Nicotinamide Adenine Dinucleotide Cofactors

References: Two key reviews: Protein Dynamics in Enzyme Catalysis, S.J. Benkovic, The Chemical Record 2, 24-36 (2002)
Two other papers on Dihydrofolate Reductase (DHFR) are Fierke et al, Biochemistry 1987, 26, 4085-92. This paper describes the steady state and pre-steady state kinetics and the entire reaction coordinate of this protein. Kraut et al, Biochemistry, 1997, 36, 586-603 This paper describes the results from 100 crystal structures.
Review: Expanding the Genetic Code Chem Comm January 1-11 (2002). Gives an excellent overview of the development of the technology that will in the not too distant future allow us to put unnatural amino acids into any protein in vivo.

Masked redox chemistry

Biochem 2003 42 1900-1909.

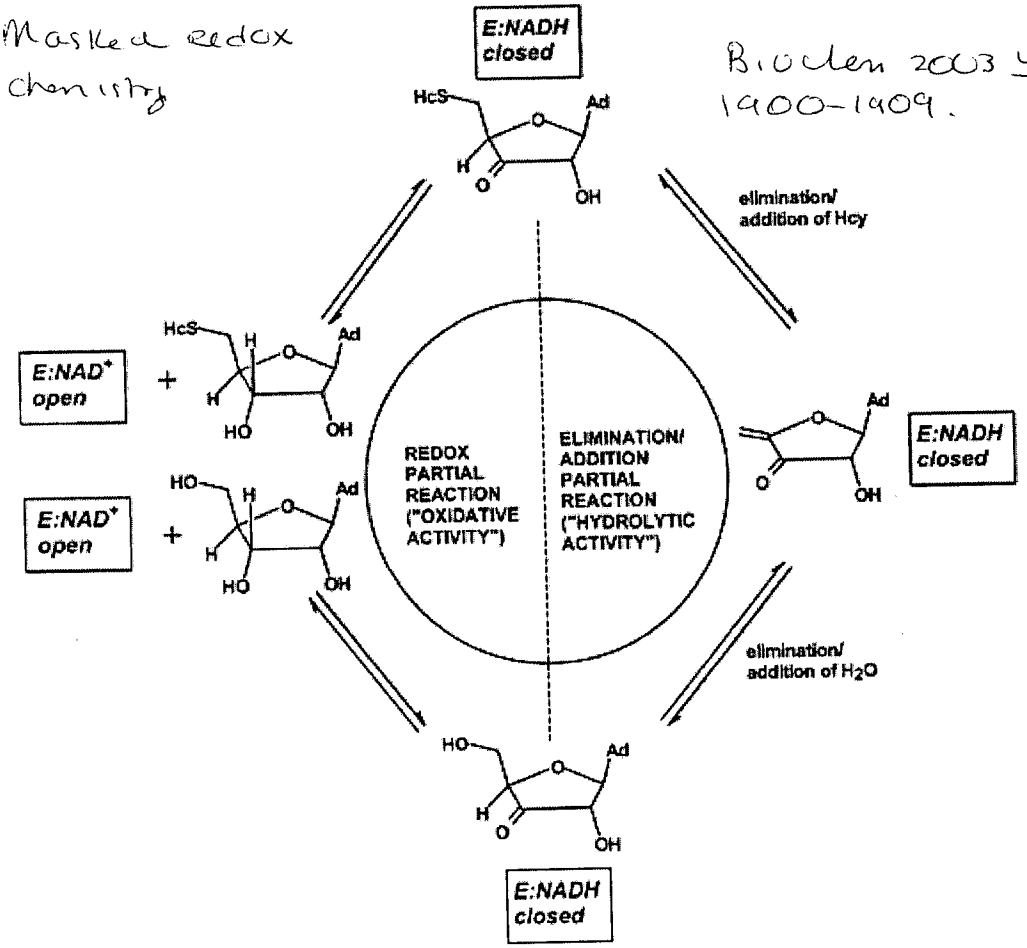
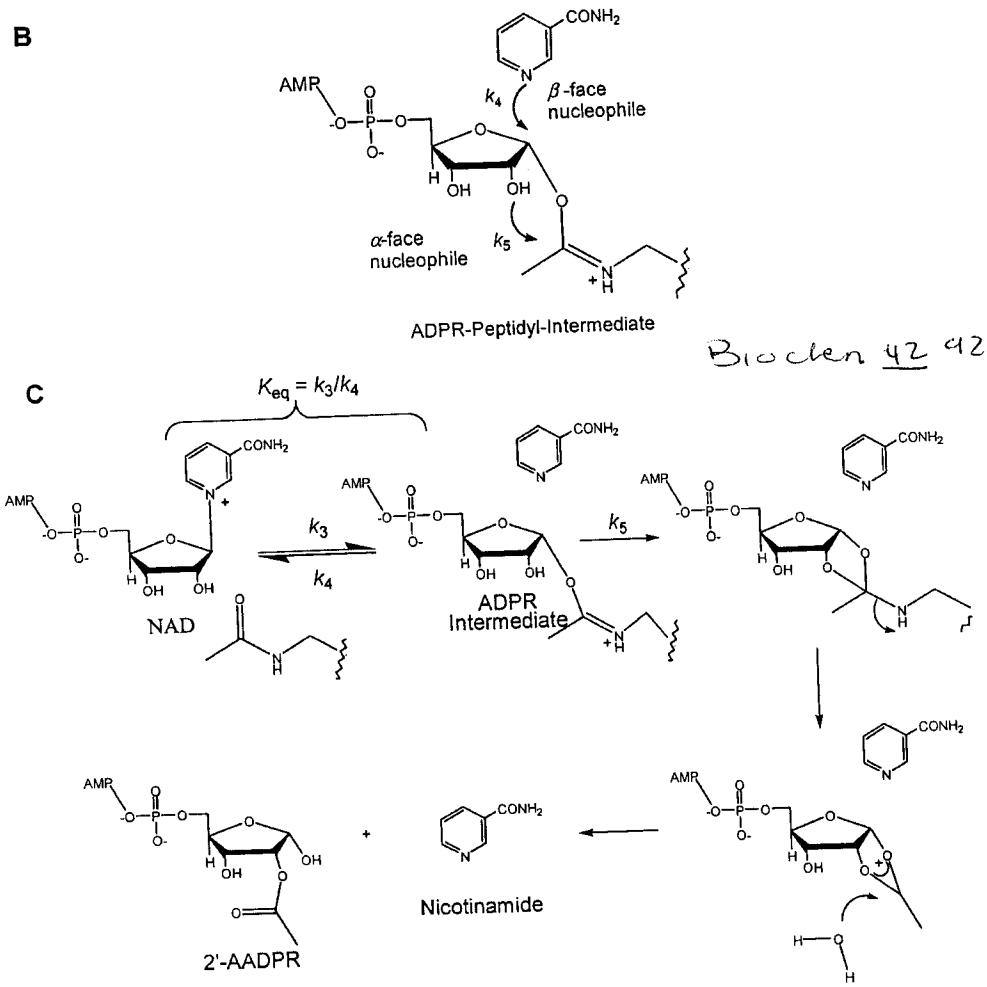


