Scott M. Ward, Asucion Delgado, Robert P. Gunsalus, Michael D. Manson

A NarX-Tar chimera mediates repellent chemotaxis to nitrate and nitrite

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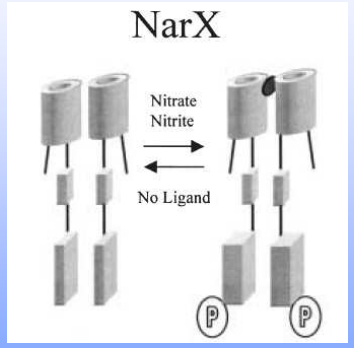
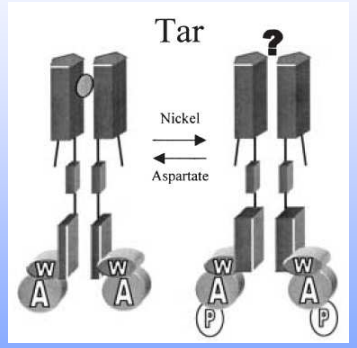
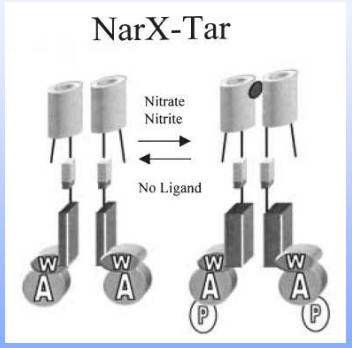






29.10.2008

Overview

- Chemotaxis and chemoreceptors in general
- Previous chimeric receptor approaches
- New approache of this paper: The NarX-Tar chimera

Chemotaxis

Attractant

Inhibition of ability of the chemoreceptor to stimulate the CheA autophosorylation

Decreasing phosphotransfer to response regulator CheY

Counterclockwise (CCW) rotation of flagella

Smooth swimming

Repellent

enhances ability of the chemoreceptor to stimulate the CheA autophosorylation

Increasing phosphotransfer to response regulator CheY

Clockwise (CW) rotation of flagella

Tumbling

Chemoreceptors

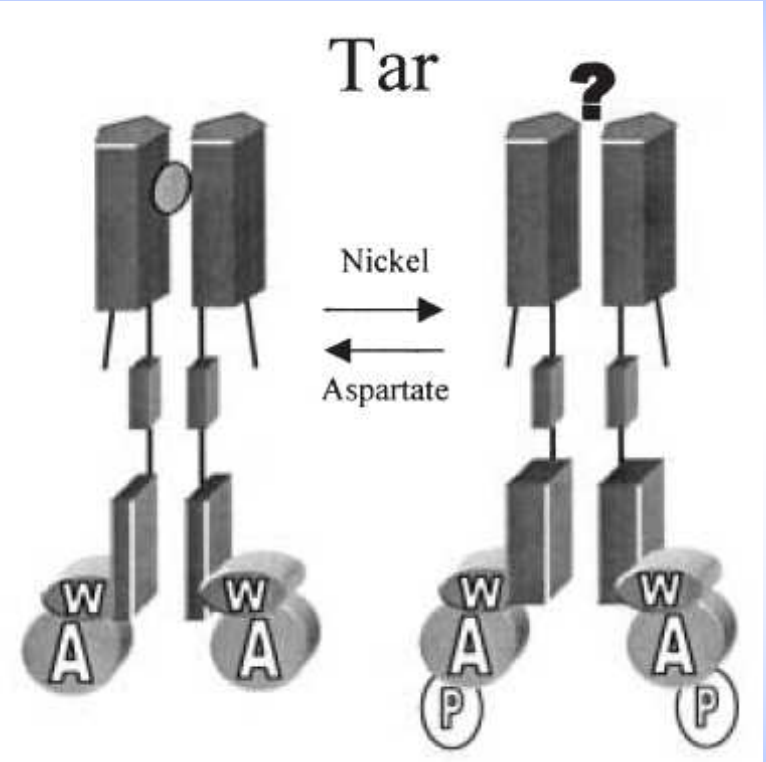
- Mainly 4 different chemoreceptors in *E. coli*

