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SAPIENZA
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ESC4
Advanced kinetics approaches to unravel protein structure and function

Rapid kinetics to dissect the mechanism of enzyme catalysis

Prof. Serena Rinaldo

LECTURE OUTILINE

Anaerobic energy metabolism in Pseudomonas aeruginosa: the nitrite reductase.

How does the enzyme cycle?

How can we use rapid kinetics?

Take home message

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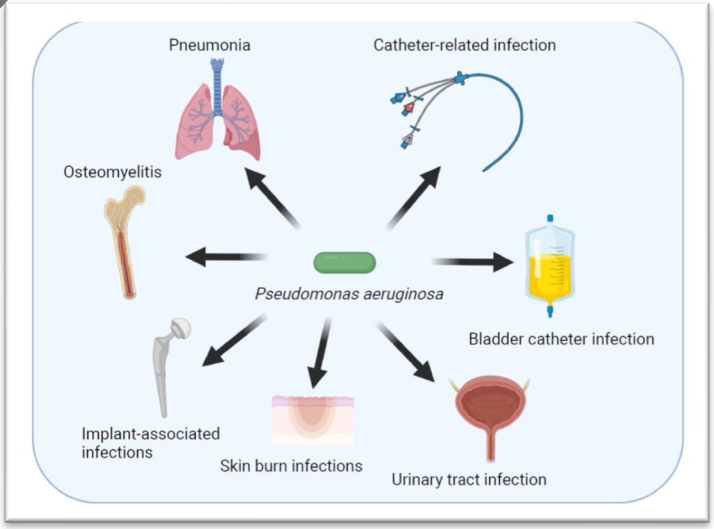
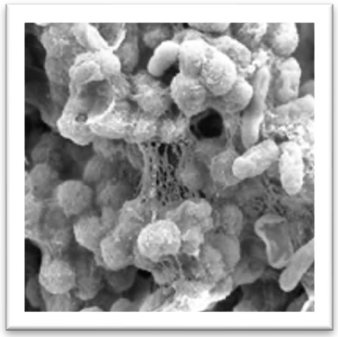
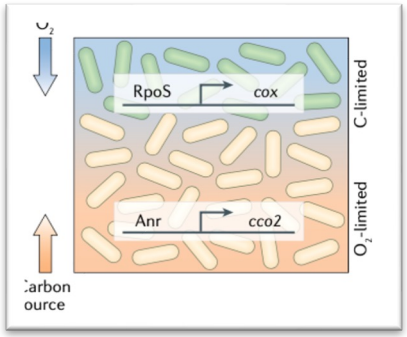
PSEUDOMONAS AND ENERGY METABOLISM

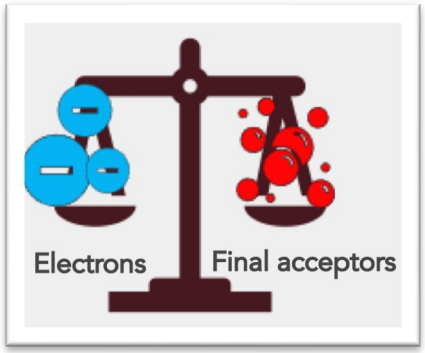


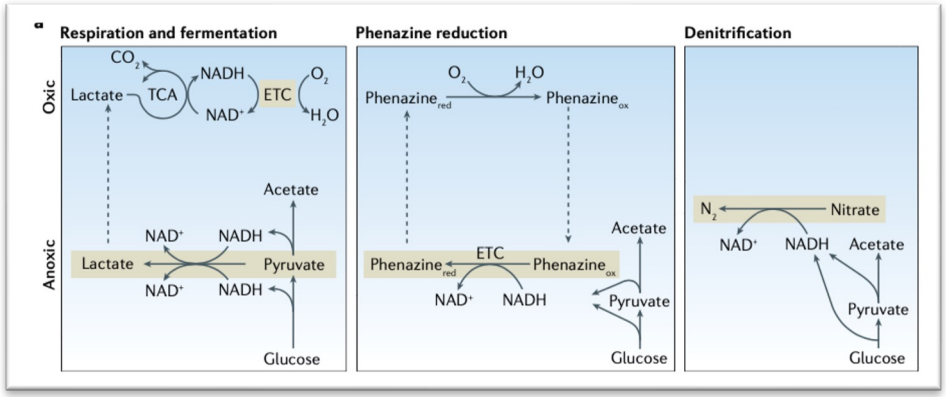
- main cause of nosocomial infections
- chronic lung infection

- formation of robust biofilm
- Oxygen and nutrient gradients take place
- Many strategies to accept catabolic electrons









Alternative respiration(s) prevents electrons:acceptors unbalancing (ROS)

REVIEWS

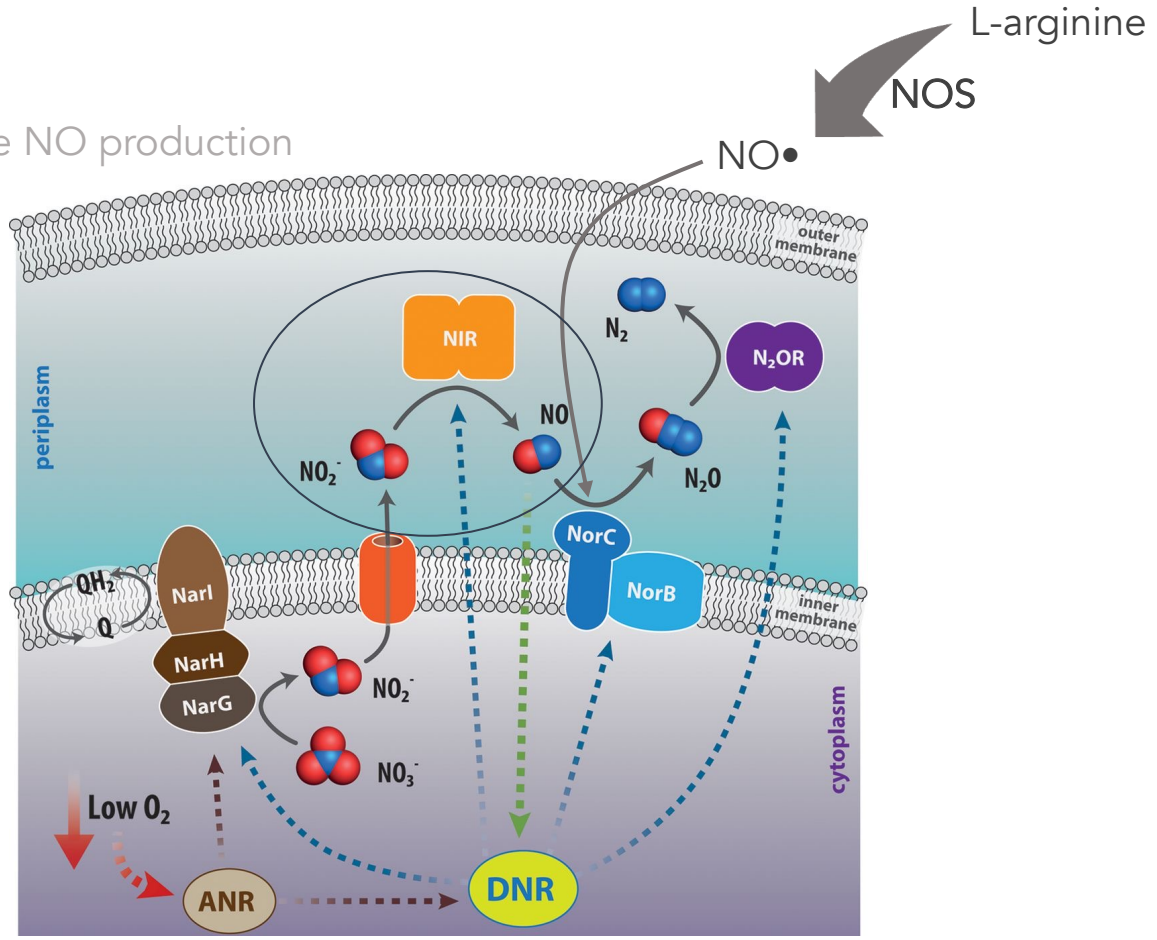
Gradients and consequences of heterogeneity in biofilms

Jeongyoung Jo, Alexa Price-Whelan and Lars E. P. Dietrich

PSEUDOMONAS AND DENITRIFICATION

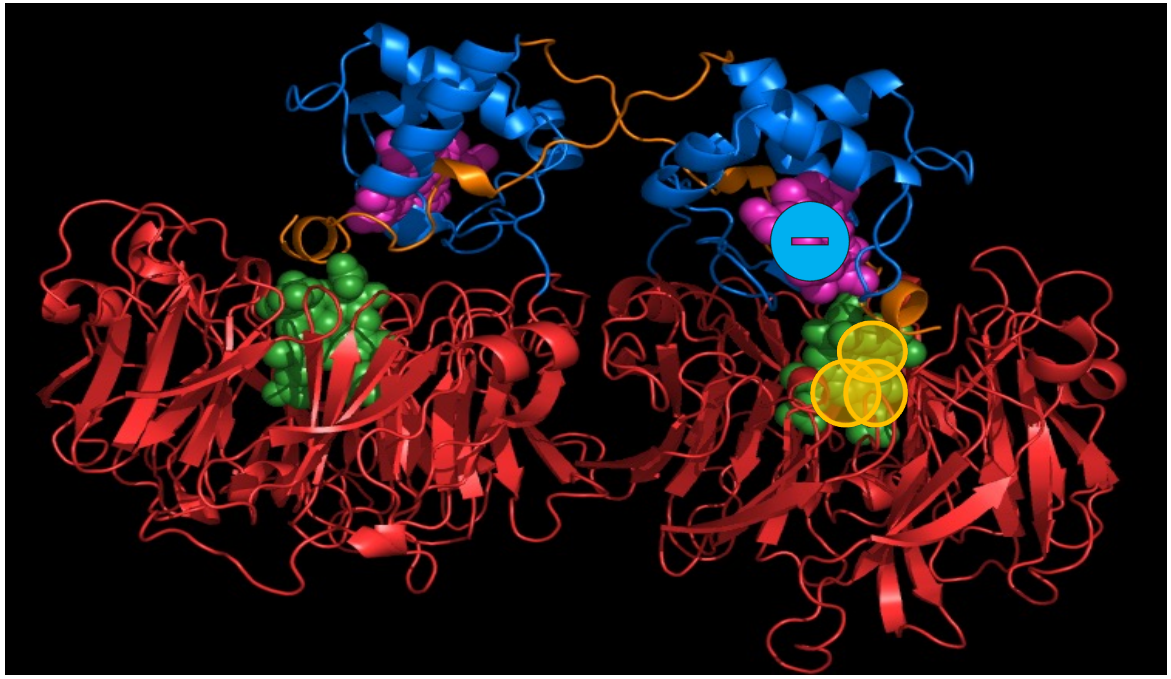
- P. aeruginosa can use denitrification under hypoxic conditions
- Denitrification is an anaerobic respiration where NITRATE is the final electrons' acceptor
- This pathway also helps against the host defense system (NO production)
- NO can modulate biofilm
- NO may target hemeproteins
- Nitrite reductase catalyzes the NO production

Can NO be produced efficiently under high reducing power?



Nitrite reductase and NO

- Two different hemes
- c-heme for electron transfer
- d_1-heme for catalysis



$$NO_2^- + e^- + 2H^+ \longrightarrow NO + H_2O$$

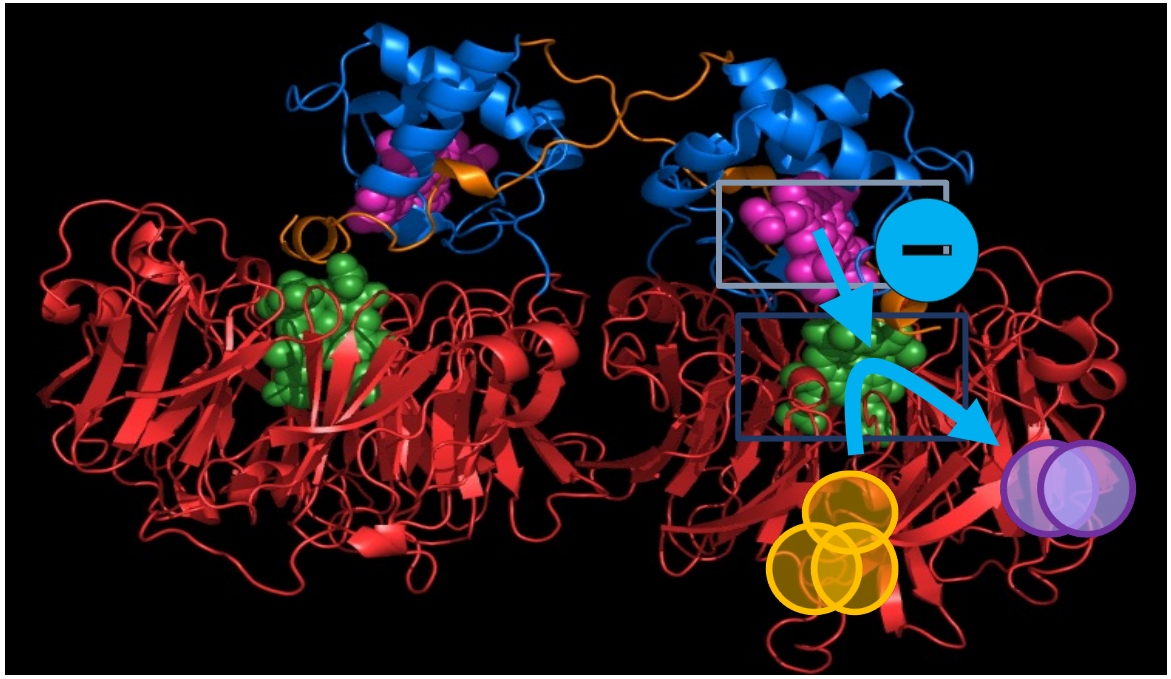
NITRITE REDUCTASE AND NO

- c-heme accepts electrons from an external donor

$$NO_2^- + e^- + 2H^+ \longrightarrow NO + H_2O$$

NITRITE REDUCTASE AND NO

- c-heme internally transfers the electron to the d_1- heme
- nitrite binds to reduced d_1-heme and catalysis occurs



