Induced Bacterial Responses

There are several LTTRs that are activated upon various stress conditions. OxyR is the master regulator upon H_2O_2 stress in *E. coli*. The oxidative condition results in a disulfide bond formation between the cysteine residues 199 and 208, altering the conformation and thus leading to activation of OxyR. Thereby, oxidized OxyR induces the transcription of the oxidative stress response genes (Zheng et al., 2001). A similar mechanism, which involves a cysteine residue, developed the LTTR OxyS in *Mycobacterium tuberculosis*. OxyS represses the expression of *katG* and its activity is inhibited by H_2O_2 stress, thereby losing its ability to bind to promoter DNA. Thus, overexpression of *oxyS* results in higher sensitivity towards oxidative stress. In this case, OxyS does not form a disulfide bond, but the oxidation of the single cysteine residue 25 is sufficient to inactivate the regulator (Li and He, 2012).

Another example for specific LTTR activation upon oxidative stress is PA2206 of *P. aeruginosa*. PA2206 is required for H_2O_2 tolerance, and also for *in vivo* lethality in a Zebrafish embryo model of infection. PA2206 alters the expression of 58 different genes, including oxidative stress and iron responsive genes, and PA2206 regulation functions independently of OxyR (Reen et al., 2013). Further, in the alphaproteobacterium *Caulobacter crescentus* the LTTR CztR has been identified, which regulates the response to several stress conditions. First, it was shown that a *cztR* ⁻ strain is sensitive to cadmium and H_2O_2, respectively. Concomitantly, the regulator is up-regulated when cells enter the stationary phase or suffer from zinc depletion (Braz et al., 2010). The LTTR HypR from *Enterococcus faecalis* is a transcriptional regulator playing a crucial role in oxidative stress response and in the intracellular survival of this bacterium within macrophages. HypR was determined as transcriptional activator and a *hypR* ⁻ strain showed decreased infectivity in a mouse peritonitis model (Verneuil et al., 2005).

Recently, the hypochlorite-specific LTTR HypT / YjiE was identified regulating several genes involved in amino acid biosynthesis and iron acquisition (Gebendorfer et al., 2012). HypT is activated by the oxidation of three methionine residues to methionine sulfoxide (Drazic et al., 2013a)

IV. Summary of the PhD Thesis

This PhD thesis contained two objectives. One objective was the characterization, the newly identified transcriptional regulator HypT, formerly known as YjiE. Therefore, its function upon hypochlorite-induced stress and the activation mechanism have been determined by biochemical methods. The second aim of this work was to elucidate the effects of HOCl stress on the metabolism of *E. coli*. HypT is a LysR-type transcriptional regulator with an atypical oligomeric state and a new activation mechanism for redox-responsive proteins. This work revealed the interplay of the oligomeric state, the target gene regulation and the post-translational modifications that lead to the activation of HypT and its regulation. HypT is an exclusive hypochlorite-specific transcription factor. Moreover, HypT forms in its active state a DNA binding tetramer that differs from the atypical dodecameric storage form of its inactive state. This dissociation is caused by various additives, including DNA itself, high arginine and salt concentrations. Activation is enabled by the oxidation of three of six methionine residues (Met 123, M 206, and Met 230) to methionine sulfoxide. Further, this work revealed that the activation is reversible and dependent on the activity of methionine sulfoxide reductases *in vitro* and *in vivo*, respectively. The substitution of these three important methionines by glutamines, mimicking the methionine sulfoxide state, generates a constitutively active mutant. This mutant is able to bind to DNA and regulates target genes also in the absence of HOCl. Importantly, this mutant exists exclusively as tetramer. The cysteine residues often play important roles in redox-sensing proteins, but do not play a direct role in HypT activity. For two of five cysteines a function could be determined. Oxidation of cysteine 4 results in a reversible loss of DNA binding ability of HypT. Nevertheless, this seems to be a phenomenon exclusive for *in vitro* assays. In contrast, cysteine 150 is crucial for the overall stability of HypT. However, a structure is crucial to identify the precise mechanism of conformational changes and the contribution of the single amino acids that are involved in stability and activation. Further, this work could show that exposure of *E. coli* to HOCl leads to alterations in the level of some metabolites determined by gas-coupled mass spectroscopy. Other metabolites were not affected by HOCl and their levels were maintained in a steady state. Moreover, it could be shown that HOCl reacts very fast and that the cell can adapt quickly, which was demonstrated by the regeneration of some metabolite levels already 20 min after stress induction. Nevertheless, the exact pathways, which are involved in this fast adaptation have still to be revealed and are part of further investigations.

PART B

Characteristics of HypT and Metabolic Responses upon HOCl stress

V. Structure, Oligomerization and Specificity of HypT

V-1 Modeled Structure of HypT

HypT belongs to the large prokaryotic family of LTTRs. Several crystal structures of truncated proteins of this family have been solved in the last years (e.g. OxyR; Choi et al., 2001; DntR; Devesse et al., 2011) whereas only a few full length structures are available (e.g. CbnR; Muraoka et al., 2003; CrgA; Sainsbury et al., 2009; TsaR; Monferrer et al., 2008). Unfortunately, until now it was not possible to generate crystals of HypT diffracting sufficiently to solve its structure by X-ray. It was possible to model the three-dimensional structure with the online available tool Phyre2 (Protein Homology/analogY Recognition Engine V 2.0; Kelley and Sternberg, 2009). This tool processes the protein sequence and compares it with over 10 million known sequences by PSI-BLAST analysis to find evolutionary relationships. By aligning with the homologous sequences, the tool can create an evolutionary fingerprint, called hidden Markov model (HMM). Further, it compares and aligns the newly created HMM of HypT with HMMs, which were created in the same way from over 65000 known structures. Using this cross-evolutionary method the algorithm is able to generate a three-dimensional structure based on known and similarly folded proteins. In addition, the tool uses the so called Poing2 *ab initio* folding simulation, which models regions of no homology to known structures by physics simulation (Söding, 2005; Jeffereys et al. 2010). The modeled structure of HypT was compared with the modeled structure of reduced OxyR as well as with the crystal structure of the reduced $OxyR^{C199S}$ mutant (PDB: 1l69; Choi et al., 2001). Both, the modeled and the crystal structure of OxyR showed high structural similarities with the modeled structure of HypT (Fig. 7), although they share only 26% sequence homology. Analyzing the prediction of the secondary structure (Drazic et al., 2013a) and the structural model (Fig. 7), respectively, the methionine residues 123, 206 and 230, which are important for HypT's activation (discussed in section IV-4), are located in respective domains and areas, which undergo structural re-arrangements upon OxyR activation. Although the model is not equitable with an experimentally determined structure, it is suitable to support and understand the function of HypT.