Tar	**Tsr**	**Trg**	**Tap**
+ aspartate	+ serine	+ ribose	+ dipeptides
+ maltose		+ galactose	
- Ni^{2+}	- leucine		
- Co^{2+}	- acetate		

high abundance

Structure of the Tar chemoreceptor

- Homodimers
- Each subunit spans the membrane twice
- N-terminal ligand recognition loop in the periplasmic space
- C-terminal signaling and adaptation domain in the cytoplasm
- Repellent binding at periplasmic N-terminal domain stimulates autophosphorylation of CheA at the cytosplasmic C-terminal domain



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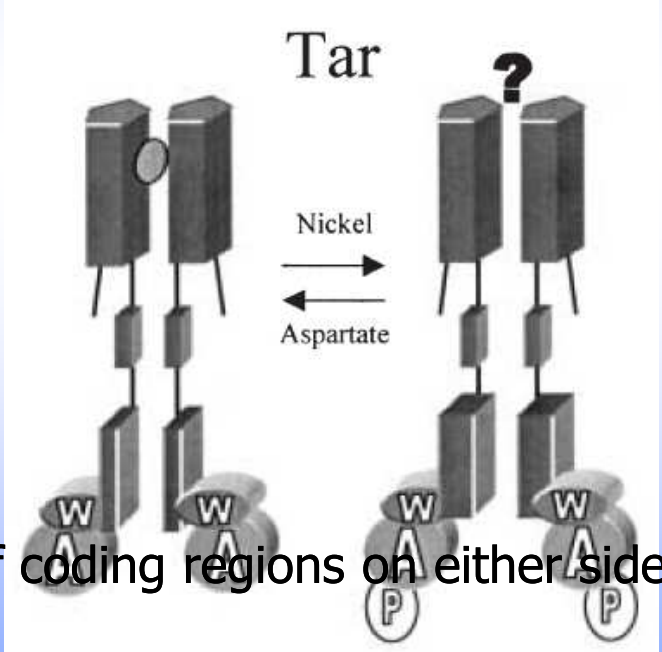
Construction of Tar-Tsr and Tsr-Tar chimera

- Fusion joint for the Tasr (Tar-Tsr) and the Tsar (Tsr-Tar) chimera:

NdeI restriction site in the cytoplasmic domain near the C-terminal end of the linker region, that connects the second transmembrane helix to the signaling and adaptation domains



➡ Reciprocal exchange of coding regions on either side of the NdeI site

Construction of Trg-Tsr and Tap-Tar chimera

- Problem: Trg and Tap natively no NdeI site

➡ Introduction of an NdeI site at the same relative position than in *tar* and *tsr* genes by site directed mutagenesis

➡ Reciprocal exchange of coding regions on either side of the NdeI site

➡ All chimera made this way have the ligand-sensing properties corresponding to the periplasmic domain of the hybrid

Construction of Tar-EnvZ and Trg-EnvZ chimera

- EnvZ is a sensor-kinase of *E. coli* for osmosensing → chemoreceptor-sensor-kinase-hybrid
- Also a homodimer and the predicted membrane topology is similar to chemoreceptors

⇨ similar architecture dictates a similar mechanism of transmembrane signaling ?

- Introduction of NdeI sites in *envZ* gene at location judged to be comparable with that of NdeI site in *tar*
- Exchange of coding regions
- For monitoring: *ompC-lacZ* fusion gene

⇨ β-galactosidase induction by Tar/Trg ligands worked quite well, but…

⇨ Problem: reciprocal hybrid not testable because EnvZ lacks a known ligand

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  - NarX transmembrane sensor kinase in general
- New approache of this paper: The NarX-Tar chimera
  - Construction of the chimera
  - Overview of used plasmids and *E. coli* strains
  - Immunoblots
  - Swarm plates
  - Behavior of unstimulated tethered cells
  - Responses of tethered cells to the addition of repellents
  - Responses of tethered cells to the removal of nitrate and nitrite
  - Repellent-in-pond capillary assays
  - Summary and conclusion

NarX transmembrane sensor kinase in general

- Sensing for nitrate and nitrite
- Regulates expression of genes for utilization of nitrate and nitrite as terminal electron receptors under anaerobic conditions (nitrate or nitrite reductase)