$$NO_2^- + e^- + 2H^+ \longrightarrow NO + H_2O$$

Ferrous heme-NO adducts are known to be very stable

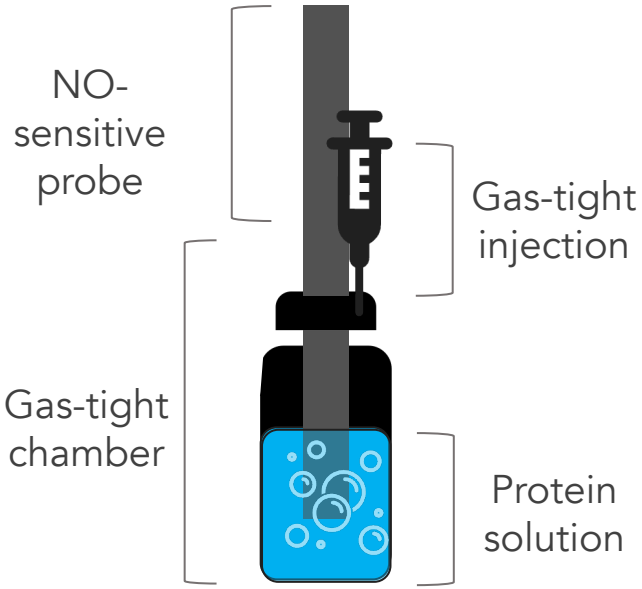
Many heme proteins are inhibited by NO

NITRITE REDUCTASE (cd_1NiR) AND NO: K_{CAT} DETERMINATION

STEADY-STATE KINETICS

(NO-sensitive electrode) pH=7.0; T=20°C

- To measure the turnover number

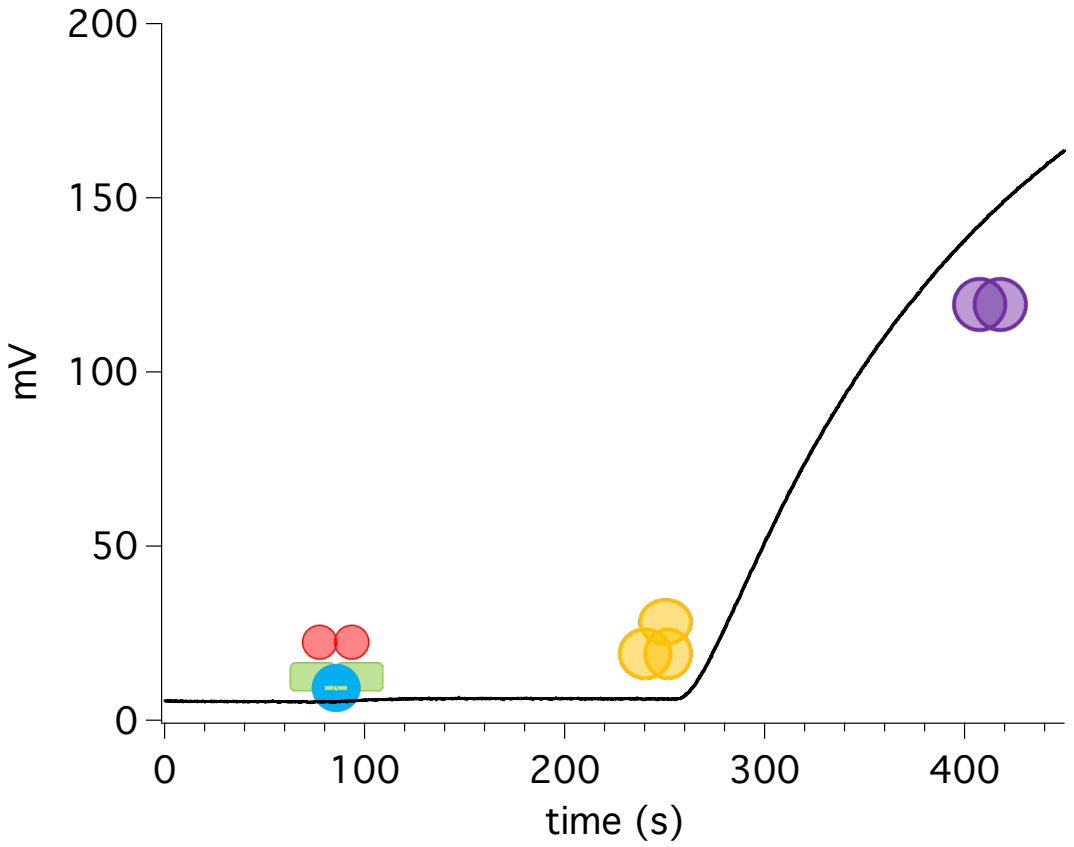


NiR

Excess reductants

Excess NO

Excess NO_2^-



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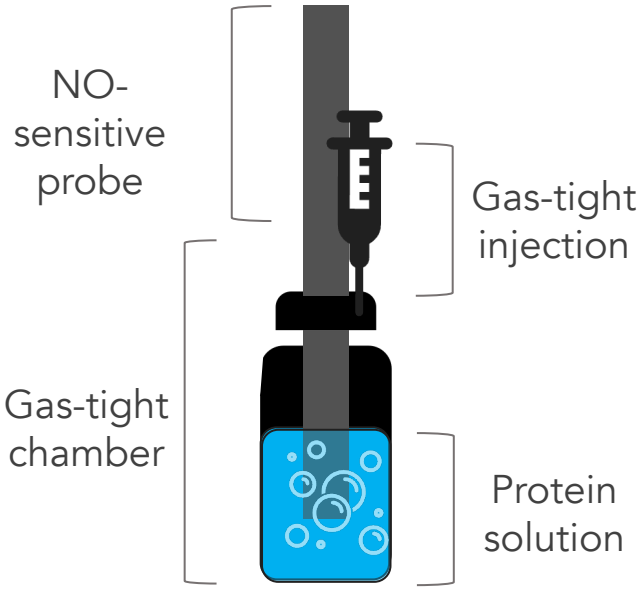

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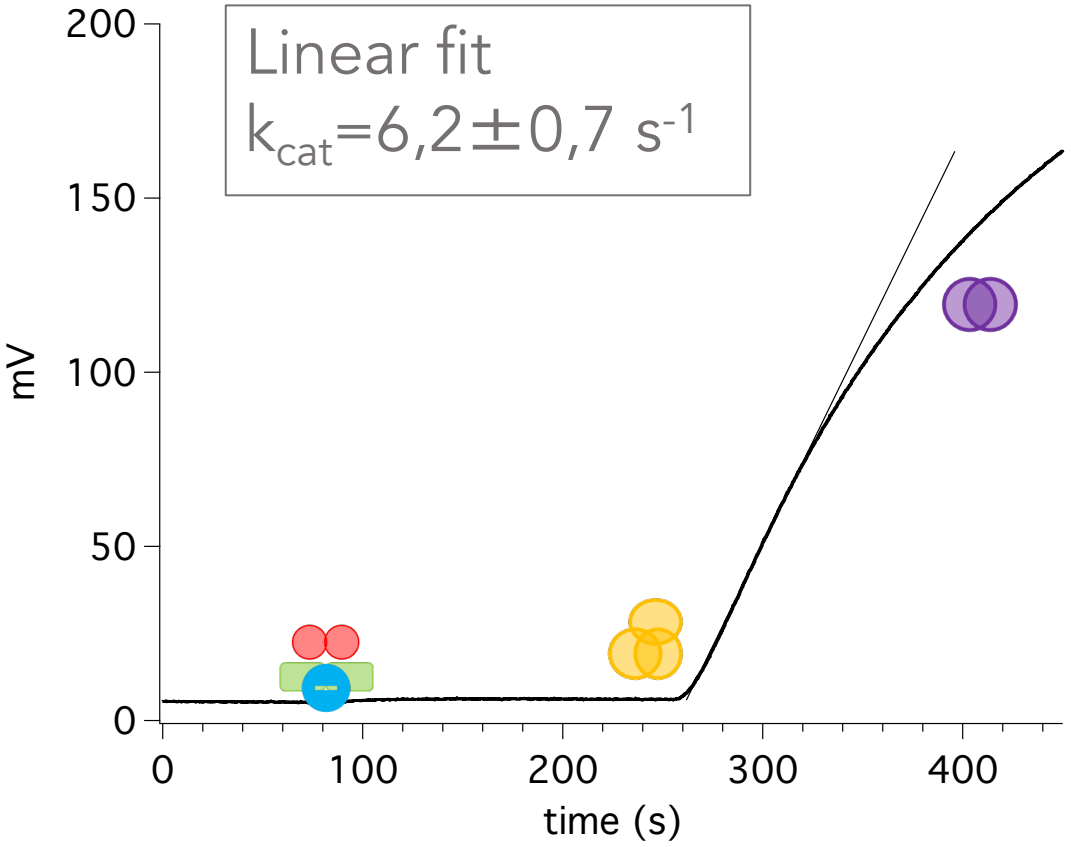
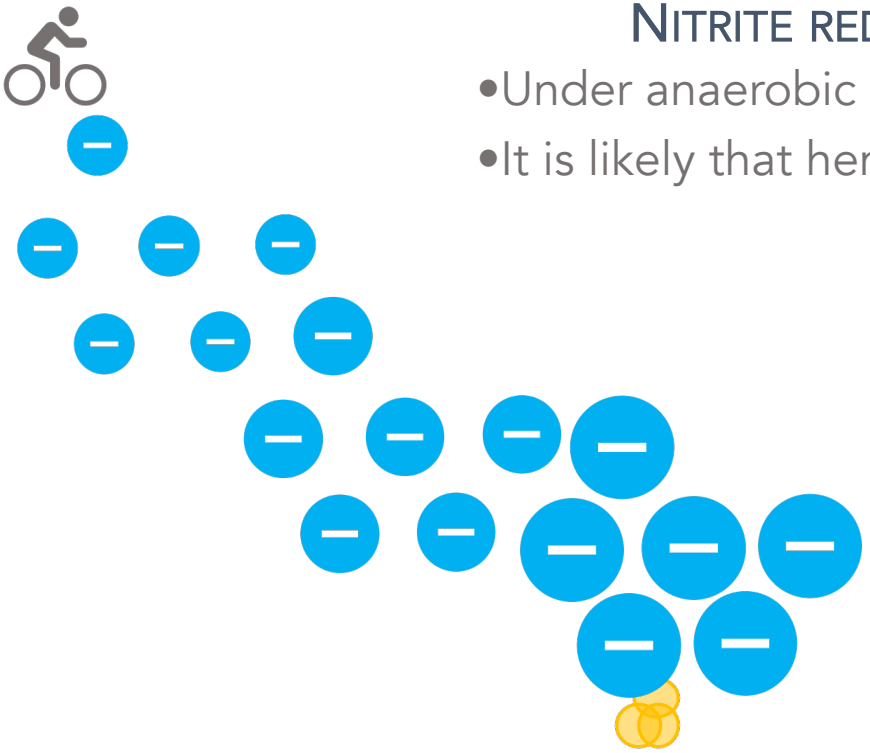
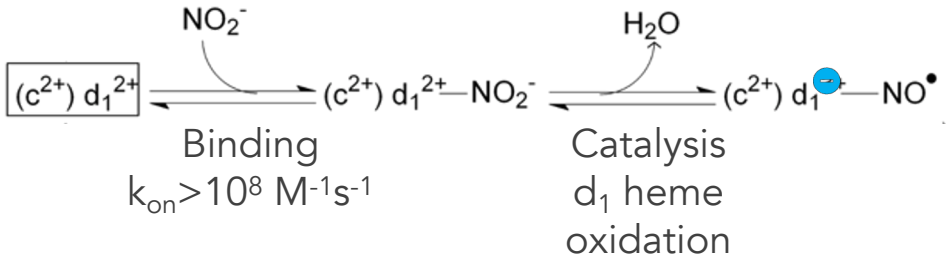


NITRITE REDUCTASE AND NO

- Under anaerobic conditions electrons accumulate
- It is likely that hemes reduction occurs before product release

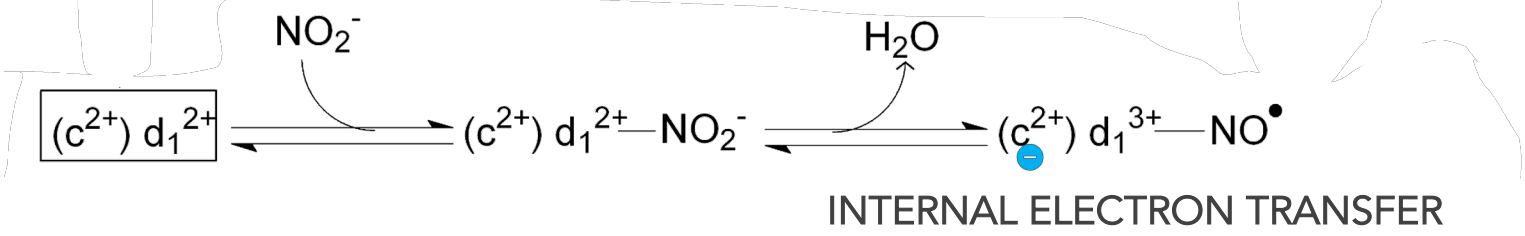




1. Product release
2. d1 heme reduction
3. c heme reduction

NITRITE REDUCTASE AND NO

Therefore, reduction of d_1 heme (d_1^{2+}) from c heme (c^{2+}) occurs BEFORE NO dissociation (product release)