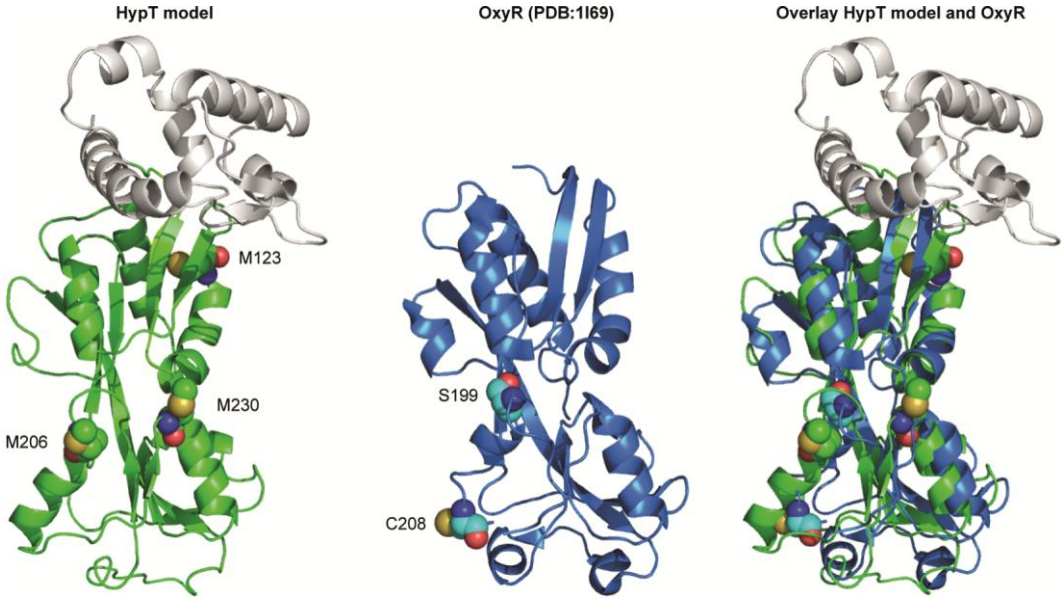

Figure 7 Structural model of HypT predicted using the Phyre 2 software (left panel). Crystal structure of OxyR (Choi et al., 2001; middle panel). Overlay of both structures for comparison (Drazic et al., 2013a; right panel). Amino acid residues that are important for activation (Met in HypT and Cys in OxyR) are indicated as spheres.

V-2 Dynamic Oligomeric State

LTTRs usually form different oligomeric states, which may change upon activation (Maddocks and Oyston, 2008). Typically this goes along with a change in DNA binding ability providing the possibility to activate or repress their appropriate target genes (Schell, 1993). HypT forms an atypical dodecameric structure *in vitro*. HypT sediments with 10 - 11 S as analyzed by sedimentation velocity runs using analytical ultracentrifugation (aUC; Gebendorfer et al., 2012). Analyzing the purified protein by transmission electron microscopy (TEM) revealed ring-like hexagonal structures with a small proportion of molecules with 5-fold/7-fold symmetry (Gebendorfer et al., 2012).

The oligomerization state of HypT can be altered by various additives *in vitro*. In the presence of target as well as non-target DNA, the purified protein dissociates into species of 3.7 and 5.5 S, likely corresponding to dimers and tetramers. TEM images of HypT incubated with DNA revealed that the protein exists mainly as tetramer, which seems to be formed by dimers. Unfortunately, the localization of the DNA on HypT was not detectable by TEM (Gebendorfer et al., 2012).

In vivo oligomerization depends on the expression level of HypT. Of note, the dodecameric state of HypT basically occurs in overexpressing cells. Besides, two additional species of 4.1 and 7.1 S were observed, respectively. Smaller oligomers have a higher abundance in cells with wild-type like expression levels (Gebendorfer et al., 2012). $HypT^{FlAsH}$ (protein competent of fluorescence; Griffin et al., 2000) cells, which were labeled before cell disruption and afterwards analyzed by aUC revealed that the abundance of smaller oligomers is relatively high compared to the dodecameric species. If the cells were treated with HOCl after FlAsH-labeling and then analyzed, a new additional species was detected at 2.6 S, likely corresponding to a monomer. Taken together, it is possible that the smaller species represent the physiological relevant species whereas the dodecamer seems to be an artificial storage form due the high HypT concentration in HypT overexpressing cells (Gebendorfer et al., 2012).

V-3 Hypochlorite-Specific Stress Response

LTTRs mediating resistance against oxidative stress are activated by different mechanism and different stress effectors (see section II-3). HypT mediates stress resistance specifically against HOCl stress. Target gene regulation and increased viability compared to a *hypT* - strain was exclusively observed when *E. coli* cells were treated with HOCl. Neither diamide, and H_2O_2, nor NH_2Cl, nor hydroxyl radicals, or methionine sulfoxide were able to activate HypT and induce a HypT-specific stress response (Gebendorfer et al, 2012; Drazic et al., 2013b). HypT regulates several target genes, which are up and down-regulated, respectively. Genes that are up-regulated in a HypT-specific manner are involved in sulfur, methionine and cysteine metabolism (e.g. *metB, metK, metN, cysH, cysK, cysN, cysPUW, sbp, sufA*), in the biosynthesis of other amino acids, or have unknown functions. The majority of genes that are down-regulated are involved in iron acquisition and homeostasis (e.g. *entC, entH, fecABCDE, fecR, fepCD, ryhB, tonB, yncE*; and *entEBA, ybiX*), or encodes transporters of sugar, peptides, and amino acids or RNA-related genes (Gebendorfer et al., 2012).

V-4 Role of Methionine Residues upon HOCl Stress

HypT contains six methionines at the positions 1, 123, 206, 230, 280, and 284 whereby the first methionine can be post-translationally processed and removed. LTQ Orbitrap mass

spectrometry experiments revealed that the methionines in HypT were oxidized to Met-SO, upon HOCl stress *in vivo*, and, albeit with a low detection frequency, di-oxidized to methionine sulfone (Met 1 and Met 123). Oxidation occurs in a HOCl-concentration dependent manner. Met 123 was found exclusively oxidized to Met-SO upon HOCl stress. The frequency of oxidation to Met-SO increased at position 1, 230 and 284 with higher HOCl concentrations. Met 206 and Met 280 could only be detected with a very low frequency in the reduced state (Drazic et al., 2013a).

Methionine residues can be substituted with glutamine to mimic the Met-SO state or with isoleucine to mimic the non-oxidizable reduced methionine state (Vogt, 2005). To test the role of Met-SO on HypT activity, methionines (except Met 1) were replaced individually by either a glutamine (M→Q) or an isoleucine (M→I). Each M→Q mutant showed wild-type like viability upon HOCl stress, indicating that all glutamine mutants are functional. Nevertheless, the individual substitution of Met 123, Met 206 and Met 230 by isoleucine led to the inability of the proteins to confer HOCl resistance and the mutants showed control-like viability (strain harboring an empty pBAD22 plasmid). In contrast, the substitution of Met 280 and Met 284 by isoleucine conferred HOCl resistance, thus these methionine residues were not important for activation (Drazic et al. 2013a).

