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Thioredoxin Reductase: analysis of kinetics and inhibition of a two-substrate enzyme, and some hints to avoid common pitfalls

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In 2008 we solved the structure of Thioredoxin Glutathione Reductase from the human parasite *Schistosoma mansoni*. This enzyme, nicknamed SmTGR, is a validated drug target and several inhibitors are known; one of them, *Auranofin*, is already in use (for other diseases).

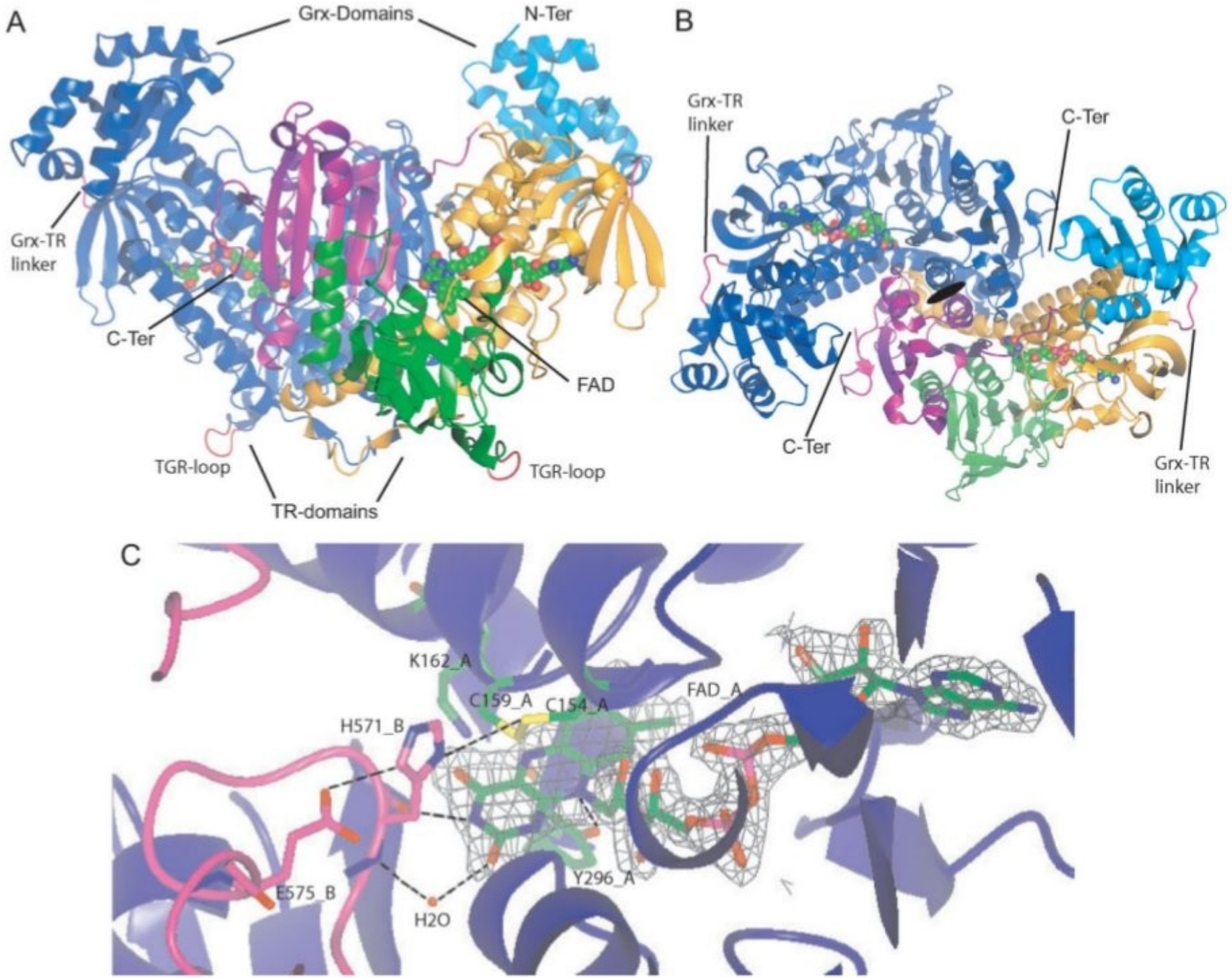
In 2009 we solved the structure of SmTGR in complex with gold, released by *Auranofin*, and demonstrated that the metal ion is the actual irreversible inhibitor, while the drug only acts as a gold carrier. In 2010 we solved the structures of all the putative intermediates of the catalytic cycle of SmTGR.

References:

Angelucci et al. (2008) *Glutathione reductase and thioredoxin reductase at the crossroad: the structure of Schistosoma mansoni thioredoxin glutathione reductase.* Proteins 72: 936-45.

Angelucci et al. (2009) *Inhibition of Schistosoma mansoni thioredoxin-glutathione reductase by auranofin: structural and kinetic aspects*. J Biol Chem. 284: 28977-28985.

