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Comparing system-specific chaperone interactions with their Tat dependent redox enzyme substrates

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Abstract

Redox enzyme substrates of the twin-arginine translocation (Tat) system contain a RR-motif in their leader peptide and require the assistance of chaperones, redox enzyme maturation proteins (REMPs). Here various regions of the RR-containing oxidoreductase subunit (leader peptide, full preprotein with and without a leader cleavage site, mature protein) were assayed for interaction with their REMPs. All REMPs bound their preprotein substrates independent of the cleavage site. Some showed binding to either the leader or mature region, whereas in one case only the preprotein bound its REMP. The absence of Tat also influenced the amount of chaperone–substrate interaction.

Structured summary—MINT-8047497: *FdhE* (uniprotkb:P13024) and *FdoG* (uniprotkb:P32176) *physically interact* (MI:0915) by *two hybrid* (MI:0018)

MINT-8046441: *HybO* (uniprotkb:P69741) and *HybE* (uniprotkb:P0AAN1) *physically interact* (MI:0915) by *two hybrid* (MI:0018)

MINT-8046375: *DmsA* (uniprotkb:P18775) and *DmsD* (uniprotkb:P69853) *physically interact* (MI:0915) by *two hybrid* (MI:0018)

MINT-8046425: *TorA* (uniprotkb:P33225) and *TorD* (uniprotkb:P36662) *physically interact* (MI:0915) by *two hybrid* (MI:0018)

MINT-8046393: *NarJ* (uniprotkb:P0AF26) and *NarG* (uniprotkb:P09152) *physically interact* (MI:0915) by *two hybrid* (MI:0018)

MINT-8046409: *NapD* (uniprotkb:P0A9I5) and *NapA* (uniprotkb:P33937) *physically interact* (MI:0915) by *two hybrid* (MI:0018)

Keywords

System specific chaperone; Twin-arginine translocase system; Twin arginine translocase; Leader sequence; Bacterial two-hybrid; Protein maturation

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1. Introduction

A subset of proteins in *Escherichia coli* are synthesized with a SRRxFLK twin-arginine (RR-) motif in their N-terminal signal peptides [1]. They are targeted and translocated post-translation-ally by the twin-arginine translocation (Tat) system in a folded state [2]. The translocon is formed by the TatABC subunits in the cytoplasmic membrane.

Of the growing list of Tat-substrates predicted and identified, we find that a number of redox enzymes also appear to have their own system specific accessory chaperone protein [3,4]. The chaperones termed REMPs (redox enzyme maturation proteins) are required for assembly, protease protection, maturation, and targeting to the translocon through a complex multi-step process [5,6]. The REMPs are not part of the final active holoenzyme complexes, but instead seem to be important in monitoring assembly processes by mechanisms unknown.

The Tat dependent redox enzymes can be separated into two groups based on their catalytic cofactors [5]. Here we investigate the molybdopterin-containing enzymes dimethyl sulfoxide reductase DmsABC, trimethylamine *N*-oxide reductase TorAC, formate dehydrogenases FdnGHI and FdoGHI, periplasmic nitrate reductase NapABC, and cytoplasmic nitrate reductase NarGHI. The respective REMP chaperones for each of the enzymes are DmsD, TorD, NapD, NarJ, FdhD, and FdhE [5,7]. The other group consists of two hydrogenases, HyaAB and HybOC, which have a Ni–Fe cofactor in their catalytic sites, where HyaE and HybE assist their maturation, respectively.

Previously our group has shown that the different RR-containing N-terminal peptides and/or preprotein enzymes subunits indeed interact with specific REMPs [7]. Here using the in vivo adenylate cyclase based two-hybrid (BACTH) system, we present a comparative study of the REMP interactions with different regions of the RR-leader containing subunits. Hybrid recombinants of the full preprotein ($Enzyme_F$, complete sequence including RR- peptide, peptidase cleavage site, and mature region), RR-leader peptides ($Enzyme_P$, RR-peptide region before the peptidase cleavage site), mature proteins ($Enzyme_M$, mature region only), and the peptidase cleavage site deleted ($Enzyme_X$) forms of RR-containing subunit were generated (Fig. 1A). The interaction between these constructs and their cognate REMPs were investigated in both wildtype and Tat subunit(s) deletion strains. We find all REMPs interact with the full preprotein of RR motif-containing subunit. However, not all REMPS interact with the RR-leader peptide or the mature protein alone. Many REMP interactions are also affected by the absence of the Tat system.