To further elucidate the role of Met 123, Met 206 and Met 230 regarding HypT activity, mutants were generated in which these methionine residues were replaced simultaneously by either glutamine ($HypT^{M123,206,230Q}$) or isoleucine ($HypT^{M123,206,230I}$). The expression of $hypT^{M123,206,230Q}$ facilitates HOCl resistance and regulates strongly the RNA levels of target genes (e.g. *metN, cysH, metB, and fecD*) upon HOCl stress. The level of regulation upon stress of the triple M→Q mutant is comparable to the levels of *hypT*-overexpressing cells, indicating that the mutant is fully functional. However, expression of the $hypT^{M123,206,230I}$ mutant renders the protein unable to confer resistance. It showed control-like viability and target gene regulation (Drazic et al., 2013a). Thus, the oxidation of all three methionine residues (Met 123, Met 206, and Met 230) is crucial for the activation of HypT.

The substitution of the three important methionines, no matter if by glutamine or isoleucine, resulted in an oligomerization state that differed from the one of wild-type and unstressed HypT (~6.7S). Instead of forming dodecamers as the wild-type HypT, the mutants formed tetramers *in vitro*. This suggests that the methionine residues are at least in part responsible for forming the quaternary structure of HypT. This hypothesis is further confirmed by the fact that *in vivo* activated HypT ($HypT\text{-}HOCl^{2.75mM}$), sediments as a single species at 6.8 S, corresponding to a tetramer (Drazic et al., 2013a).

According to the observations above, it can be assumed that methionine oxidation to Met-SO of the methionine Met 123, Met 206 and Met 230 facilitates HypT activation. Therefore, the triple M→Q substitution should generate a constitutively active HypT mutant independently of HOCl stress. To test this assumption, target gene regulation of unstressed C600 cells carrying an empty plasmid (control), overexpressing wild-type *hypT*, *hypT*^{M123,206,230Q} or *hypT*^{M123,206,230I}, respectively, was analyzed. Expression of mutants was induced by arabinose addition to follow time dependent target gene regulation. The expression of *hypT*^{M123,206,230Q} facilitates a significant target gene regulation of *metN, cysB, metB,* and *fecD* with a maximum after 20-40 min. In contrast, the RNA levels of the target genes are less or not affected by expression of the control, *hypT* or *hypT*^{M123,206,230I}, respectively (Drazic et al., 2013a).

Down-regulation of *fecD* normally facilitates the decreased import and intracellular acquisition of iron (Staudenmaier et al., 1989), concomitantly resulting in decreased unincorporated intracellular iron levels. The analysis of free intracellular iron levels *in vivo* by electron paramagnetic resonance spectrometry (EPR; Keyer and Imlay, 1997) revealed that cells expressing the constitutively active mutant HypT^{M123,206,230Q} show over 50% lower unincorporated iron levels than cells expressing the control plasmid or wild-type *hypT*, supporting the constitutive activity of the mutant (Drazic et al., 2013a). Iron plays an important role in oxidative stress (Rosen and Klebanoff, 1982; Keyer and Imlay, 1996). Ferrous iron (Fe^{2+}) can generate toxic ·OH in presence of H_2O_2 or HOCl via the Fenton reaction (Candeias et al., 1994), increasing the damaging effects of ROS / RCS induced stress. An *E. coli recA*$^-$ strain (RecA is a DNA strand exchange and recombination protein; *recA*$^-$ is strongly impaired in DNA repair) suffers from increased DNA damage upon H_2O_2 stress due to high ·OH levels formed by free iron and H_2O_2 via the Fenton reaction. To analyze the importance of iron levels in HOCl-stressed cells, viability of a *recA*$^-$ *hypT*$^-$ strain expressing wild-type *hypT* or *hypT*^{M123,206,230Q} was analyzed in a time-dependent manner upon H_2O_2 stress. The advantage of this assay is that H_2O_2 does not activate HypT, in contrast to HOCl. Cells expressing *hypT*^{M123,206,230Q} showed a two orders of magnitude higher viability upon H_2O_2 stress in comparison to the control strain or a strain expressing wild-type *hypT*, confirming that the triple M→Q mutant is constitutively active (Drazic et al., 2013a). To further support this hypothesis, various mutants were purified and DNA binding ability was analyzed by fluorescence anisotropy (FA; e.g. HypT^{M123,206,230Q}, HypT^{M123,206,230,284Q}, HypT^{M123,206230I}, HypT^{M123,206,230I}-HOCl$^{2.75mM}$). HypT^{M123,206,230Q} showed similarly high DNA binding activity towards target *metN*-promoter DNA as HypT-HOCl$^{2.75mM}$ isolated from *in vivo* stressed cells.

Most of the post-translational modifications in redox-regulated proteins are reversible (Kiley and Storz, 2004). The activation of HypT is also reversible by reducing Met-SO to

methionine (Drazic et al., 2013a). This reaction is catalyzed by the enzymes methionine sulfoxide reductases A and B (MsrA and B). MsrA and B are stereospecific reductases, MsrA reduces the S epimer of methionine sulfoxide (Moskovitz et al., 2000; Weissbach et al., 2002) whereas MsrB reduces the R epimer (Boschi-Muller et al., 2008; Ezraty et al., 2005). To test for the role of MsrA and B, wild-type cells and *msrA*$^-$ msrB$^-$ knock-out cells, which express wild-type *hypT*, were compared after stress and recovery time, including gene regulation, and MS and FA analysis of purified protein. MS analysis showed that the abundance of Met-SO in HypT decreases in wild-type cells after quenching HOCl stress and recovering cells in fresh LB medium compared to *msrA*$^-$ msrB$^-$ knock-out cells. These results were confirmed by FA and target gene regulation analysis. The ability of HypT to bind DNA decreases after recovery from HOCl stress in wild-type cells whereas HypT in *msrA*$^-$ *msrB*$^-$ remains fully activated and able to bind DNA completely. HypT-dependent regulation of MetN RNA levels in wild-type cells is relieved quickly after HOCl stress with a similar half-time $t_{1/2}$ as the control strain, carrying an empty plasmid ($t_{1/2,\ hypT}$ = 2.4 min; $t_{1/2,\ control}$ = 2.5 min). In *msrA*$^-$ *msrB*$^-$ cells the MetN RNA levels showed a protracted decrease and remained at about 70% during HOCl stress (Drazic et al., 2013a). Inactivation of HypT-HOCl$^{2.75mM}$ was also observed *in vitro*. FA analysis revealed that HypT-HOCl$^{2.75mM}$ loses its DNA binding ability when treated with MsrA and MsrB in combination with DTT. MS analysis of MsrA/B treated HypT-HOCl$^{2.75mM}$ resulted in a significantly decrease of Met-SO at the positions 1, 123, 230 and 284. Treatment of HypT-HOCl$^{2.75mM}$ with an excess of DTT alone had no effect on the DNA binding ability, further confirming that the methionine residues alone determine HypT activity (Drazic et al., 2013a).