NITRITE REDUCTASE AND NO

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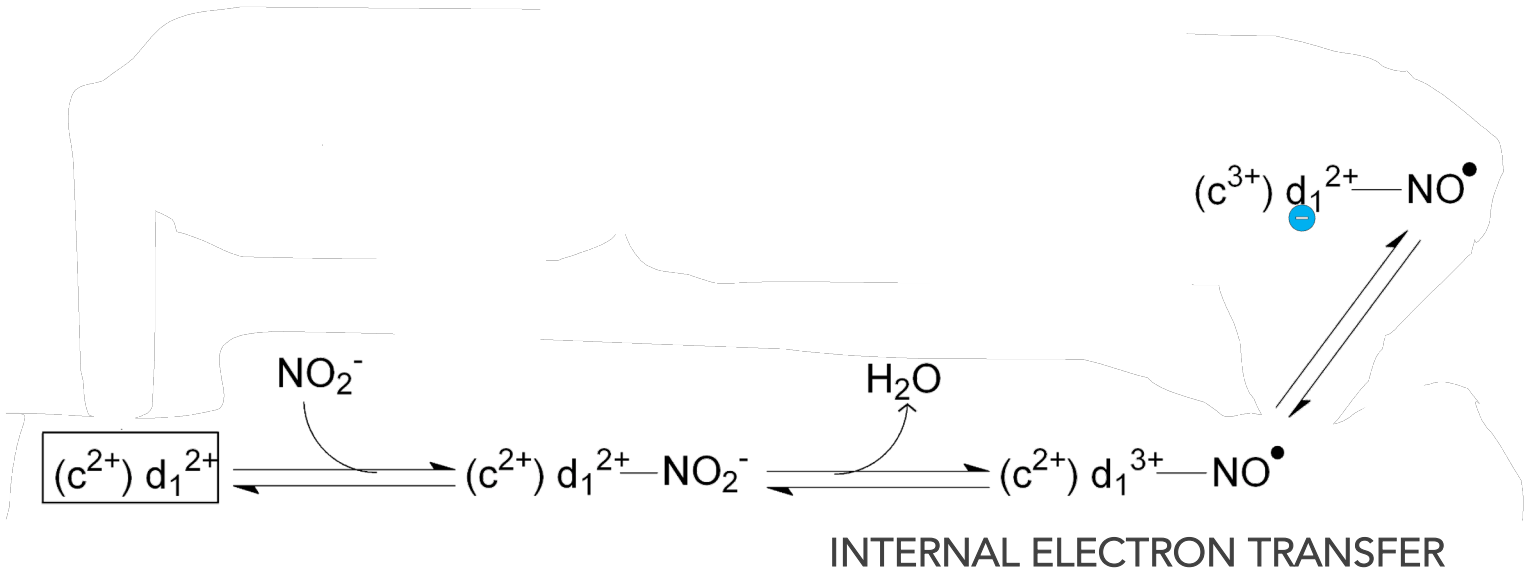
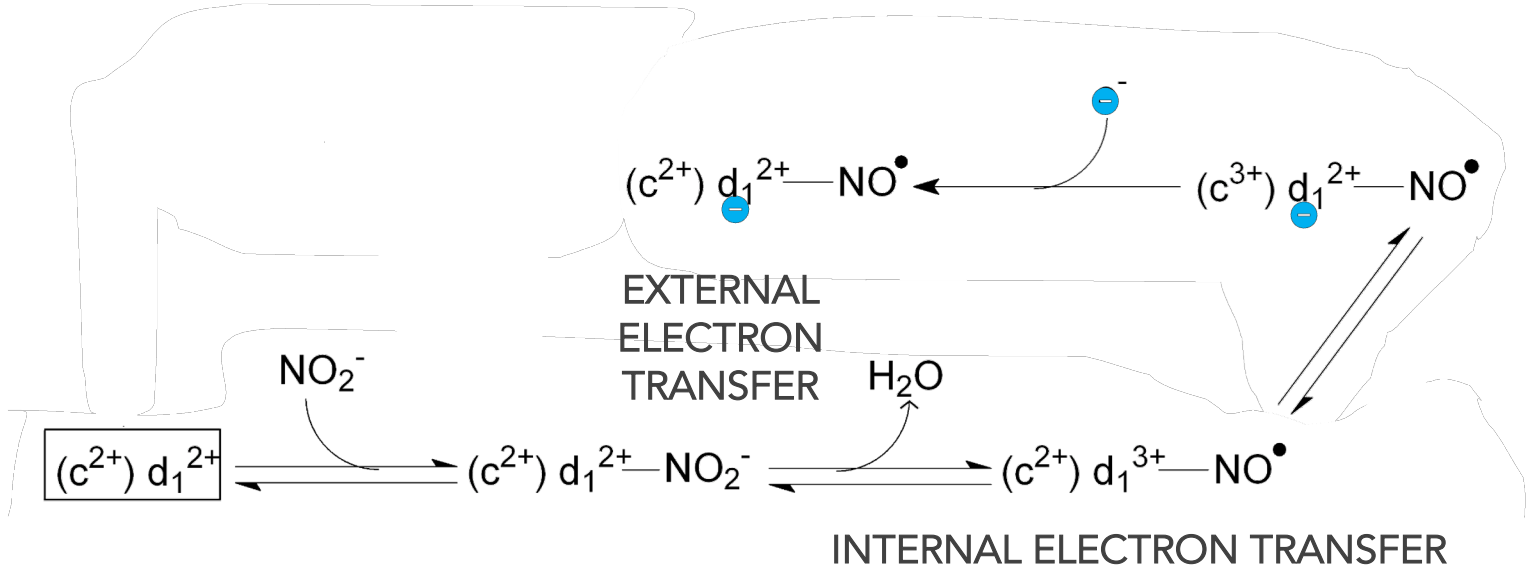


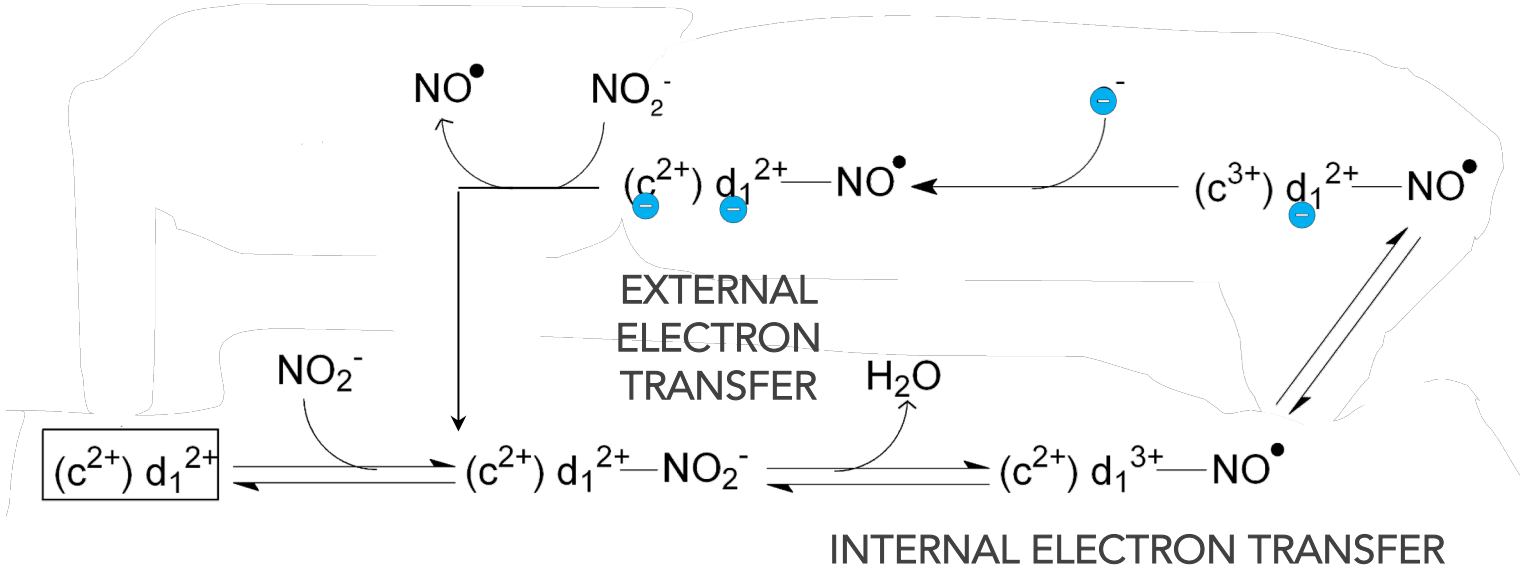
NITRITE REDUCTASE AND NO

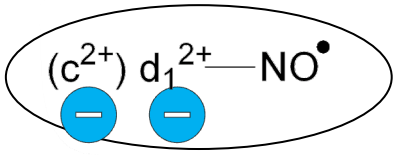
Therefore, reduction of d_1 heme (d_1^{2+}) from c heme (c^{2+}) occurs BEFORE NO dissociation (product release) and subsequent c heme reduction yields a fully reduced NO-bound adduct ($c^{2+}d^{2+}NO$).



NITRITE REDUCTASE AND NO

The fully reduced NO-bound adduct ($c^{2+}d^{2+}NO$) can release the product and a novel molecule of substrate can enter the new catalytic cycle.





Nevertheless, the fully reduced NO-bound adduct ($c^{2+}d^{2+}NO$) is supposed to be a **DEAD-END**

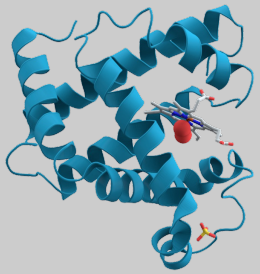
WHY?

Ferrous (Fe^{2+}, reduced) hemeproteins binds NO with very high affinity:

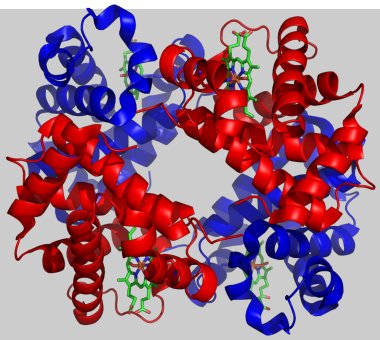
$K_D = k_{offNO}/k_{onNO} \sim 10^{-11}$ M

Very fast $k_{onNO} \sim 10^{7}$-10^{8} $M^{-1}s^{-1}$

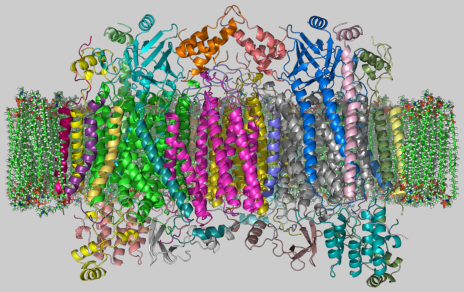
Very slow k_{offNO}:



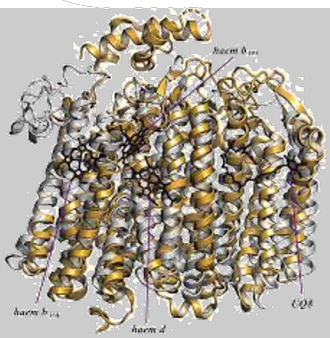
Myoglobin
$b^{2+}NO$
$k_{offNO}=10^{-4}$ s^{-1}



Hemoglobin
$b^{2+}NO$
$k_{offNO}=10^{-3}$, 10^{-5} s^{-1}



Cytochrome c oxidase
$a^{2+}NO$
$k_{offNO}=4x10^{-3}$ s^{-1}



Cytochrome bd oxidase
$d^{2+}NO$
$k_{offNO}=10^{-1}$ s^{-1}

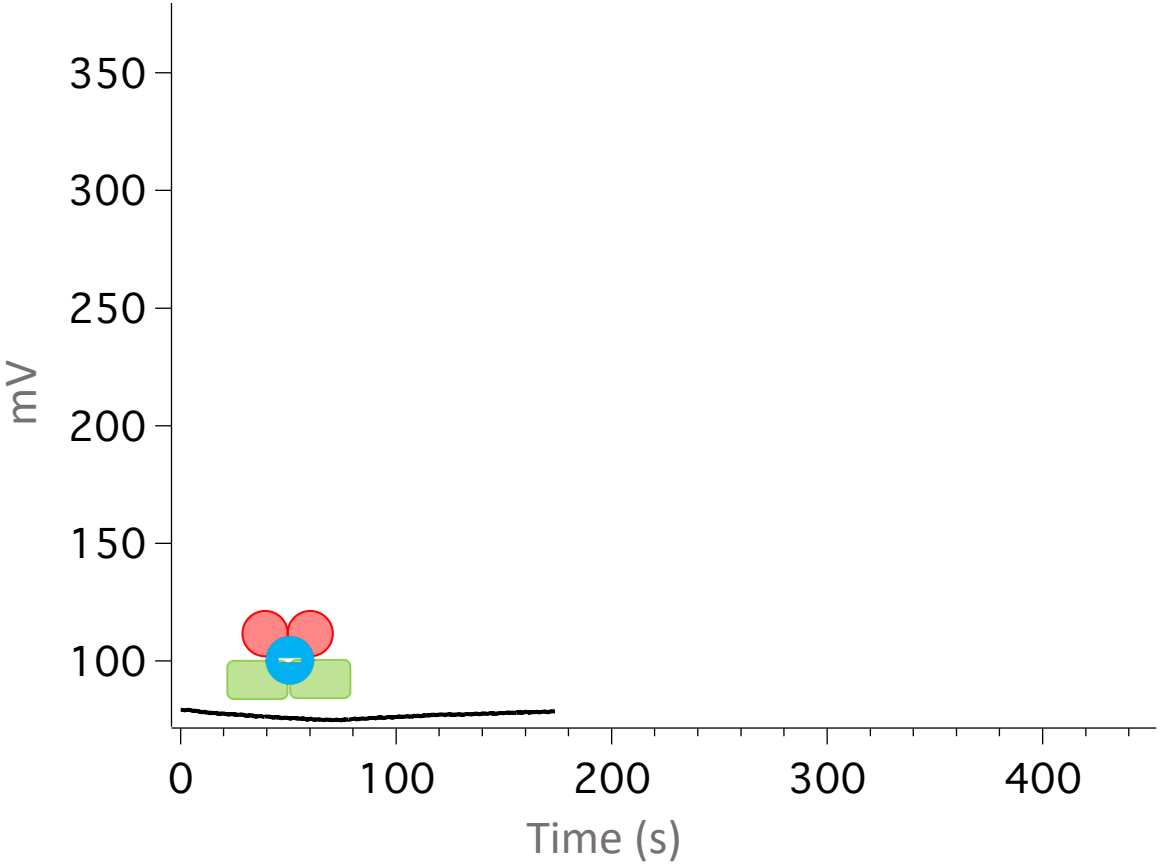
Expected k_{offNO} *vs* observed turnover