2. Materials and methods

2.1. Plasmid constructions and TatABC/E deletion mutant constructions

Strains and recombinants used and produced in this study are described in Supplementary Table S1. Recombinants of REMPs with T25- and enzyme with T18-domains of the adenylate cyclase fused to their C-termini were generated as described in [7] using primers listed in Table S2. The peptidase I cleavage sites were removed from the preprotein

sequences by site-directed mutagenesis using a Quickchange II kit (Stratagene) with the T25 fusion recombinants above as templates.

Gene deletions were performed according to the method of Datsenko and Wanner [8] to generate strains CLBTH4B (*tatA/E*), CLBTH5B (*tatBC*) and CLBTH6B (*tatABC/E*) from BTH101 (Primers; Table S3).

2.2. BACTH interaction screening and β-galactosidase assays

Assays based on the reconstitution of functional adenylate cyclase was performed as described in [7,9,10] with the following exceptions. Pre-screening of interactions was performed on LB plates containing 40 μg/ml bromo-chloro-indolyl-galactopyrano-side. Overnight cultures also contained 0.5 mM isopropyl-β-D-thiogalactopyranoside. Western blots were performed to verify accumulation of each fusion construct (not shown).

3. Results

3.1. Regions of the RR-containing subunits

To further investigate the differential interactions between the REMPs and the RR-peptides of Tat-specific redox enzymes observed in our previous study [7], further two-hybrid experiments were undertaken. DmsD was initially identified by its ability to bind to the RR-leader peptide alone [11]. We now consider potential interactions of REMPs with the mature protein. Specifically, constructs were generated to determine the interacting region(s) of the RR-signature containing substrate. The different enzyme substrate forms investigated in this study are shown in Fig. 1A ($Enzyme_P$, $Enzyme_F$, $Enzyme_X$, and $Enzyme_M$). Note that the NarG subunit contains an uncleaved vestige RR-peptide and thus no $Enzyme_X$ form was necessary. $NarG_P$ was constructed as the first 50 amino acids in NarG, while the ‘‘mature” form ($NarG_M$) starts at residue 51. This approach is aimed at providing a glimpse of the substrate form that the REMP interacts with along the translation, assembly, and targeting process pathway (Fig. 1B).

3.2. Investigating the REMP interaction sites within the catalytic subunits

Using the four enzyme forms described in Fig. 1A, the enzymes NarG, DmsA, NapA, TorA, HybO and FdoG were targeted for interaction with their respective REMPs: NarJ, DmsD, NapD, TorD, HybE and FdhE. β-Galactosidase activity (in Miller units) was used to evaluate the degree of interaction between the two partners via the reconstitution of the domains (T18 and T25) of adenylate cyclase (one fused to the enzyme substrate, while the other was fused to the REMP). The results show that interactions were observed for all enzymes with their full preprotein ($Enzyme_F$) and full preprotein cleavage site deleted ($Enzyme_X$) forms (Fig. 2). Removal of the cleavage site had no effect on the interaction with any of the full preprotein forms, which demonstrates that the interactions observed are independent of leader processing events.

The REMPs NarJ, DmsD and NapD interacted with the RR-leader of their corresponding enzymes ($NarG_P$, $DmsA_P$ and $NapA_P$). However, there was little to no interaction with the mature forms $NarG_M$, $DmsA_M$, and $NapA_M$ (Fig. 2) based on this assay approach.

Investigation of the full preprotein (RR-leader still attached) showed weaker interaction between the REMP and $NarG_{F/X}$ and $DmsA_{F/X}$, but was the same for $NapA_{F/X}$ (Fig. 2) when comparing to the interaction with the peptide form. This suggests that while the REMPs can bind the RR-peptide of NarG, DmsA and NapA, when the full preprotein is present the strength of interaction or fraction binding is reduced for NarG and DmsA only.

The REMPs TorD, HybE and FdhE show a different trend of interaction. All four forms of TorA had approximately equivalent interaction activity when tested with TorD, suggesting that equivalent binding sites exist within both the leader and mature forms of TorA. HybE interacts with the HybO mature enzyme ($HybO_M$) as well as the full preprotein forms ($HybO_X$ and $HybO_F$), but not the RR-leader peptide ($HybO_P$), suggesting the presence of one interaction site within the mature sequence of HybO, an observation also seen by Dubini and Sargent [12]. FdhE is only able to interact with the full preprotein forms of FdoG ($FdoG_F$ and $FdoG_X$) but does not interact with either the RR-leader peptide ($FdoG_P$) or mature form ($FdoG_M$) alone (Fig. 2). This suggests that the interaction of FdhE with the preprotein forms $FdoG_{X/F}$ is dependent on the presence of both the RR-leader and mature sequence but not the cleavage site.

