



**LeuT-Desipramine Structure Reveals How
Antidepressants Block Neurotransmitter Reuptake**

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structures probably reflect conformations that are relevant in native membranes.

On the basis of biochemical data and the structures of full ABC transporters determined in recent years, a conserved coupling mechanism for ABC transporters has recently been proposed that suggests that binding of ATP to the NBDs promotes an outward-facing conformation of the TMDs, whereas a release of the hydrolysis products promotes an inward-facing conformation (8, 25). ABC importers would thus acquire substrates from their binding proteins in the ATP-bound state and release the substrates to the cytoplasm upon dissociation of the ATP hydrolysis products (2). This basic two-state scenario is in agreement with the structures of Sav1866, HII470/71, and ModBC, which all visualize such states, whereas those of BtuCD and BtuCD-F reveal intermediate conformations. Although there may be differences in the detailed transport mechanism of BtuCD when compared with that of the maltose transporter MalFGK or the molybdate/tungstate transporter ModBC, BtuCD may nevertheless largely follow the common coupling mechanism of ABC transporters.

The BtuCD-F complex is stable in the absence of ATP, which is different from MalFGK or ModBC, where ATP and vanadate are required to generate stable complexes with the binding proteins (8, 26). Even though the interaction of BtuCD with BtuF is sufficient to release B₁₂ from the high-affinity binding site, the transport of B₁₂ across the membrane requires ATP both in vivo and in vitro. During a productive transport cycle, B₁₂ is probably fed into an outward-facing conformation such as that observed in the structure of BtuCD and later released into the cytoplasm

from an inward-facing conformation similar to that revealed by HII470/71. The BtuCD-F structure presented here is a conformation that is, with respect to its TMDs, an intermediate between those of BtuCD and HII470/71. This suggests that during the conversion from the inward- to the outward-facing conformation, the BtuC subunits may not alter their conformations simultaneously.

Unlike BtuCD, many other ABC transporters have pairs of TMDs with distinct amino acid sequences, which may have mechanistic consequences. The structure of BtuCD-F provides an opportunity to study conformational asymmetry at high resolution, which could prove useful for the mechanistic understanding of intrinsically asymmetric ABC transporters.

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Materials and Methods

Figs. S1 to S4

Tables S1 and S2

References

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LeuT-Desipramine Structure Reveals How Antidepressants Block Neurotransmitter Reuptake

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Tricyclic antidepressants exert their pharmacological effect—inhibiting the reuptake of serotonin, norepinephrine, and dopamine—by directly blocking neurotransmitter transporters (SERT, NET, and DAT, respectively) in the presynaptic membrane. The drug-binding site and the mechanism of this inhibition are poorly understood. We determined the crystal structure at 2.9 angstroms of the bacterial leucine transporter (LeuT), a homolog of SERT, NET, and DAT, in complex with leucine and the antidepressant desipramine. Desipramine binds at the inner end of the extracellular cavity of the transporter and is held in place by a hairpin loop and by a salt bridge. This binding site is separated from the leucine-binding site by the extracellular gate of the transporter. By directly locking the gate, desipramine prevents conformational changes and blocks substrate transport. Mutagenesis experiments on human SERT and DAT indicate that both the desipramine-binding site and its inhibition mechanism are probably conserved in the human neurotransmitter transporters.

Sodium- and chloride ion-dependent neurotransmitter transporters for serotonin (SERT), norepinephrine (NET), and dopamine (DAT) in the presynaptic plasma membrane

terminate neuronal signal transmission in the central nervous system through a reuptake mechanism (1–6). These systems have been shown to modulate mood, emotion, sleep, and

appetite (7). Depression, arguably the most prevalent psychiatric disorder, is directly associated with perturbation of serotonergic neurotransmission (8, 9), and drugs blocking serotonin reuptake have been used successfully for its treatment. One class of these drugs, tricyclic antidepressants (TCAs) such as desipramine and imipramine, binds to serotonin and norepinephrine transporters with affinities of nanomolar to tens of nanomolar concentrations and blocks transport activity (10). The response rate of patients to TCAs is typically 60 to 70% (11). More recently, highly selective serotonin-reuptake inhibitors (SSRIs) such as fluoxetine (Prozac) have also been developed and are increasingly