PROBING $C^{2+}D^{2+}NO$ REACTVITY

STEADY-STATE KINETICS

(NO-sensitive electrode) pH=7.0; T=20°C

$c^{2+}d^{2+}NO$ can be populated anaerobically starting from $c^{2+}d^{2+}$ fully reduced species



NiR

Excess reductants

Excess NO

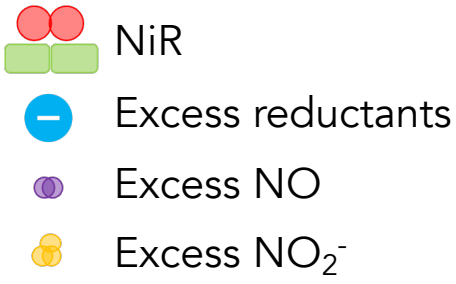
Excess NO_2^-

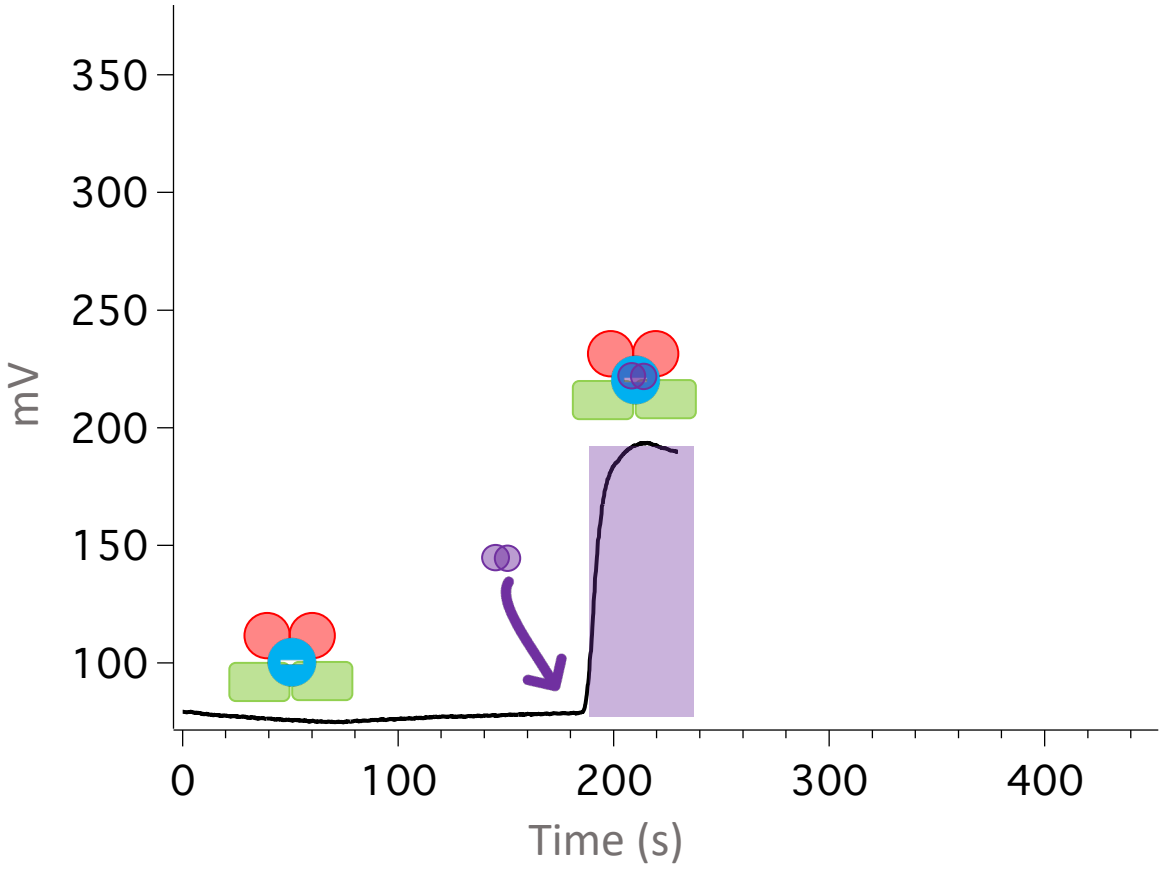
PROBING $C^{2+}D^{2+}NO$ REACTVITY

STEADY-STATE KINETICS

(NO-sensitive electrode) pH=7.0; T=20°C

- NO addition yields $c^{2+}d^{2+}NO$





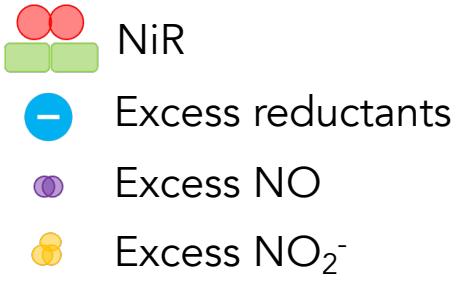
PROBING $C^{2+}D^{2+}NO$ REACTVITY

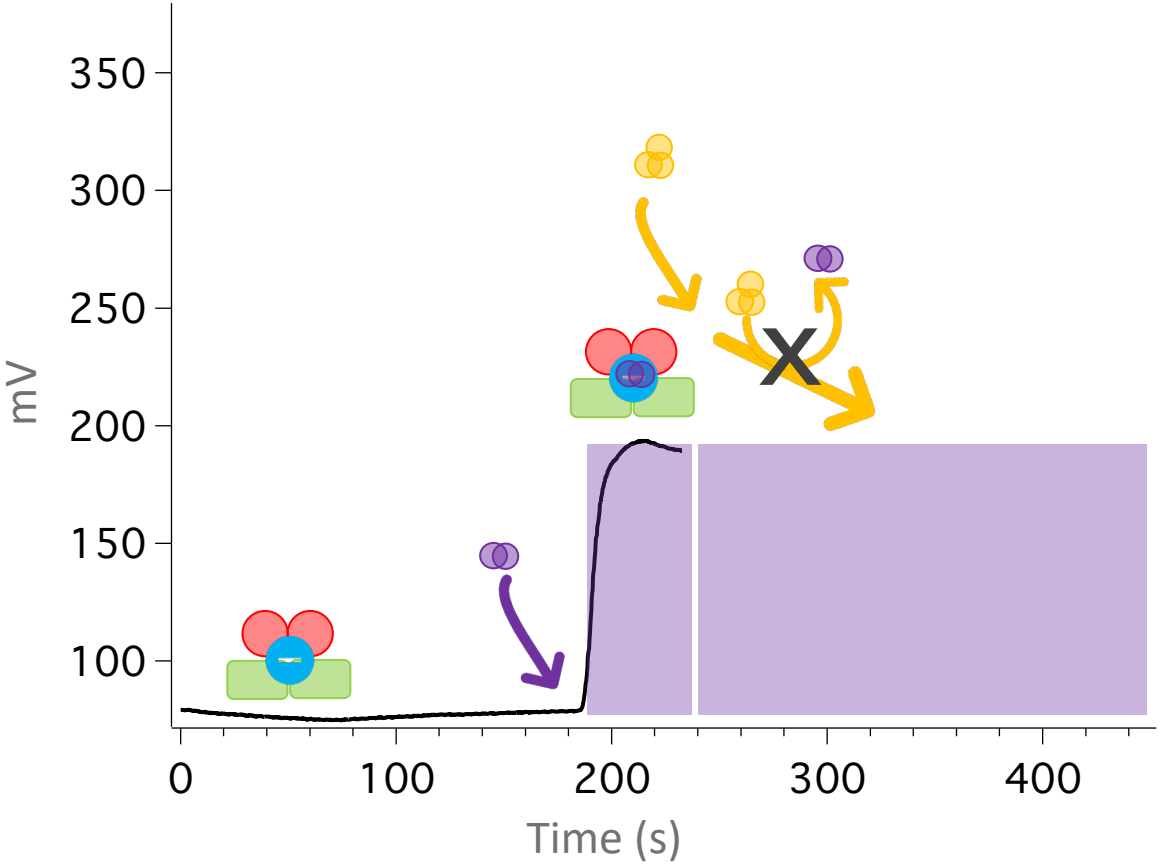
STEADY-STATE KINETICS

(NO-sensitive electrode) pH=7.0; t=20°C

- NO addition yields $c^{2+}d^{2+}NO$
- NO_2^- addition: two possible scenarios

$c^{2+}d^{2+}NO$ is a dead end state. No nitrite reduction occurs





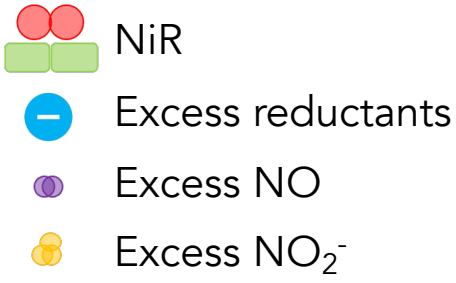
PROBING $C^{2+}D^{2+}NO$ REACTVITY

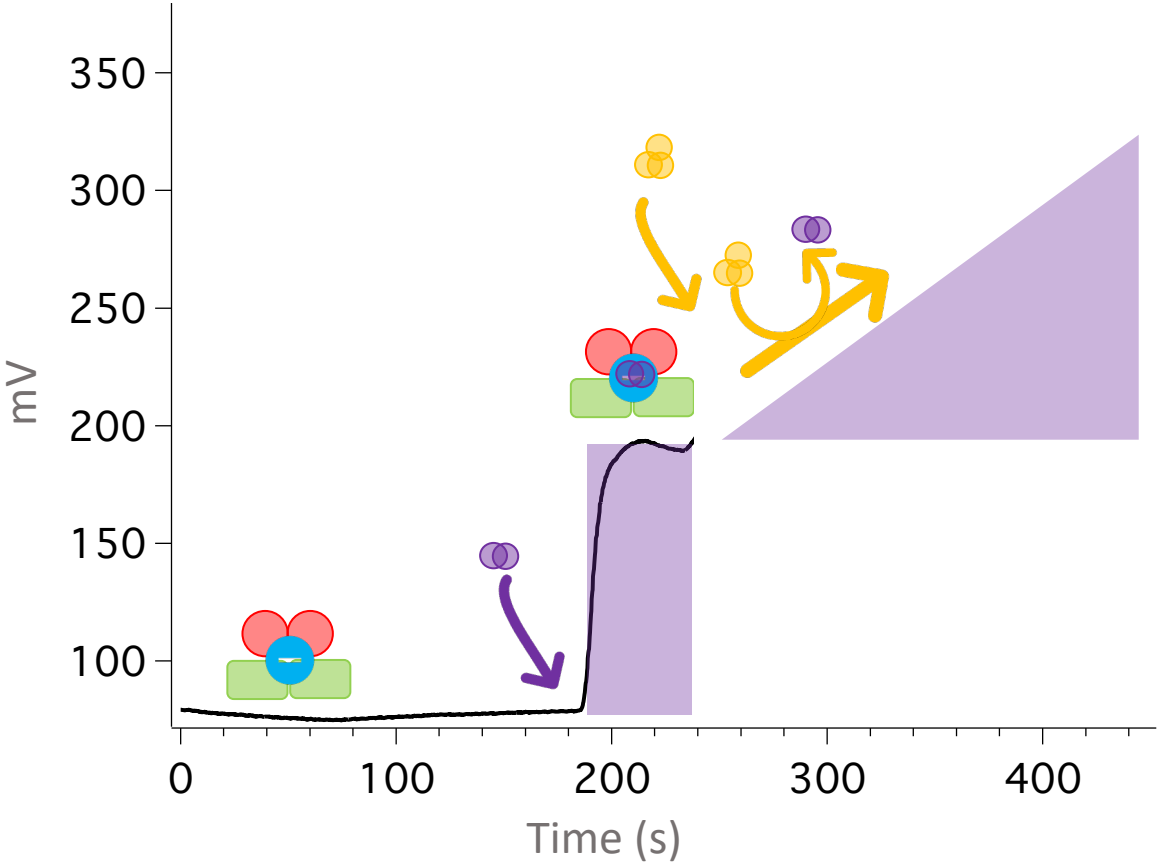
STEADY-STATE KINETICS

(NO-sensitive electrode) pH=7.0; T=20°C

- $c^{2+}d^{2+}NO$ (with excess reductant)
- NO_2^- addition: two possible scenarios