3.3. Interaction dependence of the Tat subunits

Previous observations implied that DmsD localization to the membrane is dependent on the presence of the TatBC subunits [13]. This suggested that the holoenzyme DmsA may be guided by DmsD to the translocon for Tat docking. To investigate the requirement of the individual Tat subunits for REMP–enzyme interactions, the strain for BACTH, BTH101, was engineered with deletions in *tatA*/*E*, *tatBC*, or *tatABC*/*E*. All REMP–enzyme interaction pairs described above were then assayed in these Δ*tat* mutant strains as well as in the BTH101 (‘‘wildtype”) containing the intact *tat* operon (Fig. 3).

The interaction of NarJ with the RR-vestige peptide region from NarG ($NarG_P$) was affected in such that absence of any of the Tat components reduced the activity by almost 50% (Fig. 3). However, the interaction of NarJ with either $NarG_F$ or $NarG_M$ showed no *tat* dependence. Tat dependence of DmsD interactions were not observed for any substrate form. Similarly interactions between NapD and NapA had little Tat dependence, with the exception that the interaction with the $NapA_F$ form was much weaker in a Δ*ta-tABC*/*E* background (Fig. 3).

TorD bound all four forms of TorA regardless of Δ*tat* strain (Figs. 2 and 3). However, the TorD interaction with the $TorA_P$ and $TorA_M$ forms was weaker in all deletion backgrounds, with the largest reduction in Δ*tatBC* or Δ*tatABC*/*E* (Fig. 3), implying that TatBC may play a role in the TorD–TorA interaction pathway.

An interaction could only be observed between FdhE and FdoG in the full preprotein forms ($FdoG_X$ and $FdoG_F$) and this interaction was reduced in both Δ*tatBC* and Δ*tatABC*/*E* strains (Fig. 3). HybE interacts with $HybO_M$ as well as both preprotein forms (Figs. 2 and 3). Experiments performed with the different Δ*tat* strains show a similar pattern with the exception that the interaction with $HybO_F$ was almost completely abolished in the Δ*tatABC*/*E* strain, suggesting a dependence on the complete translocon for this interaction (Fig. 3).

4. Discussion

REMP chaperones have many proposed roles in aiding the maturation of Tat-specific redox enzymes, which includes protease protection, Sec-avoidance, targeting, and as an escort through the enzyme maturation pathway. Here we have explored the interactions between RR-motif leader containing substrates of Tat-dependent redox enzymes and the REMPs of *E. coli*. Given the unique processing of redox enzymes during maturation, the various regions of the enzymes were subjected to investigation using an in vivo two-hybrid assay. Distinct differences for the REMP interactions were observed, suggesting that the maturation pathways of redox enzymes involving REMPs are more unique than previously assumed. The dependence of Tat subunits for these interactions further illustrates this uniqueness.

Six different REMP–enzyme interaction pairs were investigated in this study. All REMPs were shown to interact with the full preprotein forms of their respective enzymes (Figs. 2 and 3). However, key differences suggest either sites or modes of interaction for different REMPS are different. FdhE was only able to bind to preprotein forms of FdoG. Contrary to this, the TorD REMP was able to bind to all four forms TorA at equivalent levels. NarJ, DmsD and NapD bound to the RR-leader peptide of their substrates (NarG, DmsA and NapA) but not to the mature forms. And finally, HybE bound to the mature form of its substrate (HybO) but not the RR-leader peptide. The interactions are summarized in Fig. 4.

The complete lack of an interaction between the mature regions of DmsA and NapA with DmsD and NapD, respectively, suggest that the only interaction site is located within the RR motif-containing peptide region (Fig. 4A). Despite this similarity, the interaction between NapD and the full preprotein form of NapA ($NapA_F$) was dependent on the presence of the entire Tat complex, while the DmsD interaction with $DmsA_F$ showed no Tat dependence (Fig. 4B). Recently the idea that DmsD interacts with the TatB and/or TatC proteins was confirmed [13,14]. The data here show that interaction between DmsD and the $DmsA_P$ occurs independent of TatBC and the other Tat subunits (Fig. 3), suggesting that the interaction likely occurs prior to targeting to the Tat components.