Binding increases its autophosphorylation activity

↓

Phosphotransfer to response regulators NarL and NarP

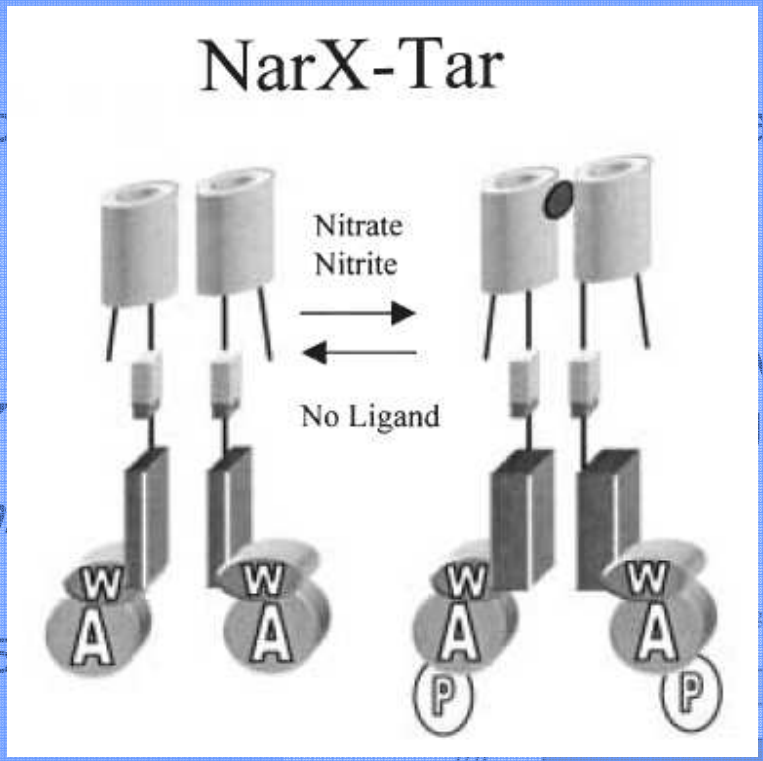
↓

NarL and NarP are positive or negative transcription factors for a number of genes involved in anaerobic respiration

Construction of a NarX-Tar chimera (Nart)

- Purpose: Construction of a hybrid receptor containing the ligandbinding domain for nitrate and nitrite of NarX and the signaling and adaptation domain of Tar

➡ Nart can serve as rep… …and nitrite

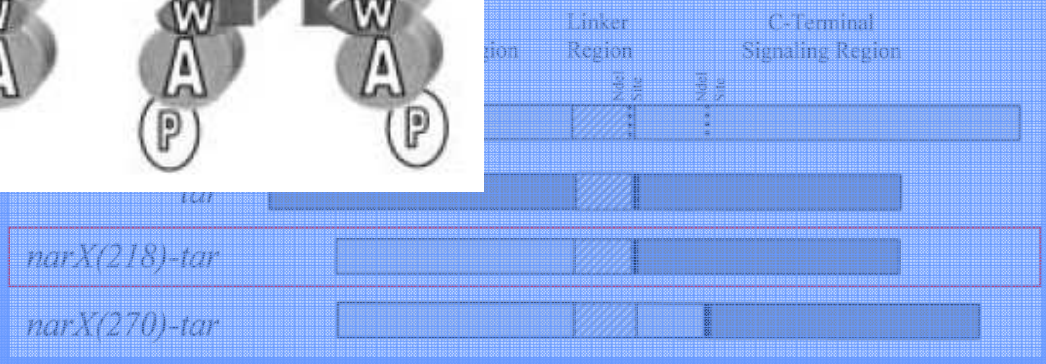


Fusion:

- Introduction of NdeI … …ve position as that introduced into *envZ* … …ions

➡ two different *narX-ta*…

  - 1-218 of *narX* + 25…
  → *narX(218)-tar*
  - 1-269 of *narX* + 257-553 of *tar*
  → *narX(270)-tar*



Overview of used parts

Plasmids:

- pAD48: containing the *narX(270)-tar* gene

⇨ no receptor expression detectable in immunoblot

- pAD56: containing the *narX(218)-tar* gene

⇨ stable expression of Nart receptor of ~55 kDa size

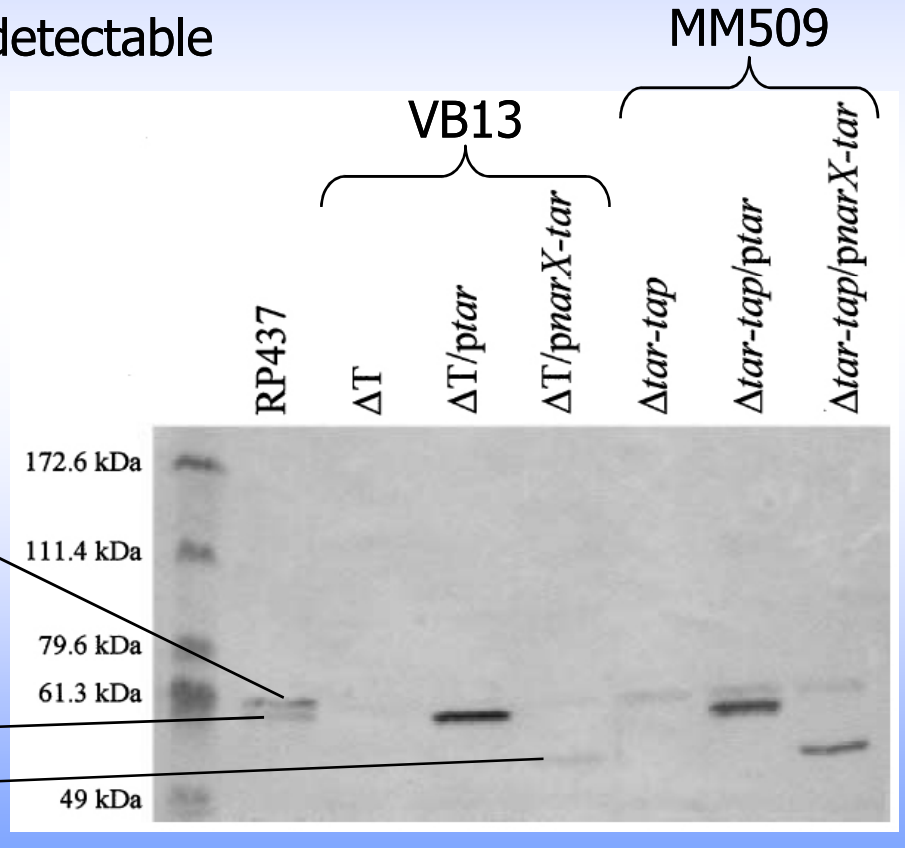
- pMK113 (p*tar*): containing *E. coli tar*-gene

E. coli strains:

- RP437: wild type: containing *tar*, *tsr*, *trg*, *tap* genes
- MM509 (Δ*tar-tap)*: containing *tsr* and *trg* genes, but lacks *tar* and *tap* genes
- VB13 (Δ*T)* : absence of all chemoreceptors

Immunoblot

- Protein extraction → protein separation by SDS-PAGE → blotting to nitrocellulose membrane → staining with antibody against conserved cytoplasmic domain of Tsr
- For *narX(270)-tar* gene: no receptor detectable
- Results for *narX(218)-tar* gene:



→ Tsr and/or Trg seems to stabilize the fusion protein Nart

Swarm plates

- Inoculation of colonies onto TB swarm plates

→ VB13: no spreading → absence of chemoreceptors renders them exclusively smooth swimming

→ VB13/pAD56: spreading colony → Nart stimulates CheA → → → tumbling

- But no formation of chemotactic rings in semi-solid TB agar containing various concentrations of nitrate or nitrite

→ Reason: Formation of chemotactic rings require metabolism of attractant/repellent to create a relative gradient

Behavior of unstimulated tethered cells

- Flagella filaments were sheared to short stubs
- Cells were mixed with antifilament antibody and tethered to glass coverslip
- Whole coverslip was put into a flow chamber

- Results:

Table 1. Rotational biases of tethered cells.

Strain	Percentage time in CCW rotation[a]
VB13	99.5 ± 0.5
VB13/pAD56	91 ± 1
MM509	71 ± 5
MM509/pAD56	20 ± 2

a. The values shown are the means of the percentage time spent rotating CCW out of 60 s, ± the standard error. Data from 20 cells were averaged for each strain.

Responses of tethered cells to the addition of repellents

- Range of tested concentration: 10^{-1} M – 10^{-6} M

Table 2. Responses of tethered cells to the addition of repellents.