Angelucci et al. (2010) *Mapping the catalytic cycle of Schistosoma mansoni thioredoxin glutathione reductase by x-ray crystallography*. J Biol Chem. 285, 32557-32567.


A
Grx-Domains
N-Ter
Grx-TR
linker
C-Ter
FAD
TGR-loop
TGR-loop
TR-domains
B
Grx-TR
linker
C-Ter
C-Ter
Grx-TR
linker
C
K162_A
C159_A
C154_A
FAD_A
H571_B
E575_B
H2O
Y296_A

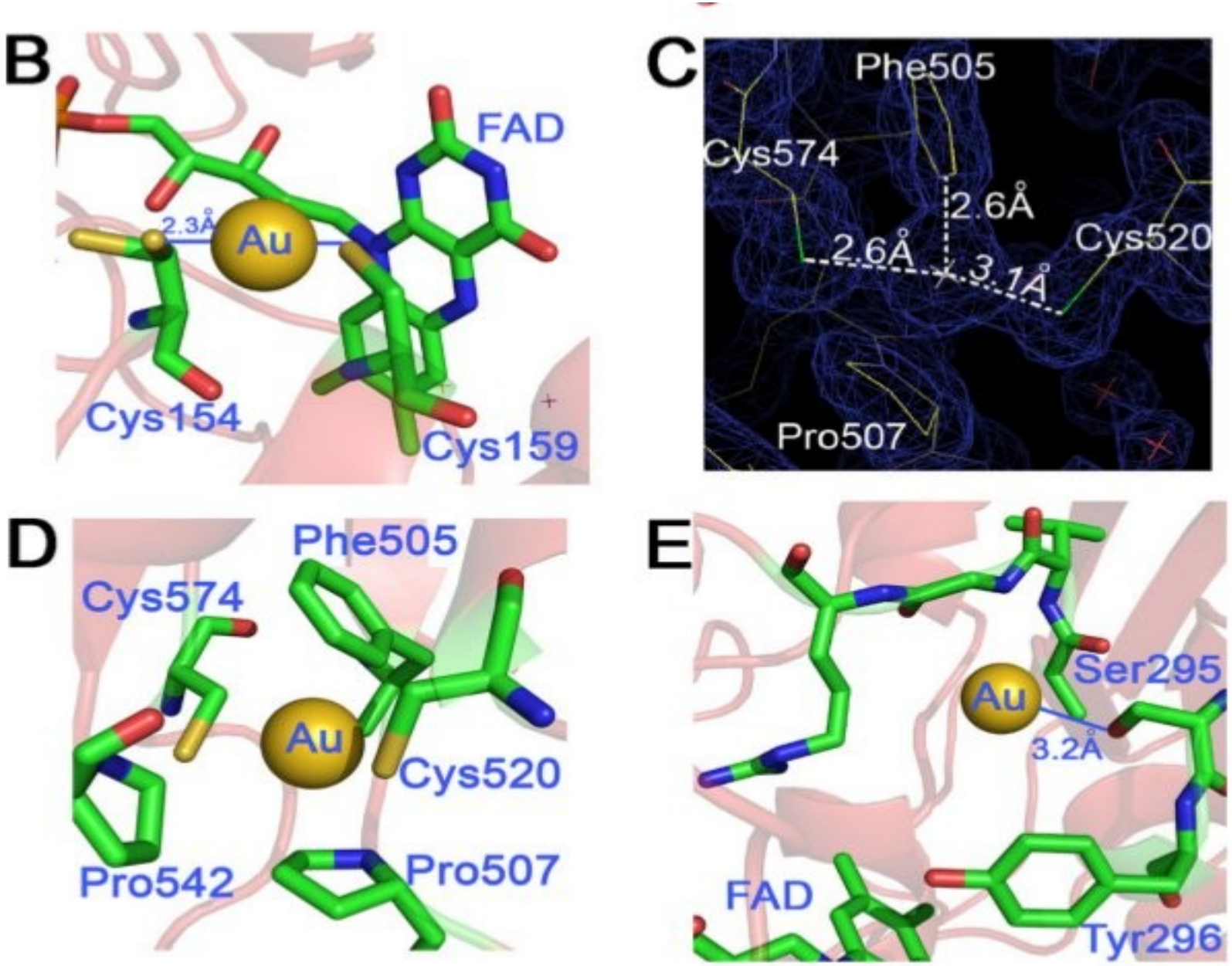
SmTGR is very similar to a Thioredoxin Reductase, except that the polypeptide sequence includes a N-terminal domain structurally homologous to a Glutaredoxin.