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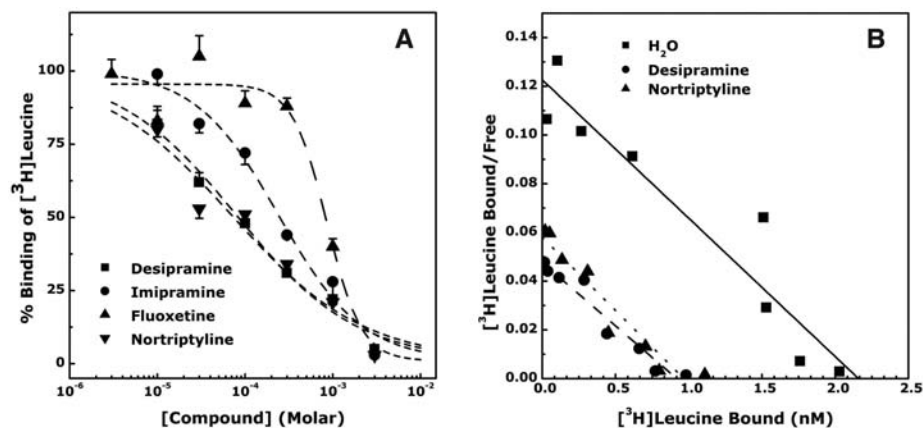


Fig. 1. Binding of various antidepressants and other compounds to LeuT. (A) Affinity of desipramine, imipramine, fluoxetine, and nortriptyline. Data are shown as means \pm SEM (vertical bars, $n = 3$). The IC_{50} values for inhibition $[^3H]$ leucine binding to LeuT were 80 ± 5 , 244 ± 12 , 858 ± 64 , and 75 ± 14 μ M, respectively. (B) Mechanism of inhibition of $[^3H]$ leucine binding to LeuT by desipramine and nortriptyline. The plot shows that desipramine and nortriptyline are not competitive inhibitors of leucine binding to LeuT. A representative experiment is shown ($n = 3$).