V-5 Role of Cysteine Residues in HypT

Besides the methionine residues, HypT contains 5 cysteines at the positions 4, 25, 150, 178, and 242, respectively. There are several stress response factors and other regulators that employ cysteine residues to trigger their activity and functionality. One example for a stress responsive factor, activated by cysteine oxidation by forming disulfide-bonds, is OxyR (Choi et al., 2001). As discussed already in section V-4, cysteines do not facilitate activation of HypT *in vivo*. To analyze potential functions of the cysteine residues, several mutants with cysteine-to-serine substitutions were generated (all possible single mutants: HypT^{C4S}, HypT^{C25S}, HypT^{C150S}, HypT^{C178S}, HypT^{C242S}; all possible quadruple mutants where only one cysteine remained: HypT^{quadrupleC4}, HypT^{quadrupleC25}, HypT^{quadrupleC150}, HypT^{quadrupleC178}, HypT^{quadrupleC242}; and a mutant completely lacking all cysteines: HypT$^{5C\rightarrow S}$). The cysteines of

HypT have an important function, despite the fact that activation of HypT is triggered exclusively by methionine oxidation (Drazic et al., 2013a). MALDI-TOF mass spectrometry analysis revealed that HypT can form disulfide-bond linked dimers *in vitro*. This dimer is formed intermolecularly between two cysteine residues at the position 4, when purified protein was oxidized *in vitro* (Drazic et al., 2013b). Interestingly, electrophoretic mobility shift assays (EMSA) revealed that disulfide-bond formation between the two Cys4 residues rendered the protein unable to bind to DNA. Under oxidative conditions, all single cysteine mutants, except the $HypT^{C4S}$, lost their ability to bind to DNA. For comparison, all quadruple mutants were still able to bind to DNA under oxidative conditions, except $HypT^{quadrupleC4}$, demonstrating that *in vitro* Cys4 exclusively impairs DNA binding in an oxidized state. The mutant $HypT^{5C\rightarrow S}$, lacking all cysteine residues, is able to bind to DNA similar to the wild-type (Drazic et al., 2013b).

To further elucidate potential roles of the other cysteine residues, the thermal stability of the single / quadruple mutants was analyzed by circular dichroism (CD). Wild-type HypT showed T_m of 59-61 °C. Similar thermal transition points can be calculated for $HypT^{C4S}$, $HypT^{C25S}$, $HypT^{C178S}$, $HypT^{C242S}$, and $HypT^{quadrupleC150}$. Exceptionally, $HypT^{C150S}$ and all quadruple mutants lacking Cys150 showed a strongly decreased thermal stability with T_m of 50.0 to 54.2 °C Drazic et al., 2013b). These results suggest that Cys150 plays an important role in stabilizing HypT. This is further supported by the modeled structured of HypT (Fig. 7), in which Cys150 seems to be imbedded in the core of the folded protein.

In summary, HypT behaves differently *in vitro* and *in vivo* due to two complete different environmental states with different experimental conditions.

V-6 Effect of Arginine and Sodium Chloride on HypT

The addition of arginine or NaCl, but not urea, led to dissociation of dodecameric HypT. The term “arginine” referes to either L-arginine or D,L-arginine; L-arginine was used for all experiments except CD and intrinsic tryptophan fluorescence analysis. HypT in storage buffer (400 mM NaCl) sediments with 10 - 11S. Already 150 to 200 mM arginine was sufficient to generate species of 8 to 9 S, likely corresponding to a hexamer. Above 250 mM arginine only species sedimenting with 3.1 to 4 S were detectable (Fig. 8A), likely corresponding to monomers and dimers. Further, addition of 800 mM NaCl generated two species (6.4 S and 9.5 S) and concentrations over 1 M NaCl facilitated further dissociation to the dimeric species (Fig. 8B). Of note, addition of up to 1 M urea showed no effect on the

oligomeric structure of HypT. To further elucidate the effect of arginine and salt on the structure of HypT, the secondary (by CD) and tertiary (by following changes of the intrinsic tryptophan fluorescence signal) structure was analyzed upon addition of increasing arginine and NaCl concentrations. The secondary and tertiary structure of HypT was not affected strongly, neither by arginine nor by high NaCl concentrations. The intensity of the intrinsic fluorescence signal increased or decreased marginally without any significant wavelength shift indicating that the hydrophobic environment of the fluorescent tryptophan residues does not change by arginine or NaCl addition. These results show that only the quaternary structure of HypT is affected by arginine and higher salt concentrations.

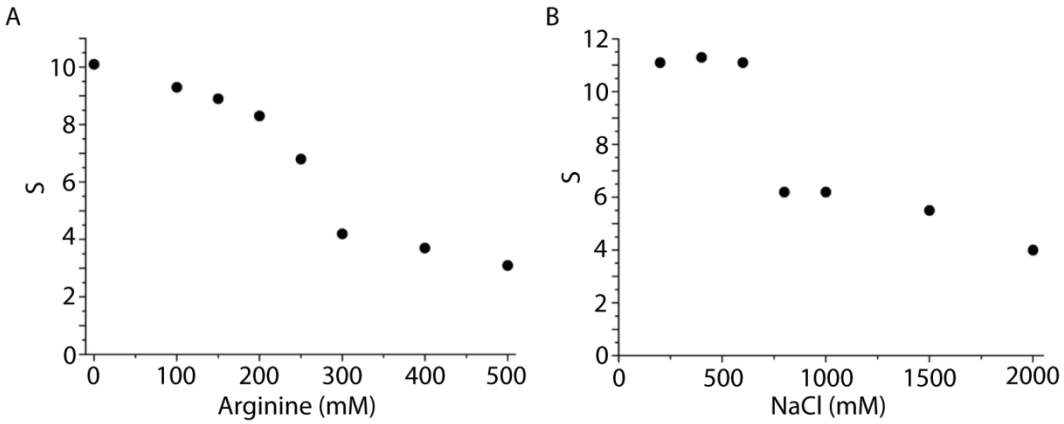

Figure 8 Sedimentation coefficient of HypT as determined by aUC. HypT was incubated in buffer containing various concentrations of A) arginine (in 400 mM NaCl) or B) NaCl. HypT was detected by absorption at 280 nm. Sedimentation coefficients were calculated using the Sedfit software.

Further, arginine and high NaCl concentrations increased the thermal stability of HypT as determined by CD spectroscopy. Increasing concentrations of arginine or NaCl led to a gradual increase in HypT's thermal stability. While the thermal transition midpoint (T_m) of HypT in the absence of additives was 60.2 ± 0.7 °C (Gebendorfer et al., 2012; Drazic et al. 2013b), it was increased to 63.7 ± 0.8 °C by 300 mM arginine and 70 °C by 2 M NaCl (Fig. 9A, B).