$c^{2+}d^{2+}NO$ is catalytically competent. Nitrite reduction occurs



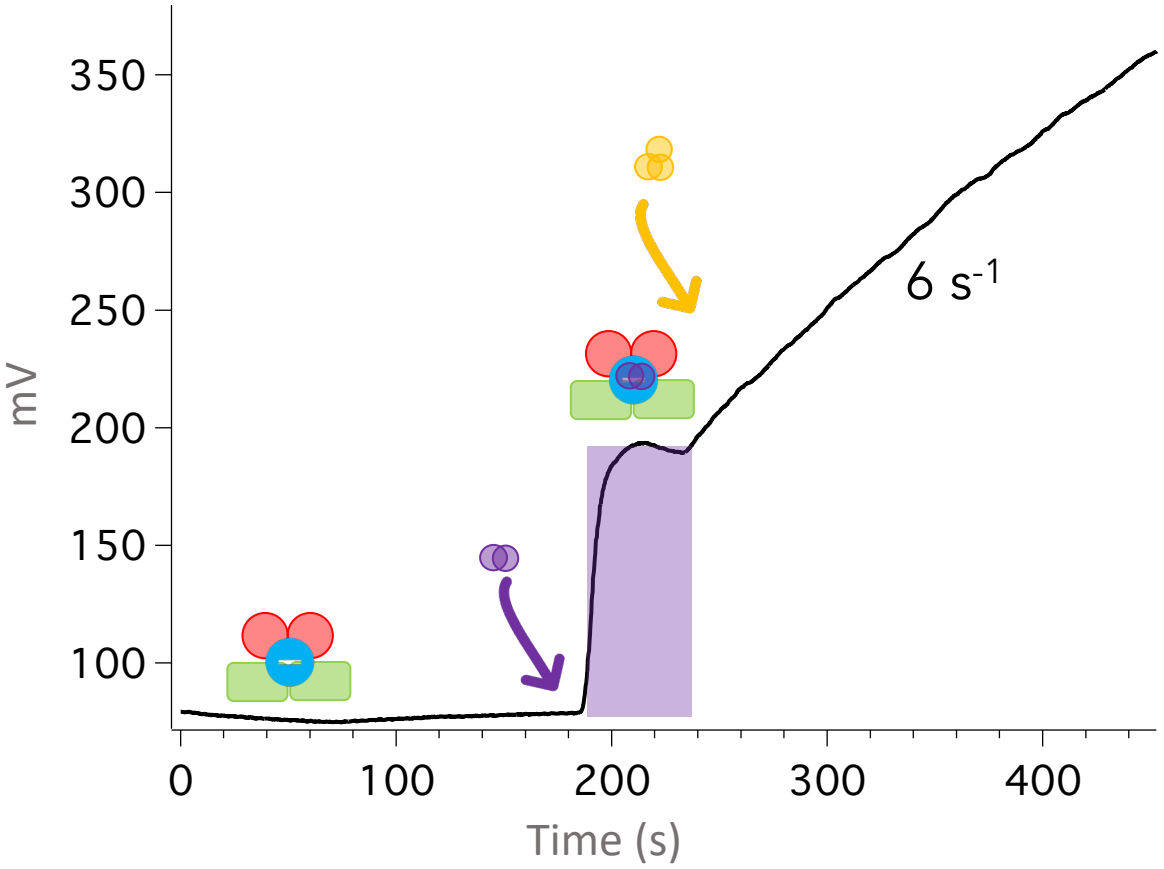


PROBING $C^{2+}D^{2+}$NO REACTVITY

STEADY-STATE KINETICS

(NO-sensitive electrode) pH=7.0; t=20°C

- NO_2^- addition yields NO production



NiR

Excess reductants

Excess NO

Excess NO_2^-

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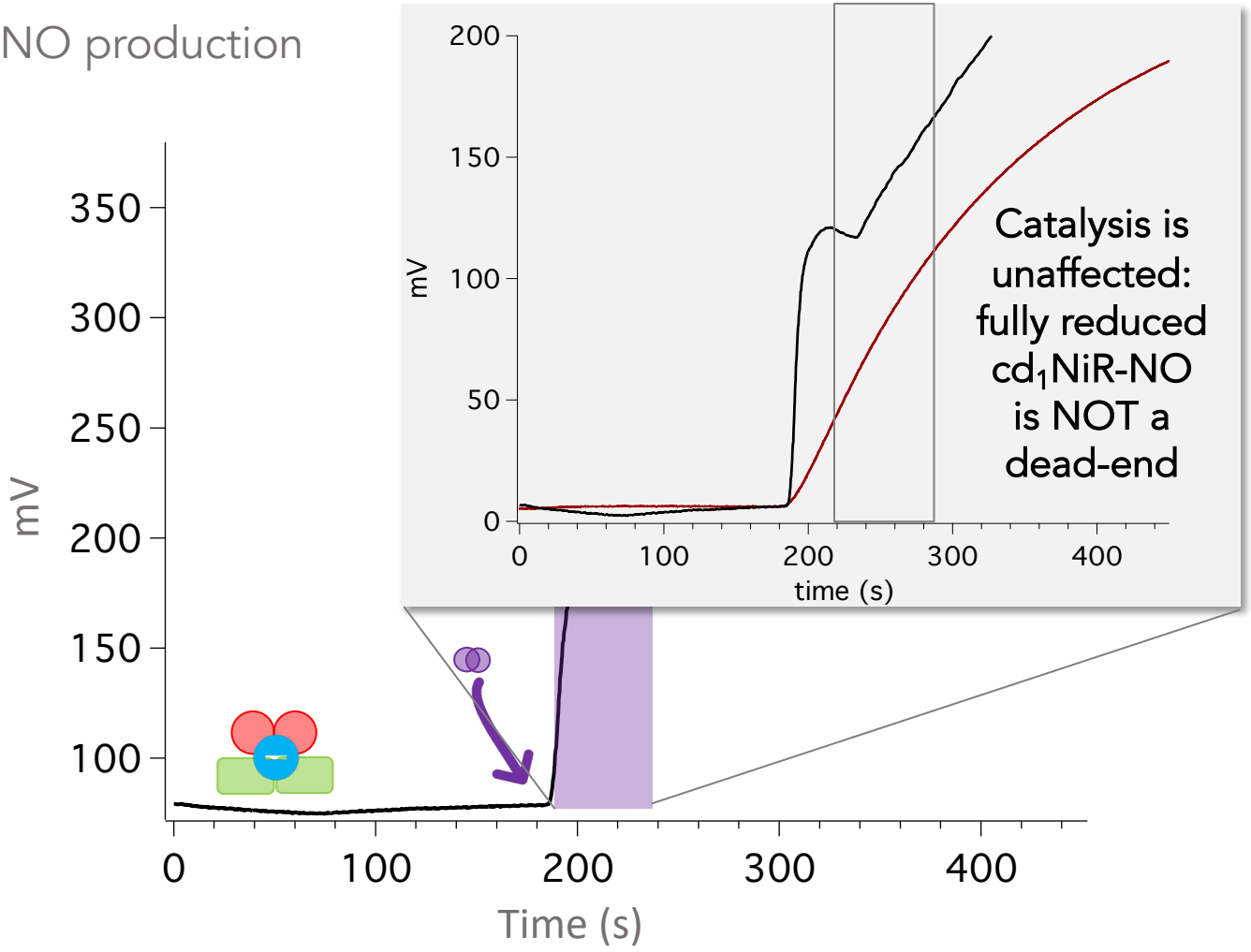


NiR

Excess reductants

Excess NO

Excess NO_2^-

NITRITE REDUCTASE AND NO

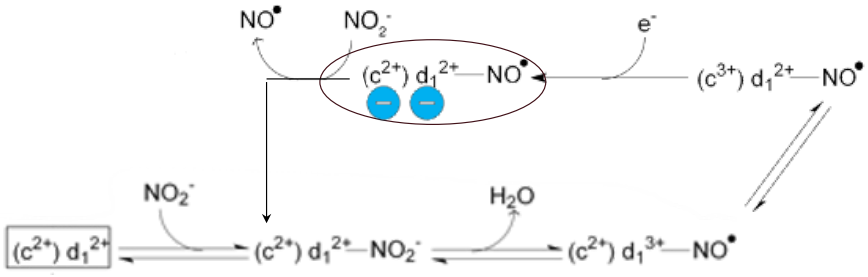
Since **$c^{2+}d^{2+}$NO is not inhibited,** it could represent a genuine on-pathway intermediate

IF

The dissociation of NO is ≥ than the rate-limiting step

$k_{offNO} \geq kcat$

Nitrite efficiently should **displace NO**, preventing NO re-binding



To verify this we have to measure the k_{offNO}

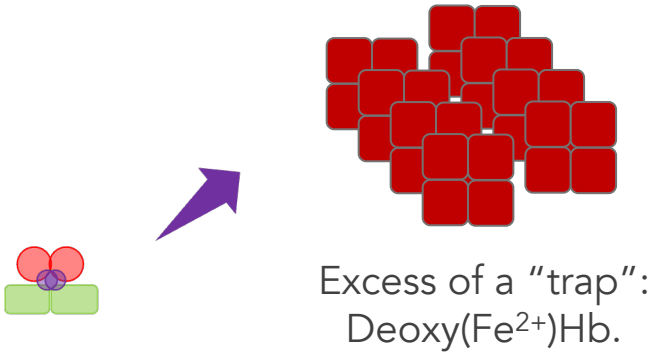
Since it is expected to be ≥ $6s^{-1}$ and dissociation rate does not depend on ligand concentration, a **rapid kinetics assay** is required.

NITRITE REDUCTASE AND NO: PROBING THE NO DISSOCIATION WITH A DISPLACEMENT REACTION

RAPID KINETICS

(Stopped-flow) pH=7.0; t=20°C

- To measure a dissociation process two possible strategies can be set:



Binding of NO to Hb is not rate-limiting:

$k_{onNOHb} \gg k_{offNONiR}$

$k_{obs} \cong k_{offNONiR}$

Excess of a "trap": Deoxy(Fe^{2+})Hb.

NiR

Excess reductants

Excess NO

Excess NO_2^-

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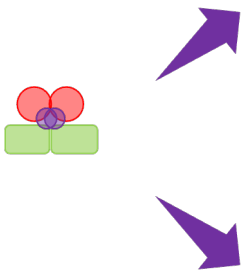

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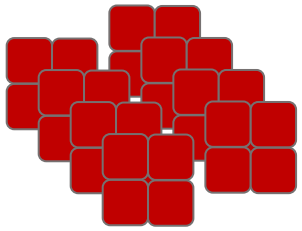

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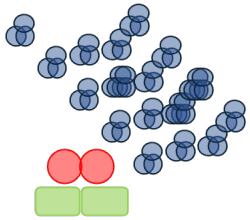


Excess of a "competitor": CN^-.

Binding of CN^- to NiR is not rate-limiting:
$k_{onCN} = 4.5*10^5\ M^{-1}\ s^{-1}$
$k_{onCN} \gg k_{offNONiR}$
$k_{obs} \cong k_{offNONiR}$

NiR

Excess reductants

Excess NO

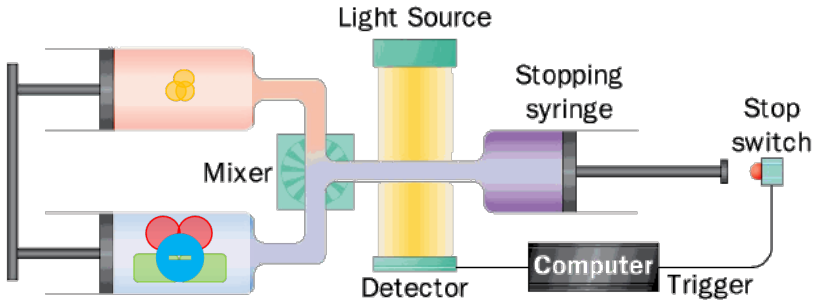
Excess NO_2^-

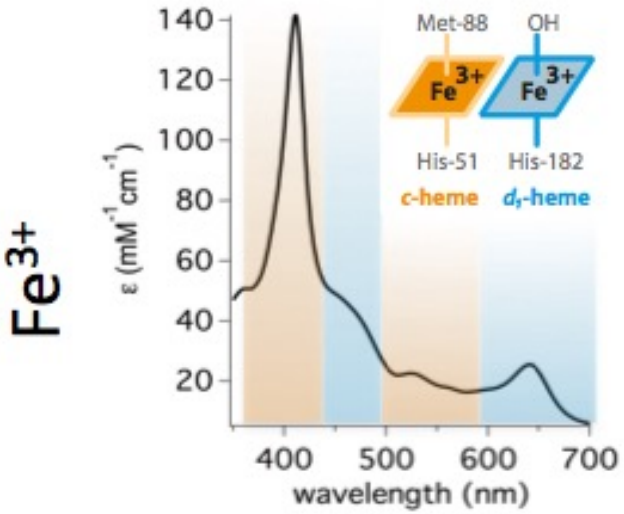
NITRITE REDUCTASE AND RAPID KINETICS

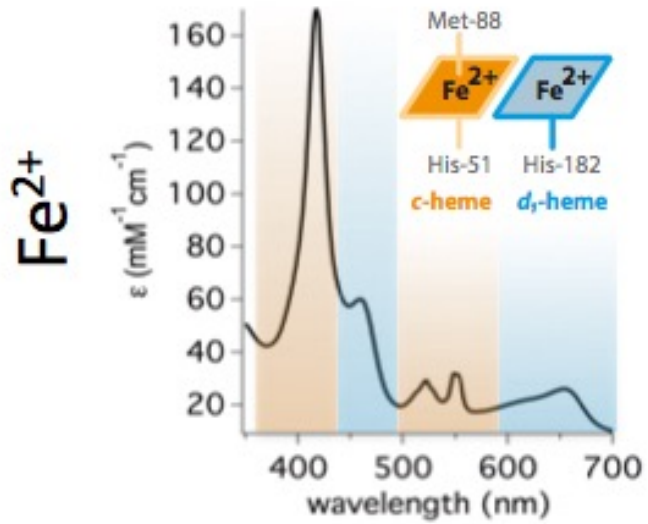
- Heme adducts display specific UV-Vis spectra