The enzyme is a homodimer; each monomer includes a Glutaredoxin domain, a NADPH binding domain, a FAD containing domain, and a C-terminal arm, responsible for shuttling electrons to its substrates. The FAD containing domain hosts a Cys-Cys couple, which is reduced by the $FADH_2$. A Sec-Cys couple is hosted on the C-terminal arm, receives electrons from the former and donates them to the substrate. The electron flow may be schematized as follows:

$NADPH \rightarrow M_1FAD \rightarrow M_1Cys_2 \rightarrow M_2Sec\text{-}Cys \rightarrow$ Trx or Grx domain

M_1 and M_2 denote the first or second monomer of the dimer and the enzyme presents a case of monomer swapping, in which the Cys couple on the FAD is used to reduce the Sec-Cys couple on the C-terminal arm of the partner subunit.

As is typical of Cys- or Sec- containing enzymes, SmTGR has several covalent inhibitors reacting with these residues. These include alkylating agents and metals capable of forming a covalent complex with S or Se.



Non-covalent, reversible, inhibitors of SmTGR (and TrxR in general) are less common, but many covalent inhibitors initially behave as reversible competitive inhibitors of the oxidizing substrate (Trx) and only slowly become irreversibly bound.

In 2011 we wrote a review on the macromolecular targets of antischistosomal therapy, where we reviewed some known inhibitors of TrxR and TGR, and we were surprised by the number of inconsistencies and “strange” results.

Two common occurrencies are as follows:
i) two K_I values are assigned to the same inhibitor, on emeasured at constant [NADPH] and variable [Trx], the other measured in the opposite way round;
ii) the same inhibitor is assigned a finite K_I value and then is declared to be irreversible.

Selected reference: Angelucci et al. (2011) *Macromolecular bases of antischistosomal therapy*. Curr. Top. Med. Chem., 11, 2012-2028.

We decided to investigate the most obvious discrepancies and, if possible to solve them. Our first step was to develop the simplest chemical model capable of explaining the enzyme's behaviour.

In the case of an oxido-reductase, such a model entails a reductive and an oxidative half cycle, each configured as a Michaelis-like model:

$TR_{ox} + NADPH \quad <==> TR_{ox}NADPH \quad --> TR_{red} + NADP^{+}$ (eq.1)

$TR_{red} + Trx_{ox} \quad <==> TR_{red}Trx_{ox} \quad --> TR_{ox} + Trx_{red}$ (eq.2)

We define:

$K_N = [TR_{ox}] [NADPH] / [Tr_{ox}NADPH]$

$K_S = [TR_{red}] [Trx_{ox}] / [Tr_{red}Trx_{ox}]$

Since our objective is to derive the simplest possible model we assume the *pseudo-equilibrium* approximation, i.e. we assume that the enzyme-substrate complexes form and decay rapidly with respect to the chemical reaction they catalyze.

Under steady state conditions the rates of oxidation and reduction are equal, i.e.:

$k_{red}\,[TR_{ox}NADPH] = k_{ox}\,[Tr_{red}Trx_{ox}]$

The equations defined above allow us to express all the intermediates of the catalytic cycle as the product of one of them, chosen as the “reference” species, with the constants and the concentrations of substrates:

Tr_{ox} = reference species

$[TR_{ox}NADPH] = [TR_{ox}]\ N / K_N$

$[TR_{red}Trx_{ox}] = [TR_{ox}NADPH]\ k_{red} / k_{ox} = [TR_{ox}]\ (N / K_N)\ (k_{red} / k_{ox})$

$[TR_{red}] = [TR_{red}Trx_{ox}]\ K_S / [Trx_{ox}] = [TR_{ox}]\ (N / K_N)\ (k_{red} / k_{ox})\ (K_S / S)$

where N stands for [NADPH] and S for $[Trx_{ox}]$.

From the above equation we construct the ***binding polynomial*** of TR, i.e. the sum of all species of the enzyme espressed as a function of the concentration of the reference species:

$$[TR] = [TR_{ox}] (K_N k_{ox} S + N k_{ox} S + N k_{red} S + N k_{red} K_S) / K_n k_{ox} S$$

It is important to keep track of the meaning of each term:

$[TR_{ox}] K_N k_{ox} S / K_N k_{ox} S = [TR_{ox}]$ is the concentration of the reference species;

$[TR_{ox}] N k_{ox} S / K_N k_{ox} S$ is the concentration of species $TR_{ox}NADPH$;

$[TR_{ox}] N k_{red} S / K_N k_o xS$ is the concentration of species $TR_{red}Trx_{ox}$;