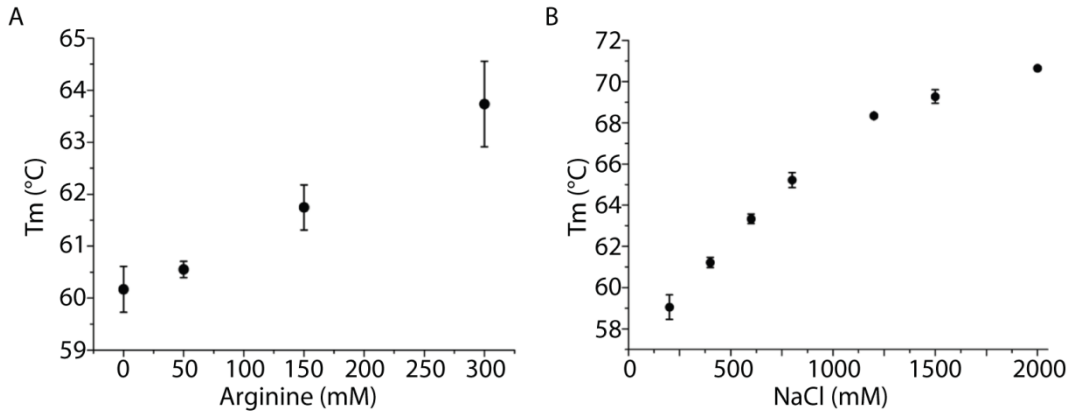

Figure 9 Thermal transition points (Tm) of HypT in different buffers determined by CD spectroscopy. A) HypT in 10 mM NaH_2PO_4, 400 mM NaCl and increasing arginine concentrations. B) HypT in 10 mM NaH_2PO_4 and various NaCl concentrations. Results are shown as mean ± SD (n ≥ 3).

Interestingly, NaCl stabilizes HypT additionally towards HOCl-induced aggregation (Fig. 10). HypT in 400 mM and 1.5 M NaCl, respectively, was treated with various molar HOCl:HypT ratios and solubility was analyzed by SDS-PAGE. At a 12:1 molar ratio of HOCl:HypT, HypT in 400 mM NaCl aggregated significantly (Fig. 10, black squares) while HypT in 1.5 M NaCl remained highly soluble (Fig. 10, red circles). With increasing molar ratios of HOCl:HypT (up to 95:1), HypT in 400 mM NaCl aggregated almost completely while HypT in 1.5 M HOCl was still about 50% soluble. Only at molar ratios of HOCl:HypT above 100:1, both proteins aggregated completely.

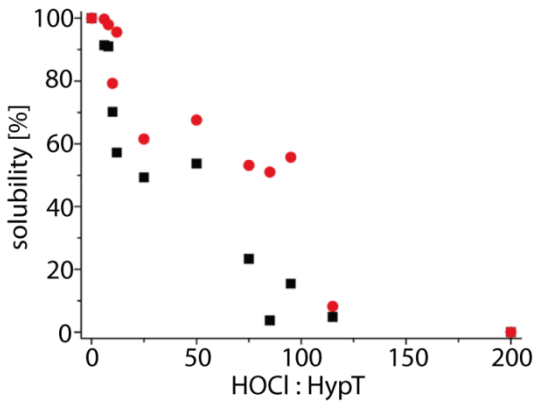

Figure 10 Stability of HypT towards HOCl-induced aggregation. HypT in 400 mM NaCl (black squares) and 1.5 M NaCl (red circles) were incubated with the indicated molar ratios of HOCl to protein and soluble and aggregated protein analyzed on SDS-gels.

Arginine-induced dissociation of HypT and the dissociation facilitated by increasing salt concentrations are reversible as determined by aUC. When HypT dodecamers were

dissociated with 300 mM arginine (2.5 to 4 S) and then arginine was diluted to a final concentration of 50 mM, the HypT dodecamers reformed, sedimenting with about 11 S. A similar behaviour was observed for NaCl dissociation. HypT dodecamers were incubated with 1.5 M NaCl resulting in smaller species of 5 to 7 S, and then buffer was exchanged back to 400 mM NaCl resulting in a species with 10 S (Fig. 11). Thus, the data suggests that the different oligomeric species of HypT are in an equilibrium governed by the arginine and NaCl concentration, respectively.

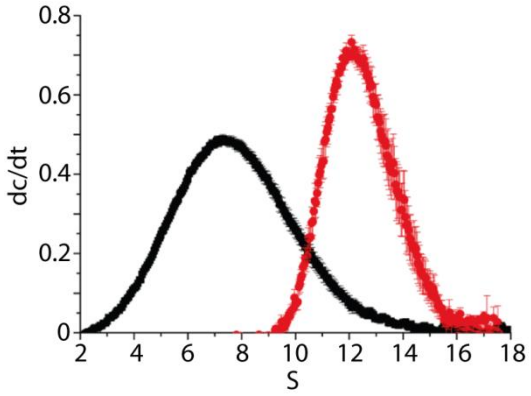

Figure 11 Analytical ultracentrifugation analysis of HypT dissociated with 1.5 M NaCl (black) and re-associated upon decreasing the NaCl concentration to 400 mM (red). Dc/dt was calculated using dc/dt+ software.

When HypT can shift from one oligomeric state to another, is it possible to form large hetero-oligomers consisting of HypT and a HypT variant? To test this, HypT variants were first separately dissociated by addition of arginine, then mixed and re-associated upon removal of arginine. Thus, mixed assemblies consisting of fluorescent labeled HypT ($HypT^{FlAsH}$) and His-tagged HypT were generated. This experimental design takes advantage of the fact that assemblies were purified via the His-tag, thus removing any homogenous $HypT^{FlAsH}$ assembly that does not contain a His-tag, and detect the presence of $HypT^{FlAsH}$ in the His-tagged assemblies via fluorescence. The simultaneous presence of fluorescence and His-tag indicates a mixed assembly. The His-tagged assembly partners are wild-type HypT (HypT), $HypT^{5C\rightarrow S}$ (Drazic et al., 2013b), and $HypT^{M123,206,230Q}$ (Drazic et al., 2013a). To test whether or not mixed assemblies were generated, samples eluted from the HisTrap column were separated by SDS-PAGE, then fluorescence was analyzed to detect $HisT^{FlAsH}$ and His-tagged HypT was detected by western blot using antibodies specific to the His-tag. When $HypT^{FlAsH}$ was titrated to $HypT^{5C\rightarrow S}$, the fluorescence intensity increased with increasing proportion of $HypT^{FlAsH}$. Similar amounts of His-tagged $HypT^{5C\rightarrow S}$ were detected in all samples, confirming that mixed assemblies were generated. The generation of mixed