TorD was the only REMP to demonstrate an equivalent level of interaction signal with both the mature and leader peptide forms of TorA, suggesting that two equivalent yet independent interaction sites exist within the full preprotein sequence of TorA – one within $TorA_P$ and another in $TorA_M$. Previous research also showed that TorD was capable of interacting with the mature region of TorA [15].

NarJ was also shown to interact with both the RR-vestige peptide and mature forms of NarG (Figs. 2 and 3) however the interaction with the $NarG_M$ was much weaker than with the $NarG_P$. This result indicated that a strong binding site responsible for NarG interacting with NarJ was located in the RR motif-region of NarG, while a second possible binding site responsible for NarG interacting with NarJ is located further into the sequence of NarG. Direct interactions between the mature region of NarG and NarJ have previously been suggested supporting that NarG may have two binding sites for NarJ. The first site somewhere within the first 15 residues [16,17] while the second site is somewhere after

residue 40 (Fig. 2 and [17,18]). Although there was an effect of absence of Tat on NarJ interacting with $NarG_P$, the effect on NarJ interacting with full and mature forms were minor. According to Blasco et al. [19], the ability of NarGH to associate with the membrane is reduced in the absence of NarJ and NarJ is also required for the membrane-bound enzyme to be activated. Reconciling our findings suggest that the interactions between NarJ and the premature form of NarG may happen prior to NarGH associating with NarI in the membrane which may be assisted by Tat. It is the NarJ–NarG vestige RR-peptide region interaction that depends on Tat as found here and [20].

The investigations here with FdhE and the mature form of FdoG rule out the possibility that the binding site previously observed [7] is within the mature region (Fig. 2). Furthermore, the results suggest that the FdoG binding site may be comprised of a short sequence from the leader and the mature region that immediately surrounds, but does not depend on the presence of the cleavage site (Fig. 4A).

No interaction was observed between the RR-leader peptide of HybO with HybE (Fig. 2 and [7]); thus the HybE binding site is likely located within the mature region of HybO. Although, the individual absence of TatA/E or TatBC did not impair HybE binding with any HybO forms. The absence of TatABC/E resulted in the loss of about 90% of the binding ability of HybE with $HybO_F$ (Fig. 3). The results suggest a different maturation pathway for this NiFe enzyme, where perhaps Tat interaction stabilizes a processing-competent enzyme.

The interaction with the four substrate forms pose interesting consequences in terms of processing of these enzymes. In accordance with the maturation model presented in Fig. 1, an interaction with form P likely occurs immediately following translation and exit from the ribosome, when the leader peptide is exposed and potentially unfolded. Docking simulations of DmsD with the DmsA leader peptide shows the leader bound to DmsD in an extended conformation without any observable secondary structure [21]. However, it is clear that this is REMP dependent, as from NMR work; NarG leader region binds to NarJ as an α-helix [17]. Forms F and X is present when translation is complete although likely different structurally at every stage prior to translocation. Form F is cleaved to remove the leader peptide resulting in form M representing the final assembled enzyme, which occurs for most enzymes during translocation across the membrane. In accordance to this, any interaction site in the M region would be present in forms F and X and have equal affinities for their REMP and processing events at the membrane was expected to have no effect on the interaction with the REMP in the cytoplasm.

This work illustrates the specificity of these REMPs and their subtle differences in substrate binding. Our study further supports the idea that each REMP is indeed a system-specific chaperone and that the nature of the substrate interaction can be more than the RR-motif peptide. Further the findings suggest that each redox enzyme system follows a different interaction/maturation pathway and degree of Tat assistance, requiring its own chaperone to facilitate its pathway toward a folded, targeted, assembled, and functional enzyme.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

REMP	redox enzyme maturation protein
Tat	twin-arginine translocase system
BACTH	bacterial two-hybrid

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi: 10.1016/j.febslet.2010.10.043.