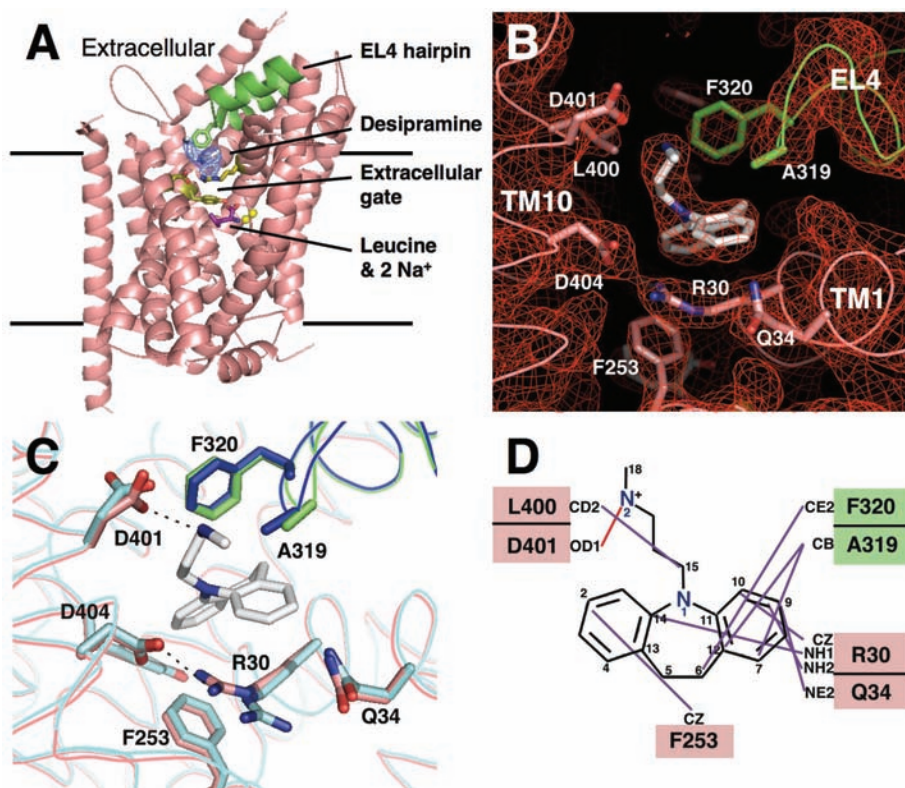


Fig. 2. Structure of the LeuT-desipramine complex and molecular mechanism of LeuT inhibition by desipramine. (A) Structure shown as ribbon diagram viewed from within the membrane plane. An $F_{obs} - F_{calc}$ map contoured at 3σ is superimposed on the structural model. The EL4 hairpin is colored green, and the rest of the protein pink. The helices TM6 and TM11 are removed for clarity. (B) $2F_{obs} - F_{calc}$ map contoured at 1σ showing the desipramine-binding site in LeuT, viewed from within the membrane plane. Residues R30, Y108, and F253 form the extracellular gate that separates the leucine substrate from the bound desipramine. (C) Local structural changes of LeuT induced by desipramine binding. The structure with desipramine bound is shown in pink and green, without desipramine binding in cyan and blue. When desipramine binds, the side chain of R30 rotates toward D404 and forms a salt bridge with the latter, and the EL4 hairpin, along with A319 and F320, is pushed toward to the extracellular space. (D) Molecular contacts between LeuT and the bound desipramine molecule. The chemical structure of desipramine is shown together with LeuT residues that are in direct contact with the drug. Residues from the EL4 hairpin are shown in the green box; residues from the rest of the protein are shown in pink boxes.

prescribed to treat depression (12). The molecular pharmacology of TCAs and SSRIs has been well defined, and their in vivo pharmacological effects appear to be mediated almost exclusively by serotonin- and norepinephrine-reuptake inhibition. Despite extensive investigations, however, whether the substrate-binding and drug-binding sites are overlapping and whether the drug inhibition mechanism is of a competitive nature remain controversial (13).

The human SERT, DAT, and NET proteins all belong to a family of transporters for amino acids and their derivatives, the neurotransmitter:sodium symporter (NSS) family (2–5, 14). Although the dopamine transporters from human, bovine, or rat are inhibited by TCAs at an inhibition constant (K_i) of micromolar concentrations, the DAT proteins from *Caenorhabditis elegans* (15) and *Drosophila melanogaster* (16) are inhibited by TCAs at a K_i of nanomolar and submicromolar concentrations, respectively (17). As bacterial NSS proteins share up to 30% sequence identity with human SERT and NET, as well as worm and fly DATs, we hypothesized that bacterial NSS proteins also have high binding affinity to TCAs and could provide opportunities for studying protein-drug interactions. We therefore chose a bacterial NSS protein, the leucine transporter (LeuT) from *Aquifex aeolicus*, to study the molecular mechanism of neurotransmitter transporter binding to TCAs (18). LeuT shares 20 to 25% sequence identity and 40 to 45% similarity with human neurotransmitter transporters. The crystal structure of LeuT, which was previously determined (19), revealed a shot glass-shaped bundle of 12 transmembrane α helices (TM1 to TM12), with a substrate leucine and two sodium ions bound at the center of the protein. The substrate-binding site is closed off from the extracellular space by a gate. There is an extracellular cavity in the protein, into which protrudes a helix hairpin formed by extracellular loop EL4.

We screened for binding of various tricyclic and other types of antidepressants to LeuT using a scintillation proximity assay (20). Several tricyclic compounds (imipramine, nortriptyline, protriptyline, amitriptyline, and doxepin) showed binding affinity (fig. S1), but desipramine bound LeuT most tightly, with a median inhibitory concentration (IC_{50}) of 80 μ M (Fig. 1A). In addition, fluoxetine also showed measurable binding (fig. S1 and Fig. 1A). Desipramine was found to inhibit leucine binding to LeuT by decreasing its maximal binding capacity without changing its binding affinity (Fig. 1B); this finding indicates a mechanism that does not involve competitive inhibition. To test if desipramine binding also inhibits substrate transport, we measured the leucine transport activity of LeuT in reconstituted proteoliposomes and found that, at a concentration of 200 μ M, desipramine indeed completely abolished LeuT's transport activity (fig. S2).