assemblies was further confirmed by analyzing the thermal stability of HypT^{FlAsH}, HypT, HypT$^{5C\rightarrow S}$, and HypT^{M123,206,230Q}, compared to the generated mixed assemblies (HypT^{FlAsH} + HypT; HypT^{FlAsH} + HypT$^{5C\rightarrow S}$; HypT^{FlAsH} + HypT^{M123,206,230Q}). In this case the T_M value was determined by the thermal stability assay (TSA) utilizing the fluorescent dye SYPRO orange that binds to exposed hydrophobic surfaces upon unfolding of proteins thus exhibiting increased fluorescence (Hawe et al., 2008). HypT and HypT^{FlAsH} have a similar thermal stability of T_m 55.5 ± 0.8 °C and 55.3 ± 0.5 °C, respectively. HypT$^{5C\rightarrow S}$ and HypT^{M123,206,230Q} showed a reduced thermal stability with a T_m of 46.7 ± 0.6 °C and 42.5 ± 0.9 °C, respectively. These values were about 4 °C lower than the T_m values determined by CD, yet both methods show a striking difference in thermal stability of HypT and the mutants. The mixed assemblies, in contrast, showed a similar thermal stability as HypT with T_m values of 55.8 ± 0.5 °C (HypT^{FlAsH} + HypT), 53.3 ± 0.6 °C (HypT^{FlAsH} + HypT$^{5C\rightarrow S}$), and 53.0 ± 1.0 °C (HypT^{FlAsH} + HypT^{M123,206,230Q}). This indicates that the HypT mutants are stabilized in a mixed assembly by wild-type HypT.

From these observations arose the question, if the oligomeric state of HypT influences its activation by HOCl, i.e. if HypT can be activated more efficiently in 1.5 M NaCl when present as tetramer *in vitro*. Of note, HypT in 400 mM NaCl, present as dodecamer, can be activated with a 6:1 molar ratio of HOCl to HypT *in vitro* when the cysteine residues are reversibly protected by S-glutathionylation prior to HOCl treatment (HypT^{GSSG}; Drazic et al., 2013a). The rate constant of this reaction is about 30 min^{-1} and the DNA-binding activity reached its maximum after one hour incubation (Fig. 12, black squares). Surprisingly, HypT that was dissociated prior to addition of HOCl was activated already at a 1:1 molar ratio to HOCl (Fig. 12, red circles). Higher ratios to HOCl did not result in HypT activation, most likely because HOCl attacks other amino acids in HypT (Pattison and Davies, 2001), thus preventing DNA-binding. Further, activation of HypT tetramers at a 1:1 molar ratio to HOCl was already completed within one minute. Given that activated HypT forms tetramers (Drazic et al, 2013a), such HOCl-treated tetramers also likely remain in this oligomeric state and are instanteanously available for DNA-binding. This makes HypT a very potent HOCl sensor.

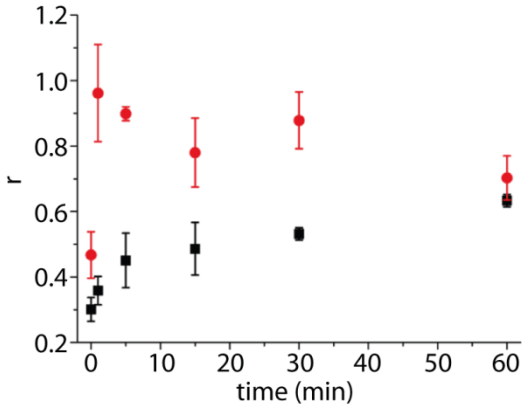

Figure 12 Time-dependent activation of HypT at different NaCl concentrations *in vitro* determined by fluorescence anisotropy. $HypT^{GSSG}$ in 400 mM NaCl was treated with a 6:1 molar ratio of HOCl:HypT (black squares) or $HypT^{GSSG}$ was first dissociated with 1.5 M NaCl, treated with a 1:1 molar ratio of HOCl (red dots), and then re-association induced. Samples were taken at the indicated time points. The control sample (time point 0) was not treated with HOCl. Shown is the mean ± SD.

VI. Metabolic Response of *E. coli* to HOCl Stress

To analyze the effects of HOCl stress on the metabolism, especially on the single metabolite level of the *E. coli* K 12 MG1655 strain, cells were cultivated in M9 minimal medium and treated with a sub-lethal concentration of HOCl (e.g. 50 µM). The stressed cells were harvested at different time points and lyophilized. Afterwards, the metabolites were extracted with deuterated methanol-d_4 and analyzed by nuclear magnetic resonance (NMR) spectrometry. The NMR samples, including 0.08 mg/ml 3-(Trimethylsilyl)propanoic acid (TSP) as NMR standard compound, were dried after the measurements with a N_2 flow and the metabolites were derivatized with 20 mg/ml methoxyamine hydrochloride (in pyridine) and 100 % N-methyl-N-(trimethylsilyl)-trifluoroacetamide (with 1 % trimethylchlorosilane) (MSTFA) for gas-coupled mass spectrometry (GCMS) analysis (Gillmaier et al., 2012).

In total, 28 metabolites could be reproducibly identified by GCMS. They were analyzed for four different time points after HOCl addition (10 min, 20 min, 40 min, and 60 min) and compared with unstressed control cells. The concentrations, relative to the dry cell mass, and their changes upon HOCl stress could be calculated and compared. Several metabolites showed significantly altered levels after HOCl stress, whereas other metabolite levels remained at a steady state level during stress. This demonstrates that the method is applicable to determine the metabolite levels, because otherwise one would expect absolutely no alterations of any metabolites or all levels would change simultaneously. Further, for a few metabolites, the levels changed after 10 min stress, but returned back to their original levels of the unstressed state. This indicates that *E. coli* adapts quickly to stress and adjusts its metabolism to reach normal metabolite levels again. The time-dependent changes of the metabolite concentrations are summarized in Table I.

Most of the amino acids that could be identified showed an increase after HOCl stress (e.g. glycine, threonine, tyrosine, and proline). The increase of amino acids could be caused by HOCl-induced degradation of proteins and concomitant release of free amino acids into the cytosol (Davies, 2005). Valine seemed to remain at a constant level. In contrast, alanine and methionine levels decreased massively over time. The decrease of methionine could result from the oxidation of free methionine to Met-SO, which is a known mechanism of direct oxidative stress defense (Levine et al., 1996; Stadtman et al., 2004). Unfortunately, an increase of Met-SO levels was not directly detected by GCMS because Met-SO is not present in the database of the GCMS analysis software. To determined Met-SO levels manually, the metabolite spectra have to be compared with a spectrum of a pure Met-SO sample. Moreover, alanine was observed in recent studies as an antioxidant that protects

cells from oxidative stress. Given that the GCMS analysis software only detects alanine and a few derivatives that do not include oxidized forms of alanine (Grosser et al., 2004), this could explain its massive decrease of alanine in stressed samples.