	Time of CW-only rotation (s) until the first CW → CCW reversal after repellent added at the indicated concentration (M)								
Leucine added	10^{-3}	10^{-4}	10^{-5}	10^{-6}	Nickel added	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Strains					Strains				
MM500	28 ± 1	10 ± 0.8	6 ± 0.8	5 ± 0.4	MM500	26 ± 2	19 ± 1	5 ± 0.4	3 ± 0.3
MM509	0	0	0	0	MM509	ND	ND	ND	ND
MM509/pTar	5 ± 0.4	2 ± 0.3	0	0	MM509/pTar	480 ± 19	350 ± 25	25 ± 2	0
MM509/pAD56	0	0	0	0	MM509/pAD56	0	ND	ND	ND
VB13	0	0	0	0	VB13	0	0	0	0
VB13/pTar	0	ND	ND	ND	VB13/pTar	740 ± 28	610 ± 26	2 ± 0.4	0
VB13/pAD56	ND	ND	ND	ND	VB13/pAD56	ND	ND	ND	ND
Nitrate added	10^{-3}	10^{-4}	10^{-5}	10^{-6}	Nitrite added	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Strains					Strains				
MM500	0	ND	ND	ND	MM500	0	ND	ND	ND
MM509	ND	ND	ND	ND	MM509	ND	ND	ND	ND
MM509/pTar	ND	ND	ND	ND	MM509/pTar	ND	ND	ND	ND
MM509/pAD56	0	ND	ND	ND	MM509/pAD56	0	ND	ND	ND
VB13	0	0	0	0	VB13	0	0	0	0
VB13/pTar	0	ND	ND	ND	VB13/pTar	0	ND	ND	ND
VB13/pAD56	84 ± 7	11 ± 1	6 ± 1	0	VB13/pAD56	10 ± 0.6	11 ± 1	6 ± 1	0

ND, not determined.

Responses of tethered cells to the removal of nitrate and nitrite

- Physiologically relevant response to repellents removal: decrease tumble frequency as concentration of repellent drops

➡ flow chamber assay with tethered cells to check response on repellent removal

Responses of tethered cells to the removal of nitrate and nitrite

Results:

- VB13/pAD56
  - Extended periods of CCW rotation (thresholds: 10^{-5} M for nitrates, 10^{-3} M for nitrite
  - Peak adaptation times (at 10^{-2} M): 1100 s for nitrate, 420 s for nitrite

- MM509/pAD56
  - Similar behavior, but lower threshold: for both 10^{-6} M
  - Peak adaptation times (at 10^{-2} M): 790 s for nitrate, 380 s for nitrite

- MM509 and MM509/pAD56
  - Removal of leucine also evoke CCW response, but much weaker
  - Peak adaption times (at 10^{-2} M): 66 s for MM509 and 79 s for MM509/pAD56

Repellent-in-pond capillary assays

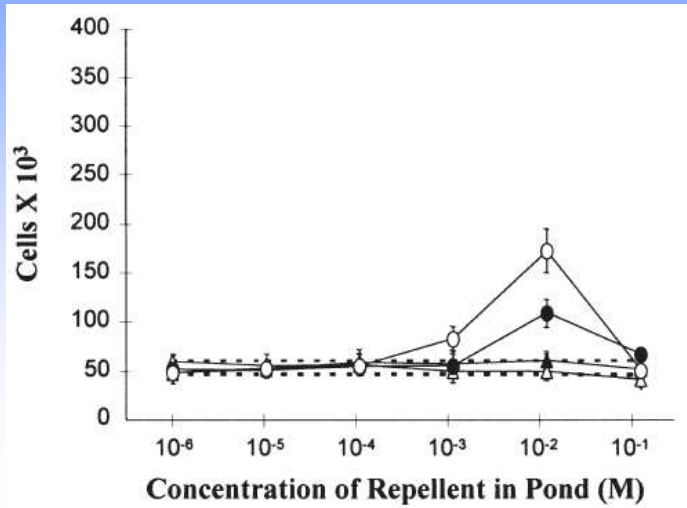
- Variant of the normal chemotaxis-assay, where cells enter a capillary as the move up a diffusion gradient of attractant
- Here: cell suspension with repellent (pond) → cells will flew into a capillary filled with repellent-free buffer

⇨ counting cells in capillary

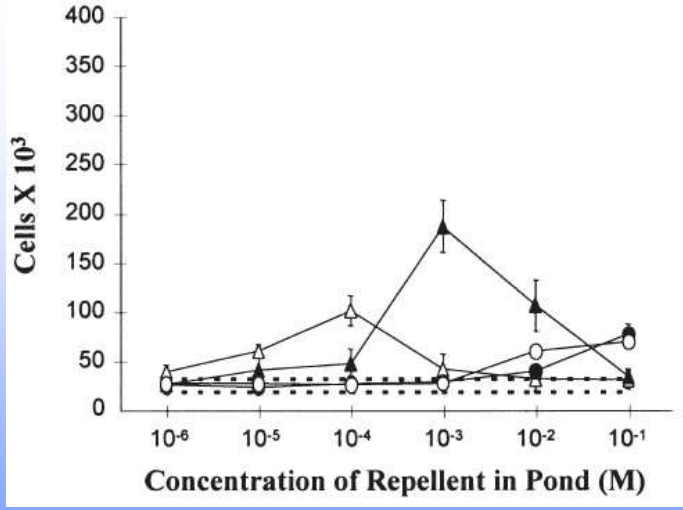
⇨ average number of cells in capillary coming out of a pond without repellent is used as reference