(Stopped-flow) pH=7.0; t=20°C

Heme iron redox state and ligands can be observed spectroscopically





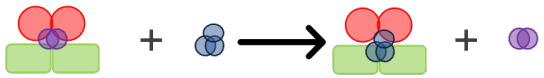


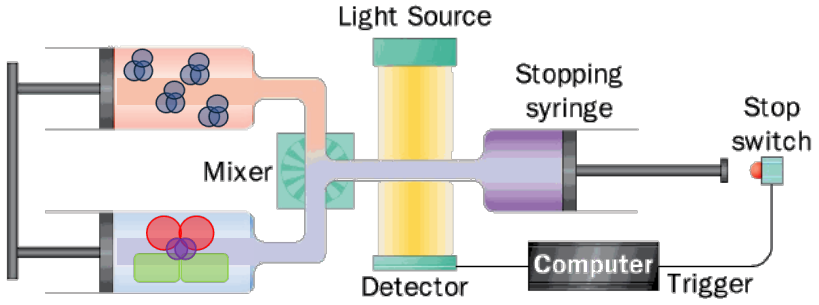
NITRITE REDUCTASE AND NO: PROBING THE NO DISSOCIATION WITH A DISPLACEMENT REACTION

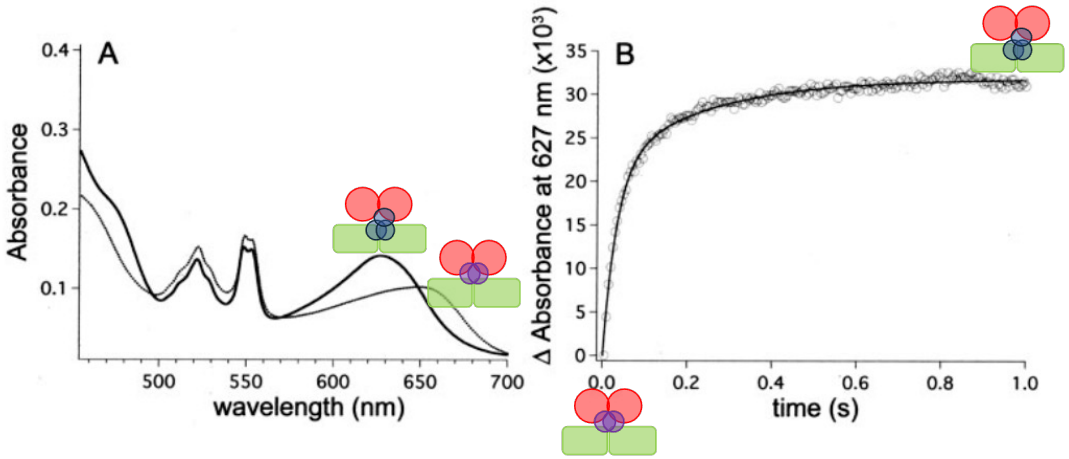
PRE STEADY-STATE KINETICS

(Stopped-flow) pH=7.0; t=20°C

- NO replacement by excess CN^-







Different spectroscopic properties

Rapid kinetics process

NiR

Excess reductants

Excess NO

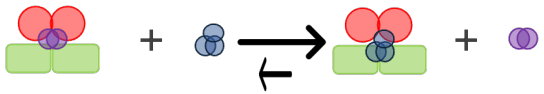
Excess NO_2^-

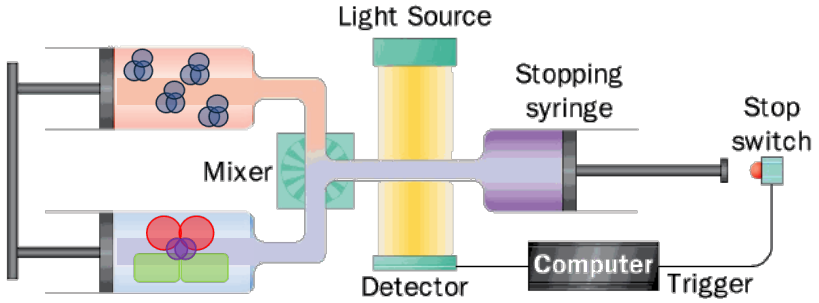
NITRITE REDUCTASE AND NO: PROBING THE NO DISSOCIATION WITH A DISPLACEMENT REACTION

PRE STEADY-STATE KINETICS

(Stopped-flow) pH=7.0; t=20°C

- NO replacement by excess CN^-





The observed rate k_{obs} accounts for mainly two processes:
1) NO dissociation ([NO]-independent)
2) CN^- binding ([CN]-dependent, under pseudo first-order conditions)
The experimental set up should move the reaction to the products. Nevertheless, two other processes participates in yielding the k_{obs}:
3) NO binding ([NO]-dependent)
4) CN^- dissociation ([CN]-independent)

NiR

Excess reductants

Excess NO

Excess NO_2^-

Therefore to extrapolate the k_{offNO} it necessary to collect more traces under **different [CN]** and fit the trend of the k_{obs} with the **replacement equation**

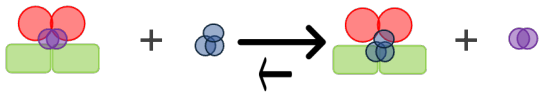
$$k_{obs} = \frac{k_{on}NO \times k_{off}CN \times [CN] + k_{on}CN \times k_{off}NO \times [NO]}{k_{off}CN \times [CN] + k_{off}NO \times [NO]}$$

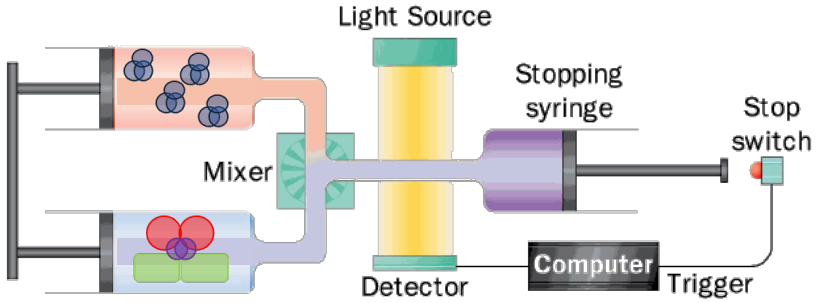
NITRITE REDUCTASE AND NO:
PROBING THE NO DISSOCIATION WITH A DISPLACEMENT REACTION

PRE STEADY-STATE KINETICS

(Stopped-flow) pH=7.0; t=20°C

- NO replacement by excess CN^-





In the replacement equation the **unknown value** should be k_{offNO}
k_{obs} is the observed rate after each stopped flow run
All the **concentration** parameters are known
k_{onCN} and k_{offCN} have been previously published
k_{onNO} can be calculated by laser photolysis, since the binding is more rapid than the dead-time of the stopped flow apparatus

$$k_{obs} = \frac{k_{on}NO \times k_{off}CN \times [CN] + k_{on}CN \times k_{off}NO \times [NO]}{k_{off}CN \times [CN] + k_{off}NO \times [NO]}$$

NiR

Excess reductants

Excess NO

Excess NO_2^-

$k_{onCN} = 4.5 \cdot 10^5\ M^{-1}\ s^{-1}$ and
$k_{offCN} = 4.3\ s^{-1}$
$k_{onNO} = 3.9 \cdot 10^8\ M^{-1}\ s^{-1}$

NITRITE REDUCTASE AND NO: PROBING THE NO DISSOCIATION WITH A DISPLACEMENT REACTION

PRE STEADY-STATE KINETICS

(Stopped-flow) pH=7.0; t=20°C

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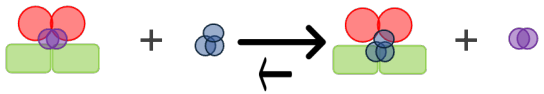
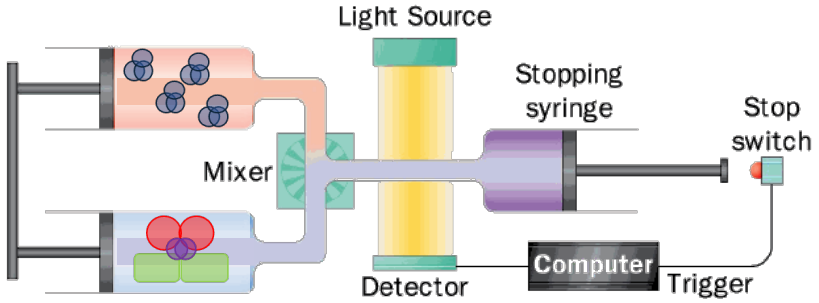
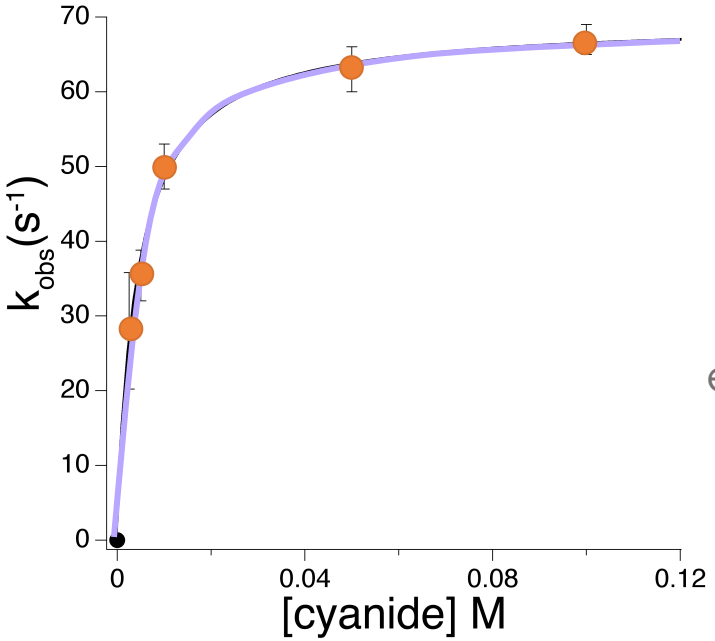






5 different mixing with different [CN^-] yields 5 different k_{obs} (±replicates)

Data fit with the replacement equation yields a k_{offNO}=70 s^{-1} (asymptote)

k_{offNO} >kcat

NiR

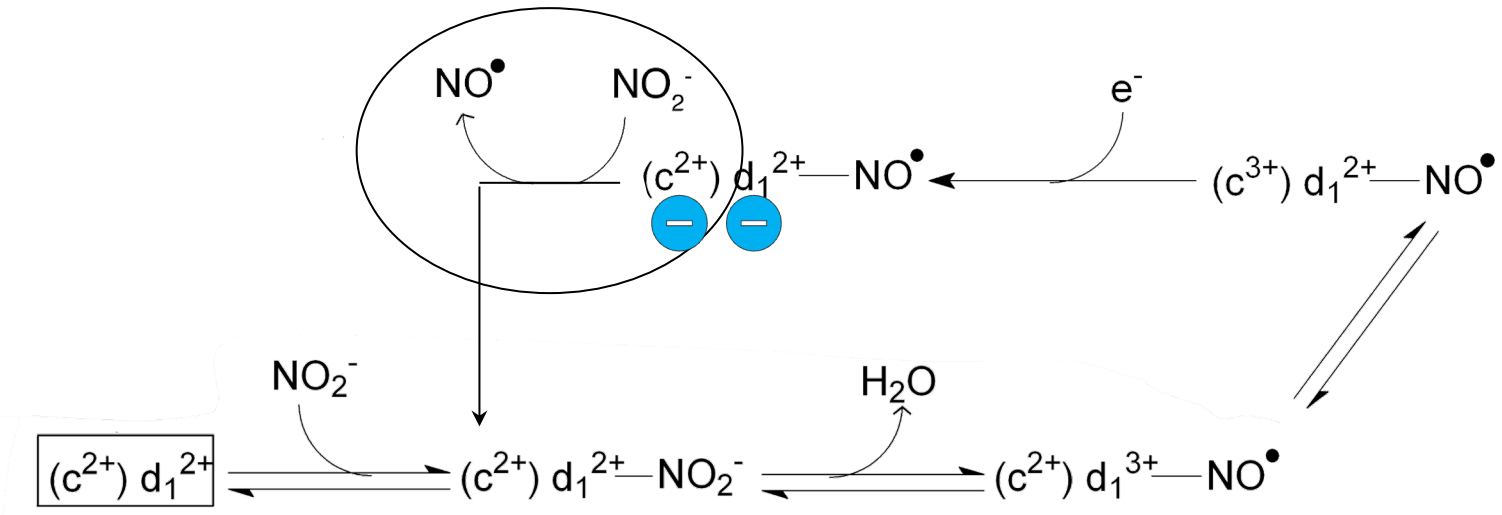
Excess reductants

Excess NO

Excess NO_2^-

NITRITE REDUCTASE AND NO

If NO dissociates from $c^{2+}d^{2+}$, can nitrite efficiently displace NO, preventing NO re-binding