Further metabolites showed a massive decrease after HOCl stress like lactic acid, phosphate, phosphoglycerol and glucose. One reason for decreased glucose levels could be that oxidative stress results in disruption of the energy metabolism (Barrette et al., 1987). Then, glucose is re-directed to the pentose phosphate pathway to generate new reductive NADPH for detoxifying reasons (Jozefczuk et al., 2010; Rui et al., 2010). In contrast, other metabolite levels increased upon HOCl stress like putrescine, urea, orotic acid and 1-monopalmitoylglycerol. While for most metabolites no role in oxidative stress has been described, putrescine, also known as 1,4-Diaminobutane, was shown to play a role in the regulation of oxidative stress defense genes. OxyR regulon genes are activated by putrescine in a concentration-dependent manner. Besides, high putrescine concentrations could even take over the role of OxyR when OxyR is inactivated by too high H_2O_2 concentrations (Tkachenko et al., 2001).

As already mentioned above, some metabolites like threonine, pyroglutamate and urea change significantly after HOCl addition, but return to non-stress levels within the time curse of the experiment. This shows that *E. coli* adapts quickly to HOCl stress and that some metabolic pathways regenerate faster than others. Moreover, this indicates that HOCl reacts quickly with biomolecules and it seems likely that all HOCl molecules have reacted after 20 min (Davies, 2005). The quick adaptation of *E. coli* cells under different stress conditions, especially oxidative stress caused by H_2O_2 treatment, was recently demonstrated and supports the described observations (Jozefczuk et al., 2010).

NMR analysis can be performed faster than GCMS analysis, yet it requires 5-fold higher metabolite concentrations than GCMS and metabolite determination is more challenging. Nevertheless, NMR spectra confirmed the results of the GCMS measurements by revealing several differences in metabolite levels over the total ^1H spectra from 0 to 10 ppm. Massive differences in metabolism such as caused by oxidative stress, especially due to modifications by oxidation, are expected to result in significantly altered spectra, not only concerning peak intensities but also peak appearance and number. Unexpectedly, this could be only partially observed in the current experiments. Some differences in peak intensities were obvious (Fig. 13). Differences in the peak landscape for different time points were also confirmed by principal component analysis (PCA), which analyzes, compares and evaluates the overall spectra of all samples (Weljie et al., 2006). Samples from unstressed cells and cells that were stressed for 5 min differ significantly. Cells that were stressed for 10 min or 20 min,

respectively, seem to have similar peak properties, suggesting that metabolism regenerates and adapts to HOCl stress already after 10 min. One metabolite that was identified by NMR is formic acid in the range of 8.5 to 8.7 ppm. The peak for formic acid massively decreased shortly after HOCl stress, but returns to its original level after 40 to 60 min. Formic acid or formate is a key metabolite in energy metabolism and is a major fermentation product of many enterobacteria (Kirkpatrick et al., 2001; Leonhartsberger et al., 2002). It plays also a role in the protection of *E. coli* against acidic stress by its conversion to H_2 and CO_2 (Rossmann et al., 1991) as well as against the bactericidal cationic peptide P2 (Barker et al., 2000). Importantly, formate is eliminated by direct oxidation with O_2 under fermentative conditions, which would explain the massive decrease after oxidative stress such as HOCl stress (Benoit et al., 1998).

Table I Time-dependent relative changes of metabolite levels after HOCl stress determined by GCMS.

metabolite	***10 min***	***20min***	***40 min***	***60min***	***tendency***
Alanine	1.38*	0.43	0.48	0.41	↓
Glycine	3.65	0.93	1.42	1.71	↑
Threonine	3.02	1.86	1.56	1.03	↑
Tyrosine	n.d.	1.06	1.14	1.21	↑
Proline	0.92	1.44	1.40	1.16	↑
Valine	0.95	0.74	1.09	1.08	Ø
Glutamate	1.09	1.66	0.99	0.55	?
Methionine	n.d.	0.81	0.70	0.63	↓
Lactic acid	1.47	0.50	0.60	0.59	↓
Succinate	1.03	1.12	1.34	1.10	Ø
Phosphate	0.85	0.40	0.50	0.43	↓
Putrescine	1.10	1.87	1.71	2.11	↑
2-Hydroxybutyric acid	0.30	0.66	0.31	0.33	↓
Uracil	0.66	1.56	0.83	0.90	?
Galactose	1.02	1.26	1.02	0.97	Ø
Glucose	0.93	1.54	0.61	0.29	↓
Pyroglutamate	n.d.	0.42	0.80	1.01	↓
Palmitic acid	1.12	1.07	0.98	1.07	Ø
Palmitelaidic acid	1.25	0.41	0.77	0.72	↓
Phosphoglycerol	1.25	0.15	0.23	0.15	↓
Tetradecanoic acid	1.08	1.31	0.51	0.22	↓
Pentadecanoic acid	1.24	1.01	1.18	1.23	Ø
Stearic acid	0.96	0.93	0.99	1.09	Ø
2-Monopalmitoylglycerol	1.19	0.78	0.94	1.28	Ø
1-Monopalmitoylglycerol	0.91	1.78	2.16	2.19	↑
Urea	n.d.	1.81	1.59	1.00	↑
Decanamide	n.d.	0.98	1.07	1.10	Ø
Orotic acid	n.d.	1.96	2.02	2.23	↑

*Level at time point zero (control, unstressed cells) was set as 1.