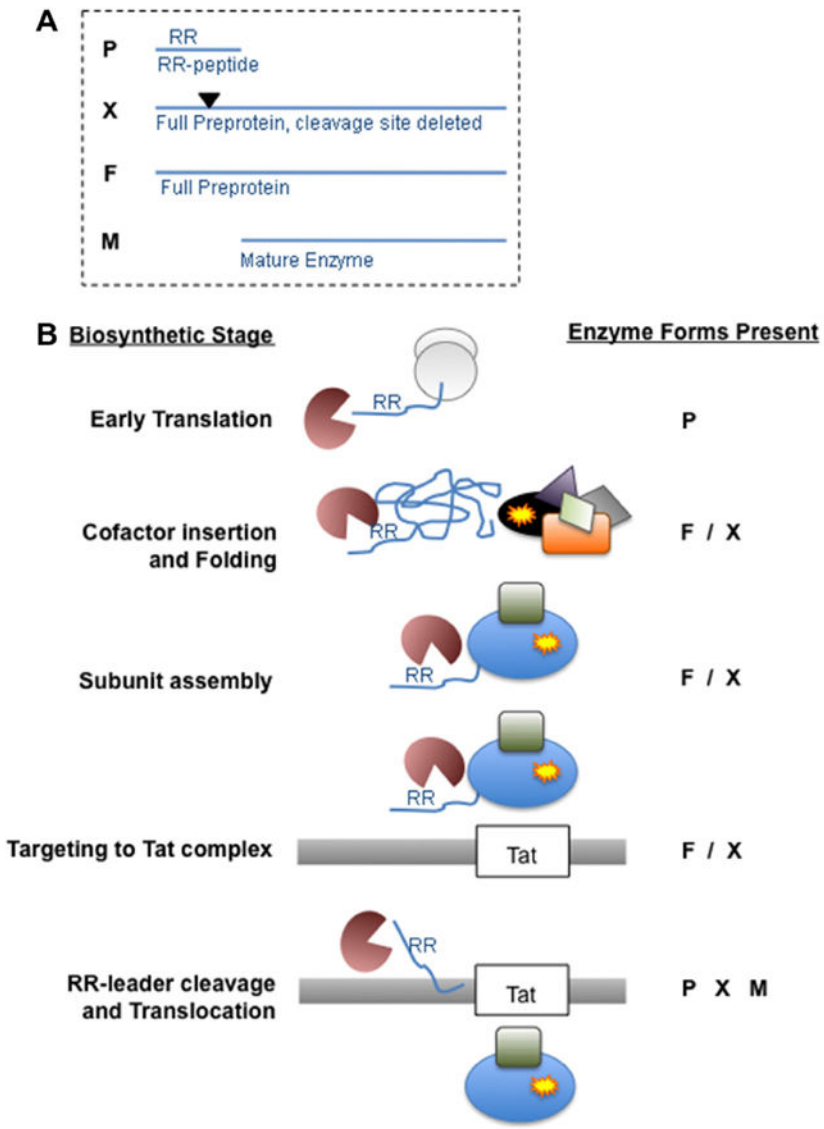

Fig. 1.

Regions of RR-leader containing redox enzymes. (A) The different regions of the substrate enzymes studied here. The translated sequence consists of the RR-containing N-terminal leader peptide (P), the leader cleavage site (triangle) within the full preprotein (F), the full preprotein with the cleavage site deleted (X), and the mature region that remains following leader cleavage and translocation (M). (B) An abbreviated model of the proposed Tat-dependent biogenesis of RR-leader containing redox enzymes. The nascent polypeptide chain synthesized with a RR-motif in its amino terminal leader would be present during early translation. The corresponding REMP chaperone recognizes a site(s) within the redox enzyme preprotein. Cofactor biosynthesis and insertion into the mature region of the enzyme as the mature polypeptide folds into its holoenzyme form. Following recruitment and assembly with any other enzyme subunit(s), the enzyme complex is targeted to the membrane and Tat translocon where the enzyme subunits are moved across the membrane with cleavage of the RR-leader peptide.

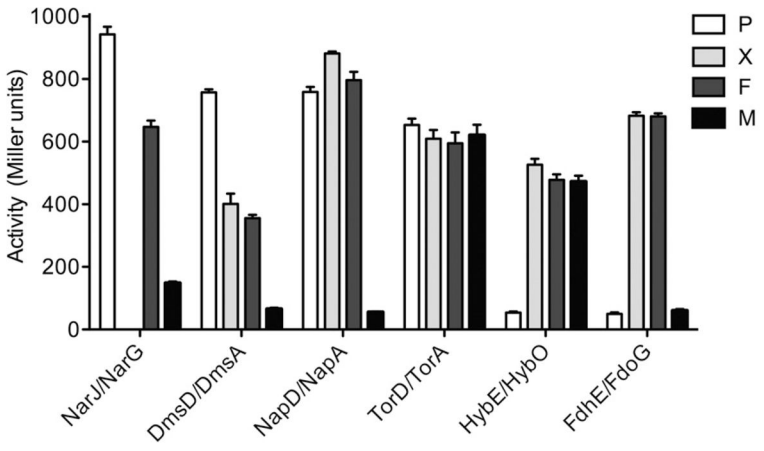

Fig. 2.