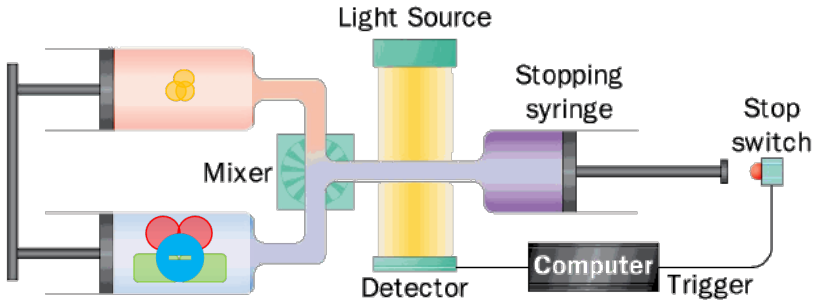


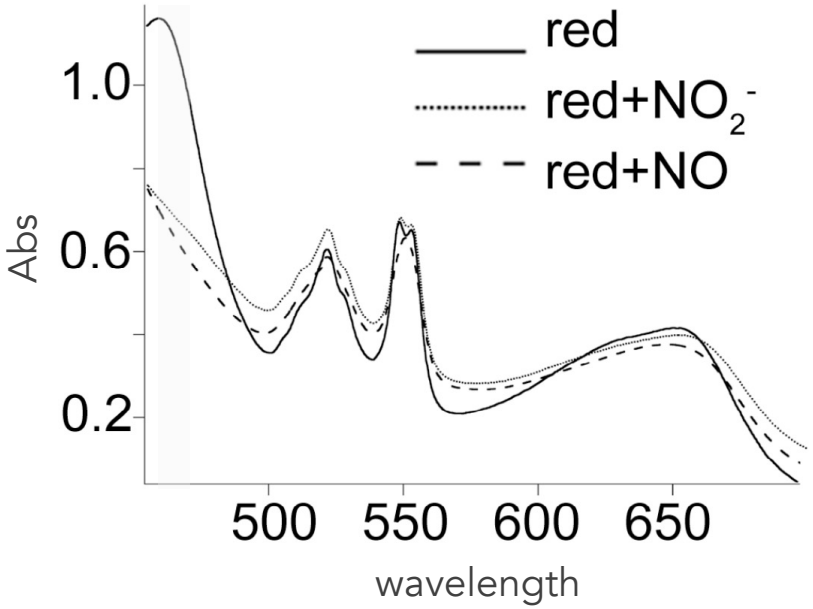
NITRITE REDUCTASE AND NO: K_{CAT} DETERMINATION AND CATALYTIC STEPS

PRE STEADY-STATE KINETICS

- Spectroscopic features of the expected species

(Stopped-flow) pH=7.0; t=20°C



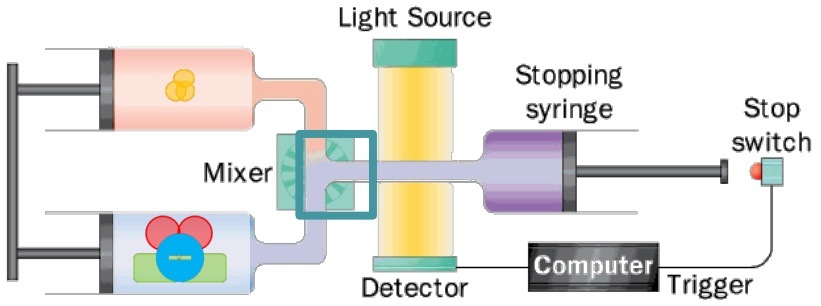


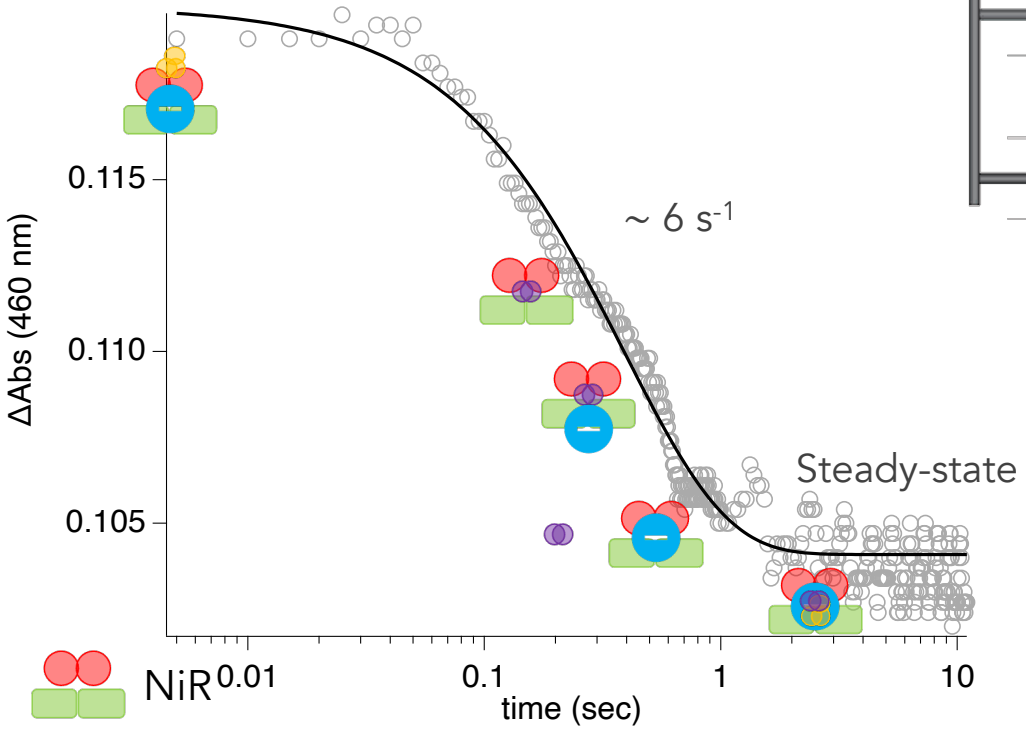
NITRITE REDUCTASE AND NO: K_{CAT} DETERMINATION AND CATALYTIC STEPS

PRE STEADY-STATE KINETICS

- Apparently, the turnover is monophasic

(Stopped-flow) pH=7.0; t=20°C





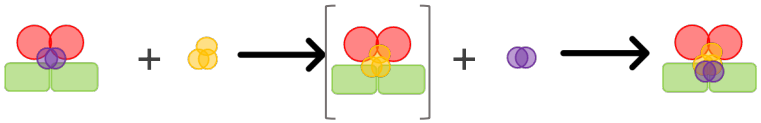
- Only the rate limiting step is observed.
- All the events before this has occurred during the mixing **(dead-time)**;
- All the events after this occurs more rapidly.
- One of the late events of the catalytic cycle is the NO release.
- NO_2^- binding has occurred in the dead-time ($k_{on}>10^8\ M^{-1}s^{-1}$)

No mechanistic hints from pre steady-state kinetics.
Is $c^{2+}d^{2+}NO$ populated during turnover?

PREFACE

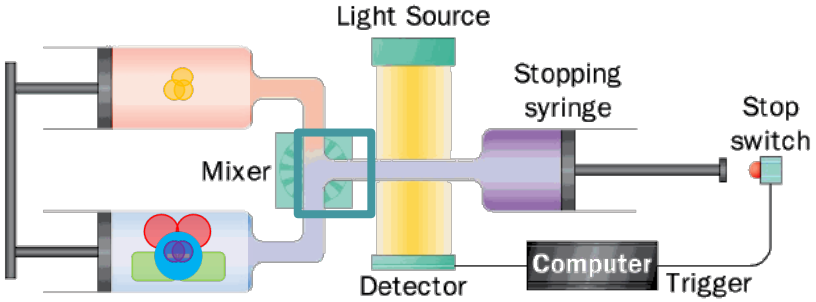
NITRITE REDUCTASE AND NO: PROBING THE NO DISSOCIATION DURING TURNOVER

PRE STEADY-STATE KINETICS:



3 events are expected:

1) NO dissociation (a single exponential at 70 s^{-1})
2) NO_2^- binding (very fast, not detectable)
3) Catalysis (a single exponential at 6 s^{-1} is expected)



NiR

Excess reductants

Excess NO

Excess NO_2^-

NITRITE REDUCTASE AND NO: PROBING THE NO DISSOCIATION DURING TURNOVER

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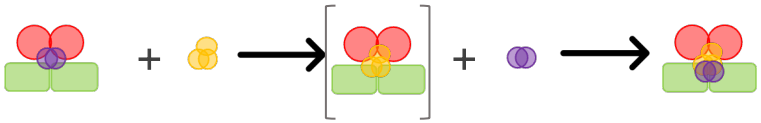

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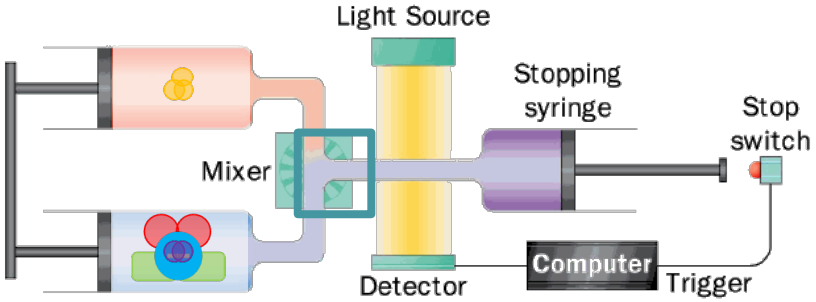

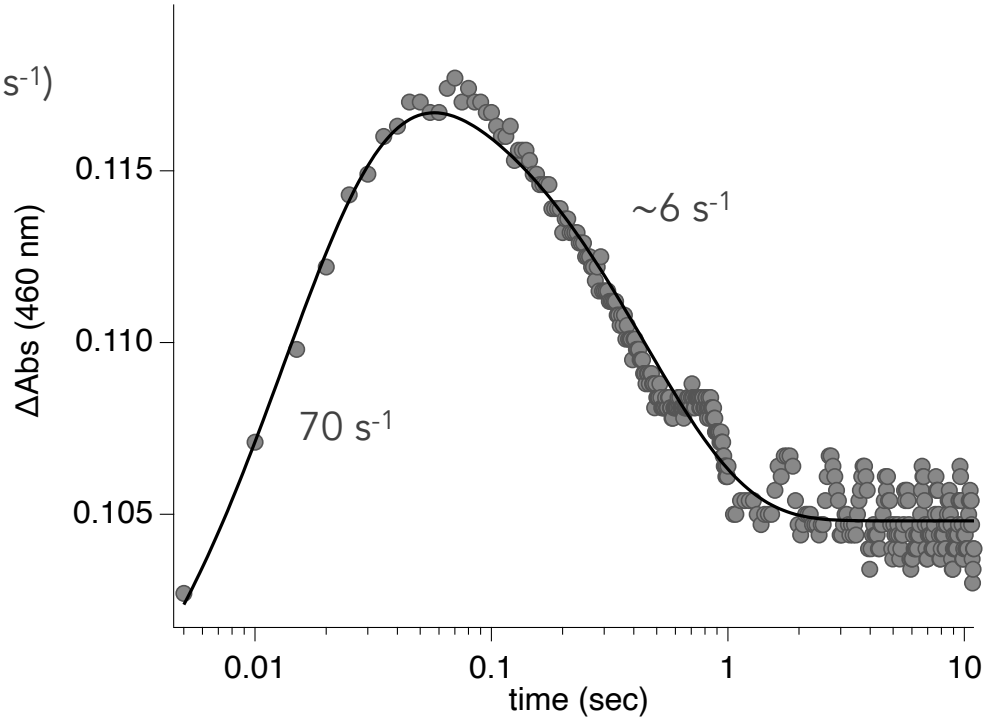

NiR

Excess reductants

Excess NO

Excess NO_2^-

Two processes are observed.
One fast and one slow.

NITRITE REDUCTASE AND NO: PROBING THE NO DISSOCIATION DURING TURNOVER

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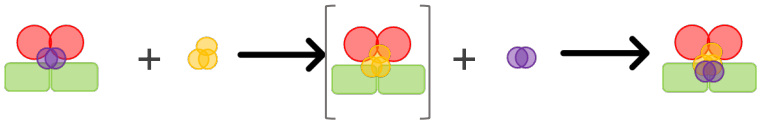

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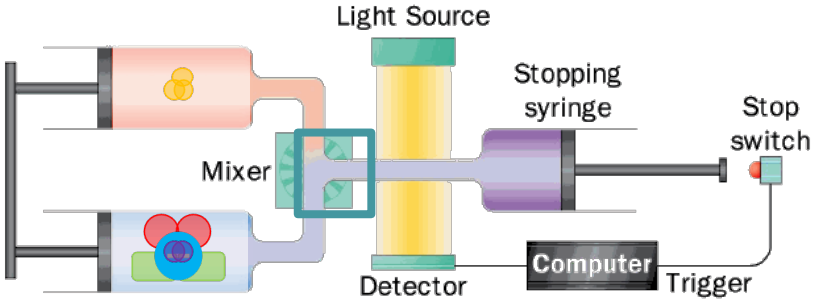

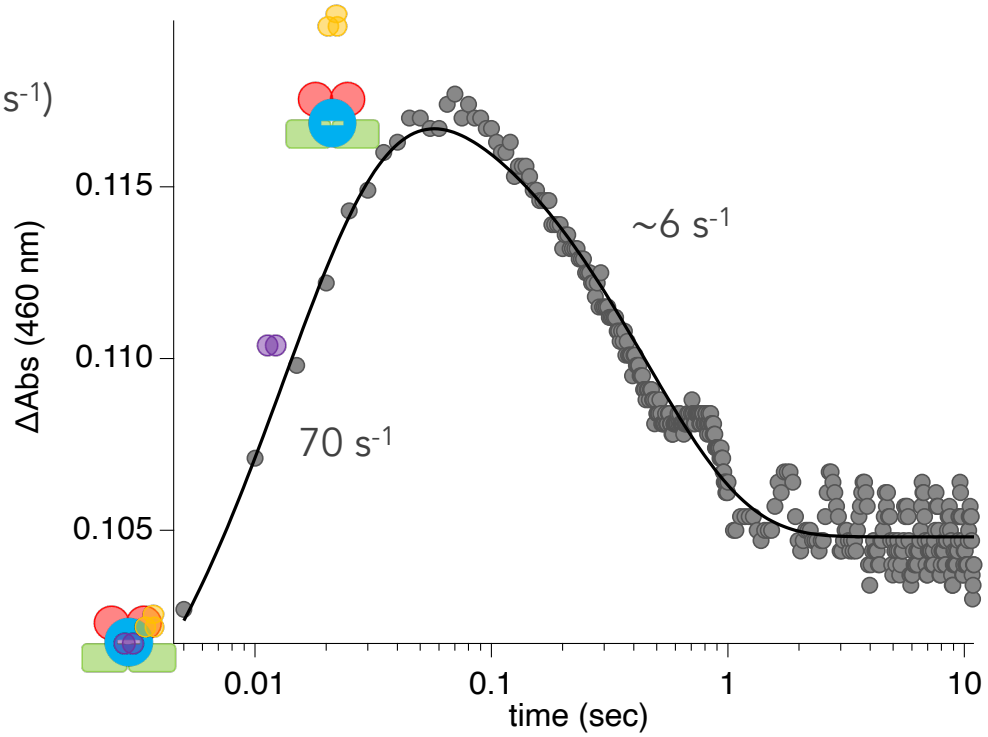

NiR

Excess reductants

Excess NO

Excess NO_2^-

The fast resembling the NO dissociation.