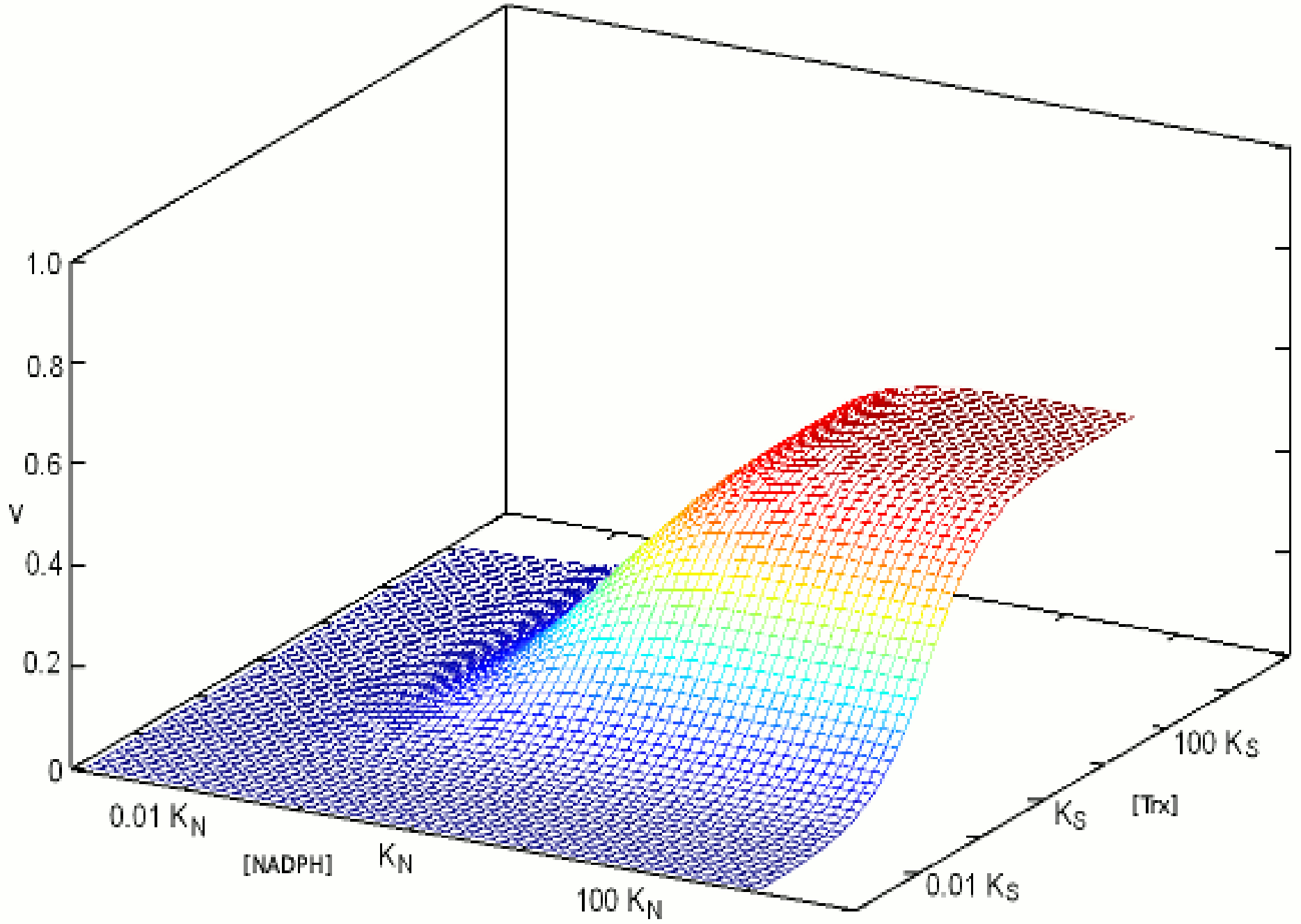
$[TR_{ox}] N k_{red} K_S] / K_N k_{ox} S$ is the concentration of species TR_{red}.

We can now define the rate of substrate transformation, which in the usual enzyme assay is measured by the absorbance change associated to NADPH oxidation:

$$V = k_{red} [TR_{ox}NADPH] = E_{tot} N S k_{ox} k_{red} / (K_N k_{ox} S + N k_{ox} S + N k_{red} S + N k_{red} K_S) \qquad \text{(eq.3)}$$

where the term $N S k_{ox} / (K_N k_{ox} S + N k_{ox} S + N k_{red} S + N k_{red} K_S)$ represents the fraction of the total enzyme in the form of the $TR_{ox}NADPH$ complex.

We remark that the equation describing the rate of substrate transformation is directly proportional to the concentration of both substrates, and to the value of both kinetic constants, as one may have expected. We also remark that this function can be applied as such, for any data set, provided that the concentration of both substrates have been systematically explored. Obviously, V is a function of two independent variables and the pertinent graph is three-dimensional:


1.0
0.8
0.6
v
0.4
0.2
0
0.01 K_N
[NADPH]
K_N
100 K_N
0.01 K_S
K_S
[Trx]
100 K_S

Enzymologists are often more familiar with the classical Michaelis and Menten representation of steady state experiments (or with its linearization, using the Lineweaver and Burk plot), and when publishing a paper one is often encouraged to use these representations rather than the three-dimensional plot depicted in the above paragraph.