Interactions between the chaperones and their cognate RR-containing subunits. The protein pairs were screened using the four forms of the substrate enzymes described in Fig. 1A. The bars from left to right are P, RR-leader peptide, white; X, full preprotein sequence with the cleavage site deleted, light grey; F, full preprotein sequence, dark grey; M, mature region, black. The degree of interaction is evaluated by cAMP-activated β-galactosidase activity that were calculated from at least three independent culture trials and assayed in each time in triplicate, indicated as standard error bars.

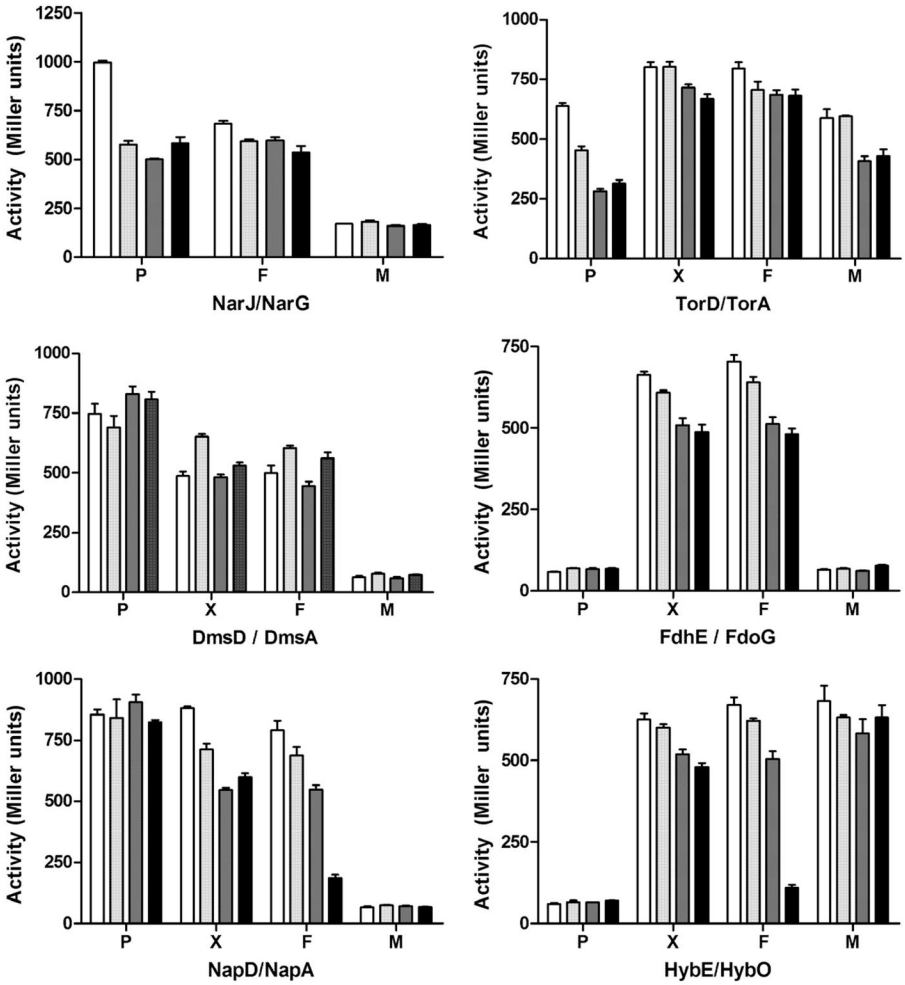

Fig. 3.

Tat-dependence of the chaperone–substrate interactions. The protein pairs as described in Fig. 2 were assayed in various *tat* gene deletion strains. Interactions were screened using the four forms of the substrate enzymes described in Fig. 1A. The bars from left to right are WT, wildtype containing all Tat subunits, white; Δ*tatA*/*E*, light grey; Δ*tatBC*, dark grey; Δ*tatABC*/*E*, black.