To investigate the molecular basis of TCA binding to LeuT, we cocrystallized the transporter with desipramine and, by directly refining the

diffraction data against the TCA-free LeuT structure (19) (table S1), determined the crystal structure at 2.9 Å resolution (Fig. 2A and fig. S3). The overall structure of the LeuT-desipramine complex (Fig. 2) is similar to that of the protein in the absence of desipramine (19), with a root mean square deviation (RMSD) of 0.2 Å for all the non-hydrogen atoms. Neither the leucine substrate nor the two Na⁺ ions had moved. However, a 5 σ $F_{\text{obs}} - F_{\text{calc}}$ (observed and calculated structure factors) electron density peak was observed at the inner end of the extracellular cavity of the protein (Fig. 2, A and B, and fig. S3), which fits well with a desipramine molecule, an interpretation consistent with the inhibitory effect of the TCA molecule on LeuT's transport activity (fig. S2) and the evidence that desipramine is not a competitive inhibitor (Fig. 1B).

The bound desipramine molecule sits at the inner end of the extracellular cavity in LeuT with its three rings tilted at a 40° angle to the membrane plane (Fig. 2B). The "tail" of the molecule, an extended methylaminopropyl chain, projects toward the extracellular space. The desipramine molecule is separated from the substrate leucine by the extracellular gate of the transporter, which consists of residues R30, Y108, and F253 (21). Note that the desipramine-binding site and the leucine-binding site are nonoverlapping, but they do share F253 as a common residue. As F253 and Q34 make contact with the first and third rings of desipramine (Fig. 2, B and D, and table

S2), respectively, R30 from TM1 forms cation- π interactions (22) with both the third desipramine ring and the phenylalanine ring of F253. On the extracellular side, desipramine is held in place by A319 and F320 of the turn of the EL4 helix hairpin, with the side chain of F320 engaging in hydrophobic interactions with the desipramine azepine ring and making contact with its tail. The desipramine tail also makes contact with residues L400 and D401 from the extracellular end of TM10. With a pK_a of 10.2 (23) (where pK_a is the acid dissociation constant) and with its nitrogen N2 atom being only 2.76 Å away from D401 (Fig. 2D and table S2), desipramine probably forms a salt bridge with the aspartate residue. In total, 393 out of 469 Å² the surface area of the desipramine molecule is buried by the protein. There is, however, still a substantial space in the protein on the extracellular side of the desipramine tail (Figs. 2B and 3A), which could accommodate the tail of other types of TCA molecules.

Although the overall LeuT structure does not change when it binds to desipramine, several residues at this binding site, as well as the backbone of the EL4 hairpin, do move (Fig. 2, A and C), by means of a typical "induced-fit" mechanism. Specifically, the guanidinium group of R30 is rotated by 170° about its C8-Ne bond toward D404, and two water molecules that were located between these two residues in the absence of bound desipramine (19) are no longer observed. As a result, R30 and D404, a pair of

conserved charged residues, form a salt bridge, effectively sealing the substrate leucine off from the extracellular space. Also in TM10, the side chain of D401 is rotated toward desipramine. Finally, the bound desipramine pushes the EL4 hairpin toward the extracellular direction by ~1 Å. Coupled with a ~16° rotation of its phenylalanine ring, F320 effectively pins desipramine in place.

The crystal structure of the LeuT-desipramine complex immediately suggests a mechanism for inhibition of substrate transport by the TCA molecule (Fig. 2A and fig. S4). The desipramine molecule is held in place by a salt bridge it forms with residue D401 and by interactions with residues of the EL4 hairpin loop. Desipramine also directly binds to the extracellular gate of the transporter and locks the gate by inducing formation of a salt bridge between R30 of TM1 and D404 of TM10. The formation of this salt bridge prevents tilting of TM1, which is believed to be required for substrate release to the cytosol (19). Thus, no substrate transport can occur. This inhibition mechanism is in contrast from the competitive inhibition of the aspartate transporter Glt by three- β -benzyloxyaspartate, in which the inhibitor binds partially to the substrate-binding site and concomitantly keeps the extracellular gate in an open position (24).