The simple recipe to obtain a more classical plot is to systematically vary the concentration of one substrate while keeping fixed that of the other: in this way a typical hyperbolic plot can be obtained, from which two parameters formally analogous to K_M and k_{cat} can be obtained; and by inverting the fixed and varied substrate, one obtains steady state parameters for both.

This procedure is widely applied, but its implications are often neglected.

Suppose that we apply the procedure to TR, and that we keep $[Trx_{ox}]$, while systematically varying [NADPH]. We obtain a hyperbola whose asymptote, ($V_{max}/E_{tot} = k_{cat,N,app}$) at saturating concentration of [NADPH] can be estimated by increasing the term N to infinity, removing from the rate equation any term that does not contain N and then simplifying what is left:

$$k_{cat,N,app} = S\, k_{ox}\, k_{red} / (k_{ox}S + k_{red}S + k_{red}K_S)$$

We observe that:
(i) $k_{cat,N,app}$ is a complex function of both kinetic constants (k_{ox} and k_{red}), K_S and S;
(ii) $k_{cat,N,app}$ cannot be considered a "constant" or a "parameter" since its contains the concentration of the fixed substrate S.

We now turn to the other parameter, namely $K_{M,N,app}$, which is defined as the concentration of the variable substrate required to achieve half the maximal rate:

$$S\, k_{ox}\, k_{red} / (k_{ox}S + k_{red}S + k_{red}K_S) =$$
$$= 2\, K_{M,N,app}\, S\, k_{ox}\, k_{red} / (K_N k_{ox}S + K_{M,N,app}k_{ox}S + K_{M,N,app}k_{red}S + K_{M,N,app}k_{red}K_S)$$

After the obvious simplifications, we obtain:
$$K_N k_{ox}S = K_{M,N,app}k_{ox}S + K_{M,N,app}k_{red}S + K_{M,N,app}k_{red}K_S)$$

and
$$K_{M,N,app} = K_N k_{ox}S / (k_{ox}S + k_{red}S + k_{red}K_S)$$

We again observe that $K_{M,N,app}$ is not a "parameter" since it is a convolution of four constants and one variable; however, $K_{M,N,app}$ is directly proportional to K_N, the real thermodynamic parameter of our kinetic scheme.

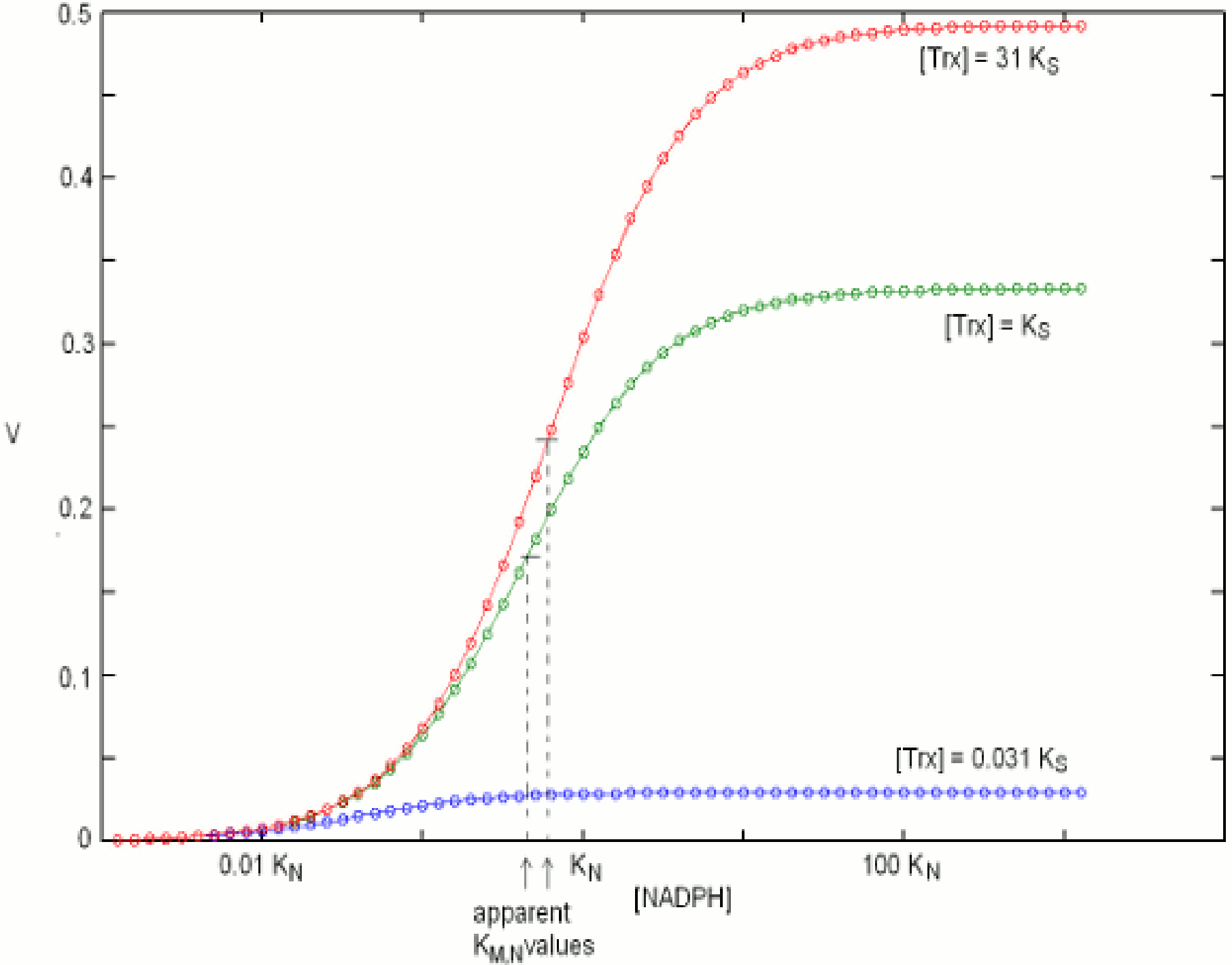
It is remarkable that our two apparent parameters bear the same relationship with the corresponding intrinsic parameter: indeed if we define the term $L = k_{ox}S / (k_{ox}S + k_{red}S + k_{red}K_S)$, it is evident that:

$$k_{cat,N,app} = L\, k_{red}$$

$$K_{M,N,app} = L\, K_N$$

This proves that each single Michaelis plot is a perfect hyperbola, but also that the apparent parameters depend on the concentration of the fixed substrate S.

We need not bother to define the other two parameters $k_{cat,S,app}$ and $K_{M,S,app}$ since the scheme is perfectly symmetric with respect to its substrates and thus these parameters are identical to the former two except that they invert K_S with K_N, S with N and k_{red} with k_{ox} and vice-versa.


0.5
0.4
0.3
0.2
0.1
0
V
[Trx] = 31 K_S
[Trx] = K_S
[Trx] = 0.031 K_S
0.01 K_N
K_N
100 K_N
[NADPH]
apparent
$K_{M,N}$values

If one can indeed obtain plausible hyperbola for a two-substrate enzyme by keeping the concentration of one substrate fixed while systematically varying the other, the parameters one obtains are complex convolutions of the desired ones, that also include the concentration of the fixed substrate. *By no means these apparent K_M and k_{cat} can be considered analogous to the corresponding Michaelis parameters for single-substrate enzymes, nor can they be compared with similar parameters obtained at a different concentration of the fixed substrate.*

Although in principle one can measure apparent values of $k_{cat,N,app}$, $K_{M,N,app}$, $k_{cat,S,app}$ and $K_{M,S,app}$ at several concentrations of the fixed substrate (Trx) and use the whole set to calculate the four intrinsic parameters of the model (K_N, k_{red}, K_S, and k_{ox}) the analysis would be statistically unsound. The best procedure is to collect the data and to analyze them globally using eq.3; then, if required one may represent the data as families of Michaelis hyperbola, as in Fig.2.

Unfortunately, even the soundest statistical analysis is model dependent

Competitive inhibitors of TR

Several competitive inhibitors of TR are known. Some of these are molecules which reversibly combine with either the binding site of Trx_{ox} or NADPH; others are molecules that after an initial reversible binding form a covalent bond and become irreversibly bound. In the latter case the inhibitor shifts from competitive to irreversible (hence non-competitive) with time. We shall consider in this lecture only reversible inhibitors (or irreversible ones incubated for too short a time for the irreversible bond to be formed).

Reversible inhibitors of TR which compete with NADPH exist: e.g. indomethacin. These are poorly specific and of limited medical interest, thus their characterization has been less extensive than that of inhibitors competing with Trx.

A well characterized inhibitor competing with Trx is methylarsonous diiodide, CH_3AsI_2, an inhibitor that initially binds reversibly to the same site of Trx but on long incubation time under reducing conditions forms a covalent bond with the active site Cys residues.

Methylarsonous diiodide is reported to have a K_I of 100 nM if its affinity is measured at constant [NADPH] and variable [Trx_{ox}], and a K_I of 250 nM if the affinity is measured in the opposite way round, i.e. maintaining a constant concentration of the competing substrate.

This is clearly an impossible finding: the same inhibitor cannot have two K_Is, depending on the measurement technique.