Repellent-in-pond capillary assays

- MM509/pMK113
  - Responses to leucine and acetate
  - No response to nitrate and nitrite

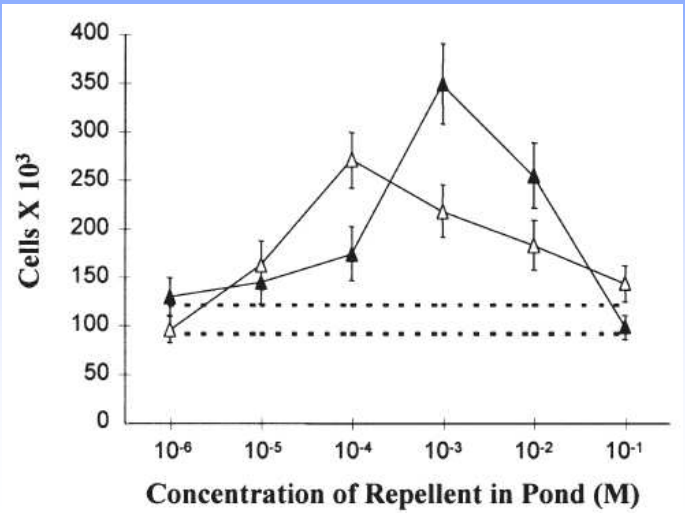


- MM509/pAD56
  - Responses to nitrate and nitrite
  - Effective at lower concentrations than leucin/acetate
  - desensitizing of leucin/acetate by Nart expression



Repellent-in-pond capillary assays

- VB13/pAD56
  - Nearly same responses to nitrate and nitrite
  - No response to leucine and acetate



Summary and conclusion

- Nitrate and nitrite evoked CW flagella rotation (tumbling) as response in MM509/pAD56 and VB13/pAD56 cells
- The responses of these cells to addition and removal of nitrate/nitrite resemble those of MM509/pMK113 and VB13/pMK113 cells to addition and removal of Ni^{2+}
- ➡ Nitrate and nitrite elicit bona fide repellent responses in cells expressing the Nart chimeric receptor
- Results of repellent-in-pond capillary assay reinforce conclusion, that NarX-Tar fusion protein mediates full functional and normal sensing, signaling and adaptation
- Nitrate elicited responses at lower concentrations and with longer peak adaptation times than nitrite
- ➡ Nitrate seems to have a higher affinity to the Nart receptor than nitrite
- ➡ Similar to the binding affinities to the naïve NarX receptor

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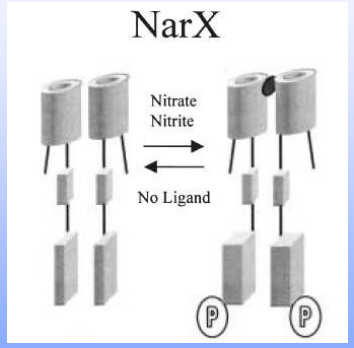

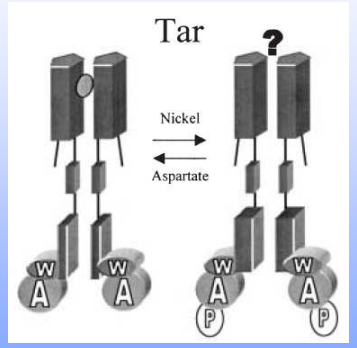

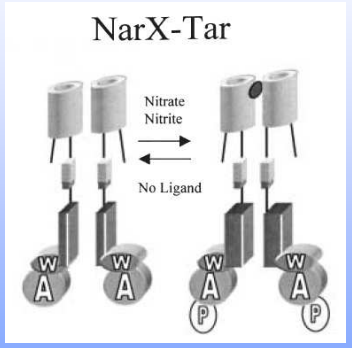

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