Human NSS proteins, as well as *C. elegans* and *Drosophila* DATs, share significant sequence homology with LeuT (fig. S5). Given that they all bind desipramine, we built three-dimensional homology models for the human proteins in complex with the drug by directly threading their sequences onto our LeuT-desipramine structure (Fig. 3). Both the residues of the extracellular

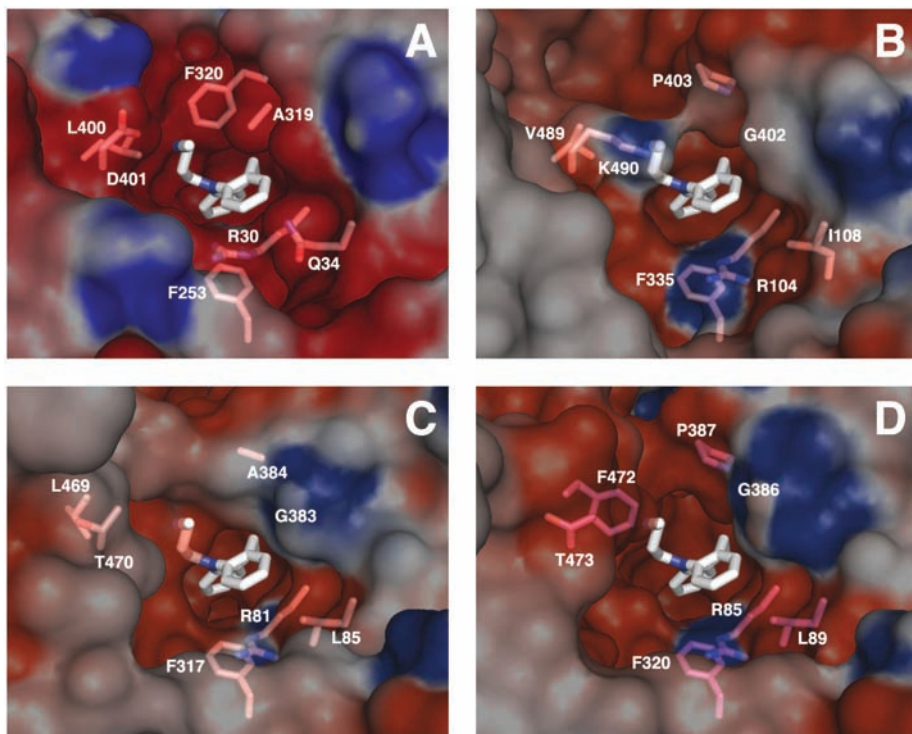


Fig. 3. Homology models and electrostatic surface potential of desipramine-binding sites in human SERT, NET, and DAT. (A) Desipramine-binding site in the LeuT-desipramine crystal structure. Homology model and electrostatic surface potential of desipramine-binding site in (B) hSERT, (C) hNET, and (D) hDAT, viewed from within the membrane plane. The equivalent residues of those in LeuT that are in direct contact with desipramine are indicated.

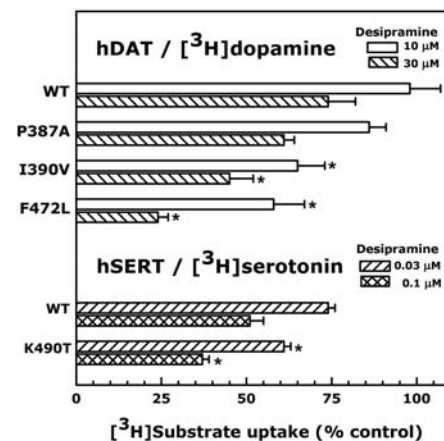


Fig. 4. Measurements of inhibition of [³H]dopamine and [³H]serotonin uptake by desipramine for human DAT and SERT mutants in HEK-293 cells. Wild-type (WT) hDAT, hSERT, and various mutant constructs are denoted on the left. Results are expressed as percentage of uptake measured with vehicle (% control). Data are shown as means \pm SEM (horizontal bars, $n = 3$ to 5). * $P < 0.05$ (compared with corresponding WT at the same concentration of desipramine, one-way analysis of variance followed by Dunnett multiple comparison test for hDAT and Student's t test for hSERT).

gate and the topology of the EL4 helix hairpin are conserved (fig. S5). The TCA-binding pocket in between is therefore likely to be conserved, and the homology models, which all show an acidic pocket at this position as in LeuT (Fig. 3, B to D), support this. The difference in binding affinity to TCAs among NSS proteins is likely to depend on sequence variations at the tip of the EL4 hairpin and at the extracellular end of TM10 (fig. S5). Indeed, previous experiments of both loss-of-function mutagenesis for human NET (hNET) (17) and gain-of-function mutagenesis (25) for hDAT in terms of TCA binding supported a binding site at just this position for the human NSS proteins. Thus, we hypothesized that both the desipramine-binding site and the inhibition mechanism of substrate uptake by LeuT were conserved also in the human neurotransmitter transporters.