Other similar esamples are:
- Epigallocatechin-3-gallate which inhibits rat TrxR with K_I 64 and 92 uM;
- Trifluoperazine which inhibits Leishmania TryR with K_I 22 and 31 uM;

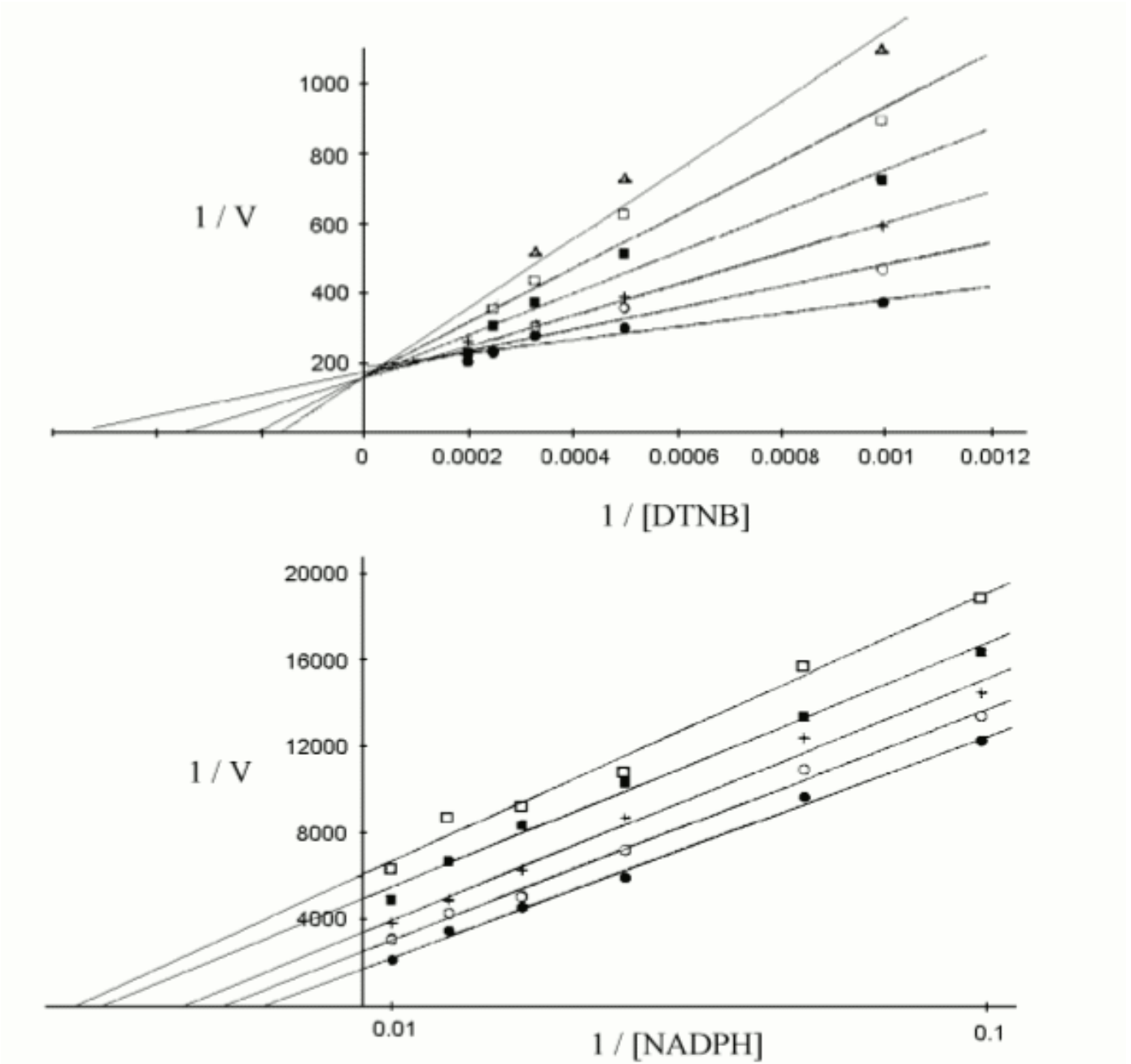


Fig. (3). Inhibition of mouse liver TrxR by methylarsonous diiodide, CH_3AsI_2 (redrawn and modified from Figs. **2** and **3** of [45]).

We want to understand how the authors arrived to estimate two K_Is and which is the good one.

To do so we write down the following (simplified) kinetic model:

$TR_{ox} + NADPH <==> TR_{ox}NADPH \dashrightarrow TR_{red} + NADP^+$ (eq.4)

$I\text{-}TR_{red} + Trx_{ox} <==> I + TR_{red} + Trx_{ox} <==> I + Tr_{red}Trx_{ox} \dashrightarrow I + TR_{ox} + Trx_{red}$ (eq.5)

which makes it plain that one and only one K_I can be defined for this system.

We do not consider binding of the inhibitor to the oxidized enzyme since, even though binding is possible, it does not (or is expected not to) interfere with binding of NADPH and reduction of the active site; thus TR_{ox} would be indistinguishable from $I\text{-}TR_{ox}$.