NITRITE REDUCTASE AND NO: PROBING THE NO DISSOCIATION DURING TURNOVER

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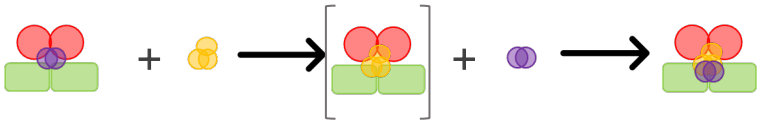

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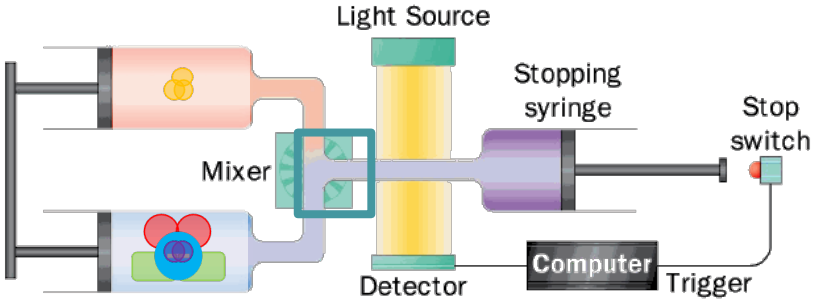
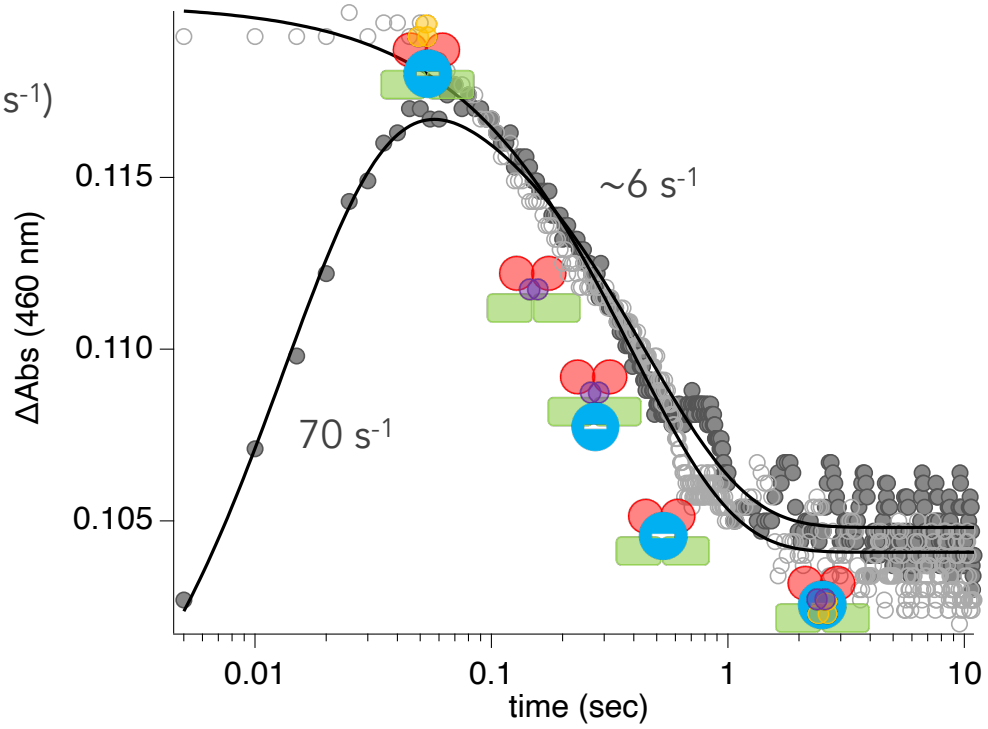




NiR

Excess reductants

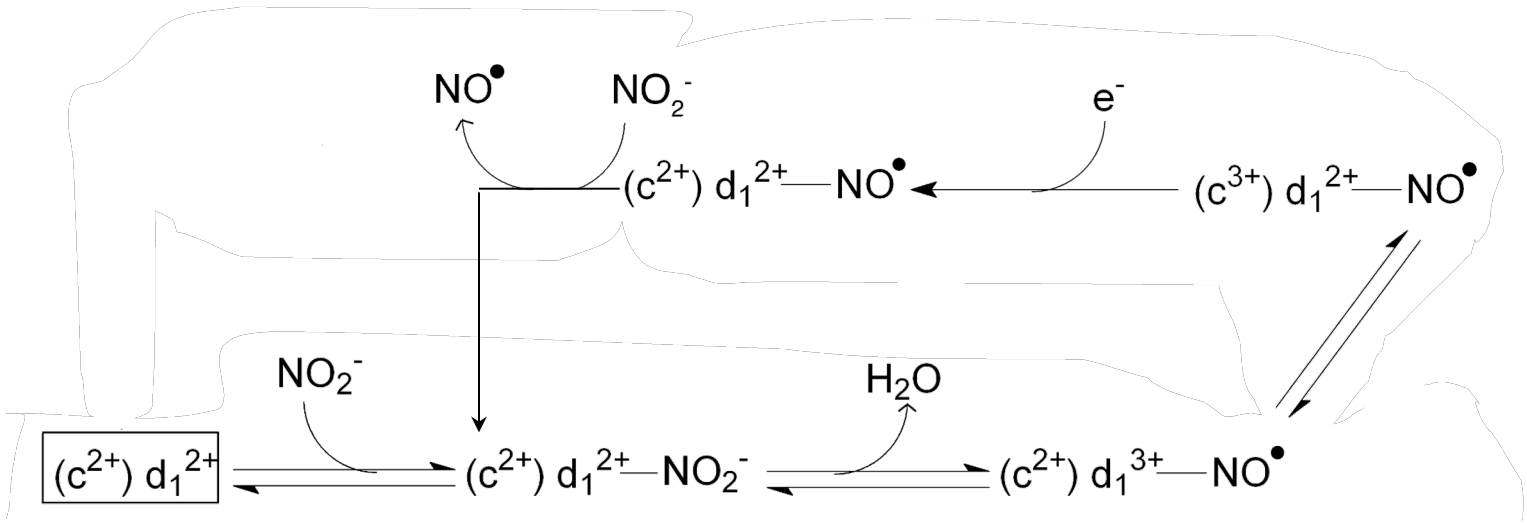
Excess NO

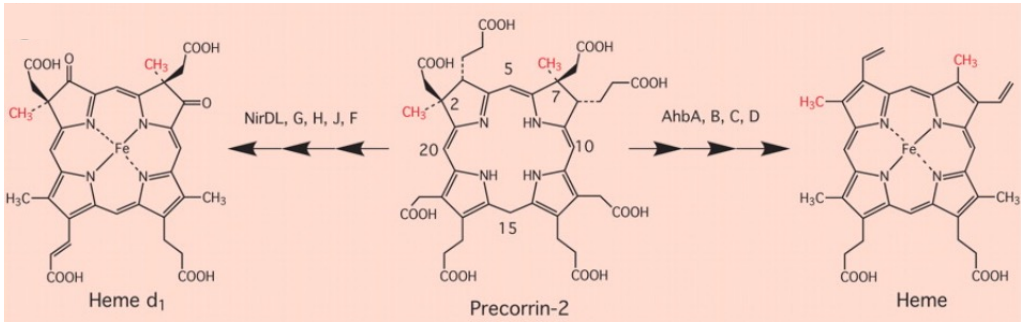
Excess NO_2^-

The slow phase superposes with the turnover phase.

NITRITE REDUCTASE AND NO

cd1NiR displays a unique reactivity with NO as compared to the other hemeproteins.
WHY (mechanistically)? It is a unique feature of the d_1-heme found only in denitrifiers.

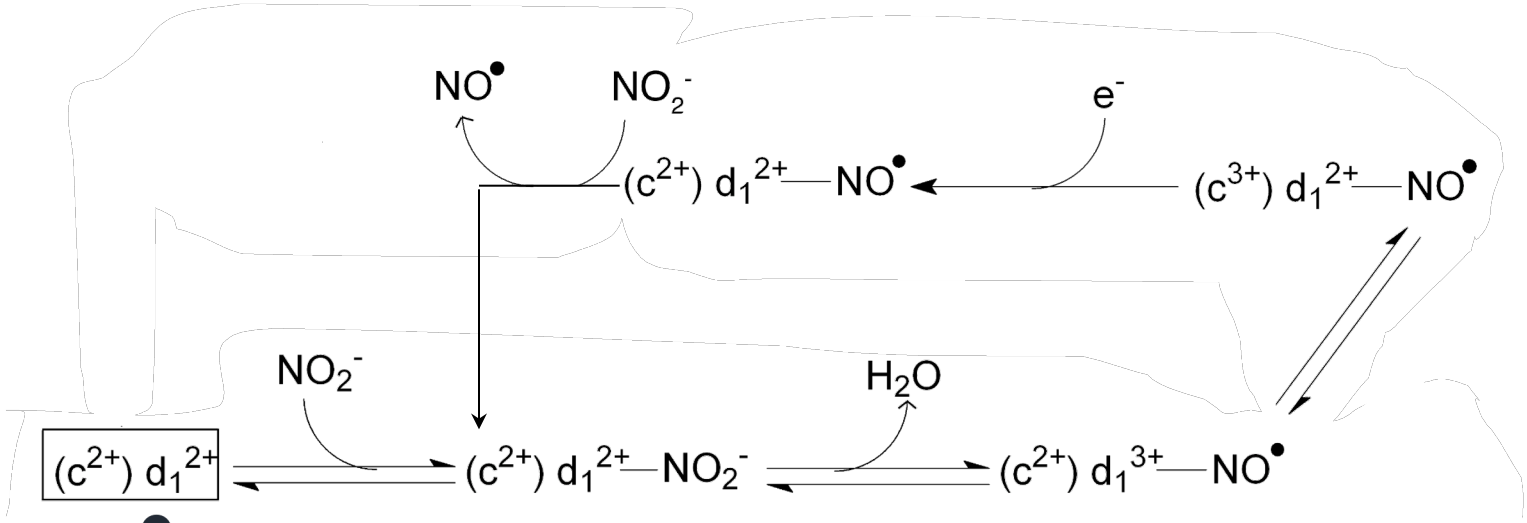


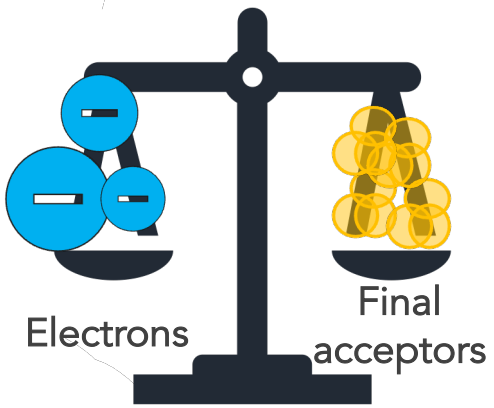


NITRITE REDUCTASE AND NO

cd1NiR displays a unique reactivity with NO as compared to the other hemeproteins.

WHY (biologically)? Coupling nitrite and electron sensing to populate the catalytically competent species.





No free ferrous enzyme, no unwanted reactivity risk.

Control of respiratory rate in response to the cell reducing power

TAKE HOME MESSAGE

- Nitrosative stress can be controlled by tuning the respiratory rate
- Reactivity of hemeproteins with ligands may deeply diverge from hemoglobin's behaviour

K_{off} determination allowed us to:

- Demonstrate that NO can be also released without a downstream scavenger: it may work as a signal
- Understand why the d_1-heme has evolved specifically in denitrifiers
- Find that the balancing of the reducing power and electron acceptors availability is a common strategies in the diverse respiration
- NO is productively released by the NO-producing enzyme…

…after all *"The obvious is that which is never seen until someone expresses it simply"*.

Khalil Gibran

Thank you for your attention!

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serena.rinaldo@uniroma1.it