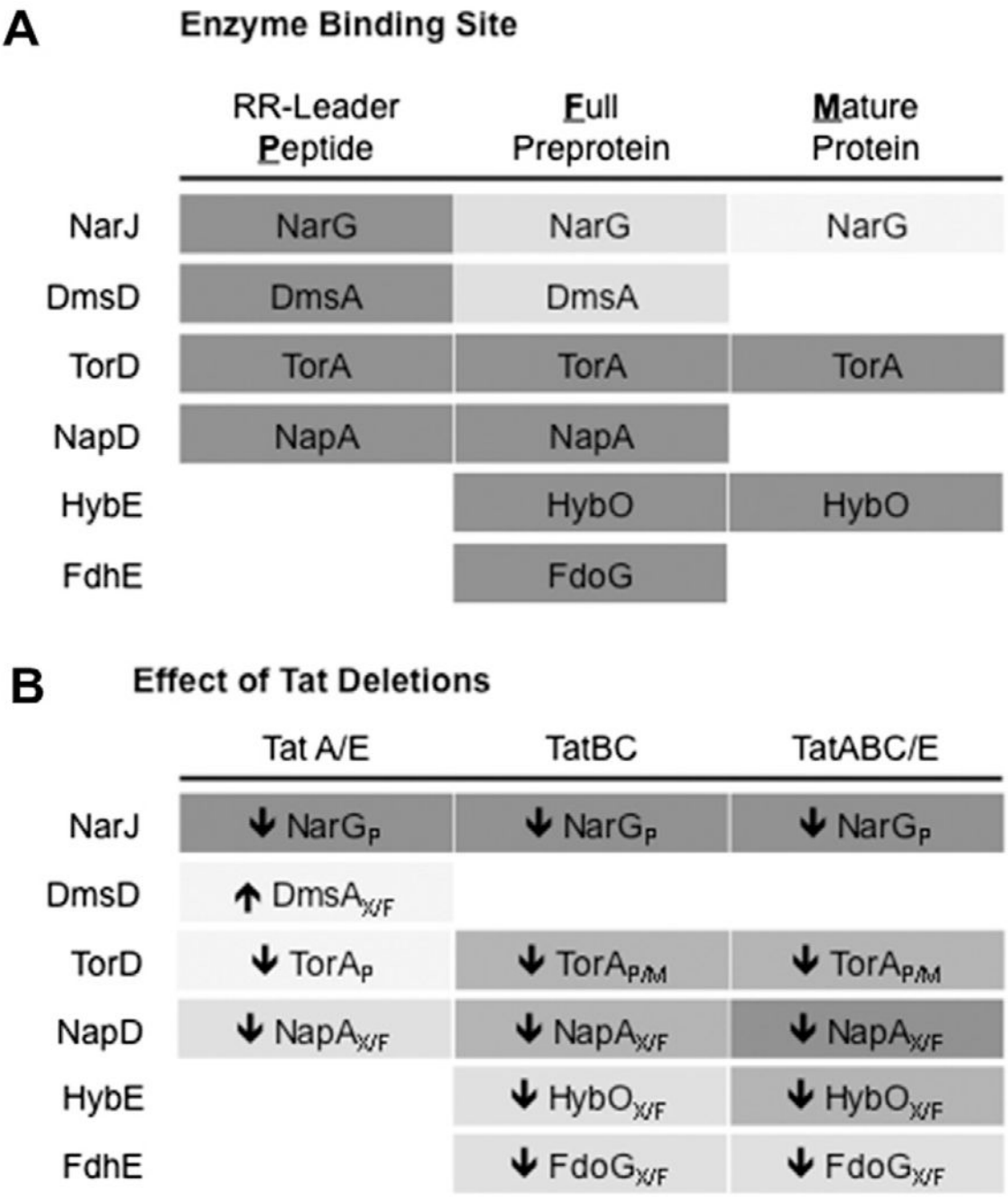

Fig. 4.

Summary of interaction sites of enzyme substrate forms and their degree of dependence on Tat. (A) The REMP interaction site for each enzyme as determined from BACTH results in Fig. 2. (B) The effect of various *tat* deletions on the interactions between the REMP and enzyme interaction site as compared to interaction in wildtype. Darker the shading the greater the interaction activity or effect.