The rate of NADPH oxidation for the reaction scheme of eqs. 4 and 5, detremined using the method illustrated above, results:

$$V = E_{tot}\, Nk_{red}\, Sk_{ox}\, K_I / (Sk_{ox}K_NK_I + NSk_{ox}K_I + Nk_{red}K_SK_I + Nk_{red}SK_I + Nk_{red}K_S[I]) \qquad \text{(eq.6)}$$

One can easily verify that in the absence of the inhibitor (i.e. [I]=0) eq.6 reduces to eq.3.

Eq.6 is a function of three variables, and its parameters can be determined by non linear least squares regression from any extensive set of V=f(S,N,[I]).

However, since in the literature data are often analyzed as Michaelis- or Lineweaver- plots in which either substrate is kept constant, we may want to know how these look like.

If one keeps constant [NADPH], and varies the concentration of the competing substrate Trx at different concentrations of the inhibitor, one obtains a set of Michaelis plots in which:

$$k_{cat,S,app,I} = Nk_{red}\, k_{ox} / (k_{ox}K_N + Nk_{ox} + Nk_{red}) = k_{cat,\,S,\,app}$$

The k_{cat} is obtained, as usual, by increasing S to infinity, by removing all terms that do not contain it, and by simplifying those which are left. We remark that $k_{cat,S,app,\,I} = k_{cat,S,\,app}$, consistent with the expectation that at infinite concentration of Trx, I is displaced: the competitive inhibitor is removed form the active site by the competing substrate, thus the kcat of the enzyme is unchanged (see Fig.3, upper panel).

By contrast, as expected, the competitive inhibitor changes the apparent K_M of the competing substrate, exactly as it occurs in single-substrate enzymes:

$$K_{M,S,app,I} = K_{M,S,app}\,([I] + K_I) / K_I$$

Regression of $K_{M,S,app,I}$ vs. [I] yields a straight line with slope $K_{M,S,app} / K_I$ and intercept $K_{M,S,app}$; thus K_I = intercept / slope.

This is the standard procedure one follows in the case of single-substrate enzymes (even though it is not statistically sound).

Explaining the lower panel of Fig.3 is a little more difficult: after all, the data and lines in the upper panel are identical to those one would obtain in the case of competitive inhibition of a single-substrate enzyme, whereas those in the lower panel of the same figure configure quite an atypical case of non-competitive inhibition.

We derive from eq.6 the values of $k_{cat,N,app,I}$ and $K_{M,N,app,I}$ for experiments carried out at constant S:

$$k_{cat,N,app,I} = k_{red}Sk_{ox}K_I / (Sk_{ox}K_I + k_{red}K_SK_I + k_{red}SK_I + k_{red}K_S[I])$$

$$K_{M,N,app,I} = K_N\, k_{cat,N,app} / k_{red}$$

We remark that:

(i) the term $k_{cat,N,app,I}$ is a function of [I] (the non competing substrate cannot displace the inhibitor), i.e. the inhibitor lowers the apparent k_{cat};

(ii) since $k_{cat,N,app} < k_{red}$, $K_{M,N,app,I} < K_{M,N,app}$, i.e. the inhibitor increases the apparent affinity of the enzyme for the non-competing substrate.

If we compare the apparent kcat in the presence of the inhibitor:
$k_{cat,N,app,I} = k_{red}Sk_{ox}K_I / (Sk_{ox}K_I + k_{red}K_SK_I + k_{red}SK_I + k_{red}K_S[I])$

with the one we found in the absence of the inhibitor:
$k_{cat,N,app} = S\, k_{ox}\, k_{red} / (k_{ox}S + k_{red}S + k_{red}K_S)$

the following relationship is immediately apparent:
$1 / k_{cat,N,app,I} = 1 / k_{cat,N,app} + K_S[I] / Sk_{ox}K_I$

the above relationship implies that the plot of $1 / k_{cat,N,app,I}$ vs. [I] is a straight line with slope $K_S / Sk_{ox}K_I$ and intercept $1 / k_{cat,N,app}$. If one wrongly assumes this system to behave as a non competitive inhibitor on a single substrate enzyme, one estimates K_I as intercept / slope, and obtains:

$K_{I,believed} = K_I (k_{ox}S + k_{red}S + k_{red}K_S]) / k_{red}\, K_S$

Now we can answer our original question: if the two-substrate enzyme is treated as a single-substrate one by keeping either substrate constant, one obtains two estimates of the affinity of the inhibitor:

i) when the varied substrate competes with the inhibitor: K_I

ii) when the competing substrate is kept constant, and the non-competing substrate is varied:

$$K_{I,believed} = K_I (k_{ox}S + k_{red}S + k_{red}K_S) / k_{red} K_S =$$
$$= K_I [1 + (k_{ox}S + k_{red}S) / k_{red} K_S]$$

and we remark that $K_{I,believed} > K_I$ (as found by many unaware researchers).

References:

Saccoccia et al. (2014) *Thioredoxin reductase and its inhibitors*. Curr. Protein Pept. Sci. 15: 621-646.
http://biochimica.bio.uniroma1.it/didattica/dottorato/Brallo2015.pdf