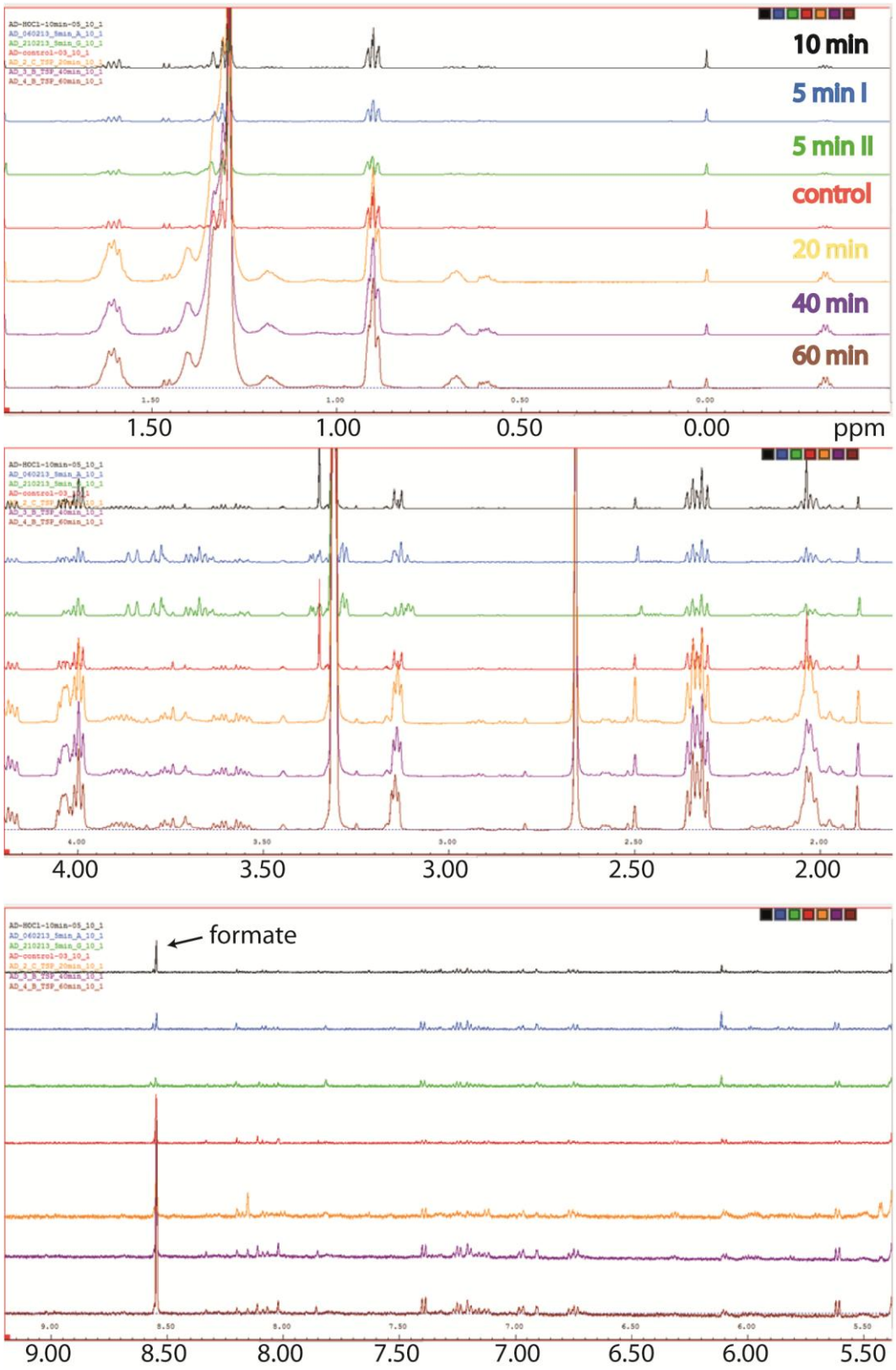

Figure 13 ^{1}H NMR spectra of metabolites from *E. coli* MG 1655 cells isolated by d_4-methanol extraction. Cells were cultivated in M9 minimal medium to an OD_{600} 0.4 – 0.5 and 400 ml cell suspension was harvested, and left untreated (control, red) or stressed with 50 µM HOCl. Duration of stress: 5 min (green and blue), 10 min (black), 20 min (yellow), 40 min (mangenta), 60 min (brown).

VII. Perspectives

Although several aspects of HypT, considering oligomerization, target gene regulation and activation mechanism, have been investigated and described in this work, several questions remain. The exact binding mechanism is still a dark horse, which means that a nucleotide consensus binding sequence has to be found. Further, the regions and amino acids involved in DNA binding have to be determined. The genes regulated by HypT are known (Gebendorfer et al., 2012), nevertheless if these genes are directly regulated by HypT binding or indirectly by intermediate gene regulation is not clear. One method to solve these problems would be the ChIP-on-chip technology. Both, consensus nucleotide sequence and promoter regions directly bound by HypT could be determined by this assay (Sala et al., 2009).

Another interesting point is the solution of the three-dimensional structure of HypT by X-ray crystallography. This could finally confirm the oligomerization of the active state as well as elucidate the conformational alterations that occurr when HypT is activated by methionine oxidation. Co-crystallization of activated HypT together with target promoter DNA would be of interest, first to determine the exact binding mechanism and second to facilitate the understanding of HypT's target gene regulation.

Albeit, several metabolites could be identified by GCMS, the overall picture is still incomplete. More metabolites should be identified by either NMR data analysis or by other metabolite extraction methods. As HOCl reacts very fast with cellular components and metabolic changes occur in comparable time periods, samples should be prepared for shorter stress periods and compared to the already evaluated time points. Moreover, to identify the alterations of the metabolic pathways, samples, prepared with isotope labeled carbon, have to be prepared and the metabolites and the position of the incorporated heavy carbon atoms determined. Further, a comparison between different strains could complete the overall picture. Of interest would be the comparison of an *E. coli* wild-type strain with a *hypT*$^{-}$ strain. To complete the analysis, metabolite levels of the strain expressing the constitutively active mutant *hypT*^{M123,206,230Q} could be helpful to understand the general oxidative stress response of *E.coli* cells on the metabolite level.

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IX. Abbreviations

ABS	activation binding site
aUC	analytical ultracentrifugation
CD	circular dichroism
CRP	cAMP receptor protein
DNA	deoxyribonucleic acid
DTT	dithiothreitol
GCMS	gas chromatography coupled with mass spectrometry
GSH	γ-L-glutamyl-L-cysteinyl-glycine
HMM	hidden Markov model
HOBr	homobromous acid
HOCl	hypochlorous acid
LDL	low density lipoprotein
LTTR	LysR-type transcriptional regulator
Met-SO	methionine sulfoxide
MS	mass spectrometry
MsrA/MsrB/fRMsr	methionine sulfoxide reductase
MSTFA	N-methyl-N-(trimethylsilyl)-trifluoroacetamide
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NMR	nuclear magnetic resonance
RBS	regulatory binding site
RCS	reactive chlorine species
RNA	ribonucleic acid
ROS	reactive oxygen species
SOD	superoxide dismutase
T_m	thermal transition point
TSP	3-(Trimethylsilyl)propanoic acid
(w)HTH	(winged) helix-turn-helix

PART C

Publications

X. List of Publications

I) Gebendorfer, K.M., Drazic, A., Le, Y., Gundlach, J., Bepperling, A., Kastenmüller, A., Ganzinger, K.A., Braun, N., Franzmann, T.M., Winter, J. (2012). Identification of a hypochlorite-specific transcription factor from Escherichia coli. *J Biol Chem 287*, 6892-6903

II) Drazic, A., Miura, H., Peschek, J., Le, Y., Bach, N.C., Kriehuber, T., Winter, J. (2013). Methionine oxidation activates a transcription factor in response to oxidative stress. *Proc Natl Acad Sci USA 110*, 9493-9498

III) Drazic, A., Tsoutsoulopoulos, A., Peschek, J., Gundlach, J., Krause, M., Gebendorfer, K.M., Winter, J. (2013b). Role of cysteines in the stability and DNA-binding activity of the hypochlorite-specific transcription factor HypT. *PLoS One 8,* e75683

IV) Drazic, A., Gebendorfer, K.M., Mak, S., Steiner, A., Krause, M., Bepperling, A., Winter, J. Tetramers are the active species of the HOCl-specific transcription factor HypT. *Epub ahead of print*

V) Drazic, A., Winter, J. The physiological role of methionine oxidation by reactive oxygen species. *Biochimica et Biophysica Acta: Proteins and Proteomics, Review, accepted*

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Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, dass ich die vorliegende Arbeit selbständig verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe.

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Die vorliegende Arbeit wurde noch keiner anderen Prüfungsbehörde vorgelegt.

München, den 25.09.2013 _____

Adrian Drazic