We tested the above two hypotheses by mutating key residues at the presumed TCA-binding sites in the human neurotransmitter transporters SERT and DAT, followed by measuring their transport inhibition by desipramine in human embryonic kidney cells. The IC_{50} values of desipramine for inhibiting uptake by DAT, SERT, and NET are 82,000 nM, 64 nM, and 4.2 nM, respectively (10). We performed gain-of-function mutagenesis in terms of desipramine binding for both hDAT and hSERT proteins by mutating their key residues to those found in the sequence of hNET, the protein with the highest affinity to desipramine. We focused on residues at the tip of the EL4 hairpin and the extracellular end of the TM10 (Fig. 2, B and D, and fig. S5). As a control experiment, we first mutated P387 in hDAT (the equivalent of F320 in LeuT) to an alanine. Because either a proline (as in *C. elegans* DAT) or an alanine (as in hNET) at this position can confer nanomolar affinity for TCAs, a proline \rightarrow alanine mutation should not further increase hDAT's affinity for TCAs. Indeed, hDAT-P387A mutant showed little increase in inhibition compared with the wild-type at either 10 or 30 μ M desipramine (Fig. 4), with no change in IC_{50} (table S3). However, when I390 in the EL4 hairpin (equivalent of G323 in LeuT) and F472 in TM10 (equivalent of L400 in LeuT) were individually mutated into their corresponding residues in hNET (valine and leucine, respectively), the IC_{50} for inhibition of [3 H]dopamine uptake by desipramine decreased by 63% and 79%, respectively (Fig. 4 and table S3). Similarly, in hSERT when K490 (corresponding to D401 in LeuT) was mutated into a threonine as found in hNET, a 51% decrease in IC_{50} of inhibition of [3 H]serotonin uptake by desipramine was observed (Fig. 4 and table S3). Such gain-of-function mutagenesis data clearly demonstrate that desipramine binds to the same site in both hDAT and hSERT (Fig. 3, B and D) as it does in LeuT (Fig. 3A) and inhibits transport activity in the same manner (Fig. 2A).

Taken together, our results show that the TCA-binding site is probably conserved from bacterial to mammalian NSS proteins. Therefore,

in the human Na^+/Cl^- -dependent neurotransmitter transporters SERT, NET, and DAT, it is likely that TCAs, as well as SSRIs, also bind between the extracellular gate and EL4 hairpin, thereby inhibiting neurotransmitter reuptake at the synapse. In combination with homology modeling (26) and molecular docking, the identification of this drug-binding site will allow studies on the interactions of SERT-, NET-, and DAT-specific inhibitors (27) with these transporters and may aid in the structure-based design of more effective neurotransmitter-reuptake inhibitors as antidepressants. As membrane proteins constitute 60 to 70% of the targets for small-molecule drugs (28), the strategy employed in this work can probably be used in studying protein-drug interactions for other systems.

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Cysteine Redox Sensor in PKG α Enables Oxidant-Induced Activation

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Changes in the concentration of oxidants in cells can regulate biochemical signaling mechanisms that control cell function. We have found that guanosine 3',5'-monophosphate (cGMP)-dependent protein kinase (PKG) functions directly as a redox sensor. The α isoform, PKG α , formed an interprotein disulfide linking its two subunits in cells exposed to exogenous hydrogen peroxide. This oxidation directly activated the kinase in vitro, and in rat cells and tissues. The affinity of the kinase for substrates it phosphorylates was enhanced by disulfide formation. This oxidation-induced activation represents an alternate mechanism for regulation along with the classical activation involving nitric oxide and cGMP. This mechanism underlies cGMP-independent vasorelaxation in response to oxidants in the cardiovascular system and provides a molecular explanation for how hydrogen peroxide can operate as an endothelium-derived hyperpolarizing factor.

Oxidant molecules can cause cellular damage, dysfunction, and disease, but also play crucial roles in homeostatic maintenance

of healthy cells and tissues (1–3). The modification of proteins by oxidant species with a coupled alteration in function allows cells to