Figure 1 The catalytic cycle of AdoHcy hydrolase resolves into two partial reactions: a *redox partial reaction* occurring at the beginning and end of the cycle and thus spanning an *elimination/addition partial reaction*, which effects the fission/formation of the C-5'-S bond and the formation/fission of the C-5'-O bond. The free enzyme is in the NAD^+ form and possesses an *open structure* in which the substrate-binding and cofactor-binding domains are in relative motion. After binding of free substrate and oxidation, the enzyme is in the NADH form and possesses a *closed structure* in which the two domains are closed against each other to isolate the active site from the external environment.



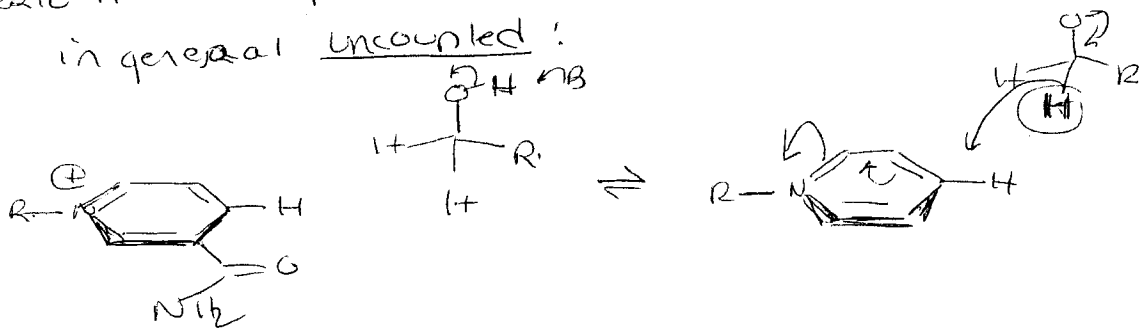
Biochem 42 9250 (2003)

[a] Abbreviated reaction scheme for Sir2 deacetylation reactions. The competitive nucleophilic attacks on the Sir2 ADPR-peptidyl intermediate occur from both stereochemical faces (A and B). The top face of the ribosyl ring is designated β, and nicotinamide nucleophilic attack at C1′ leads to re-formation of β-NAD$^+$. The bottom face of the sugar is designated α, and the hydroxyl group attacks the α-amidate group from the same face to generate deacetylation products. The rate constants for the two competing nucleophilic attacks are shown as k_4 for exchange and k_5 for deacetylation (B). Reactions of Sir2 intermediates at saturating nicotinamide concentrations are shown with binding steps omitted (C).

(↑)

Proposed Novel Mechanism for Histone Deacetylase

Generic H⁻ transfer Mechanism: (↓) H⁻ and H⁺ transfer are in general uncoupled:

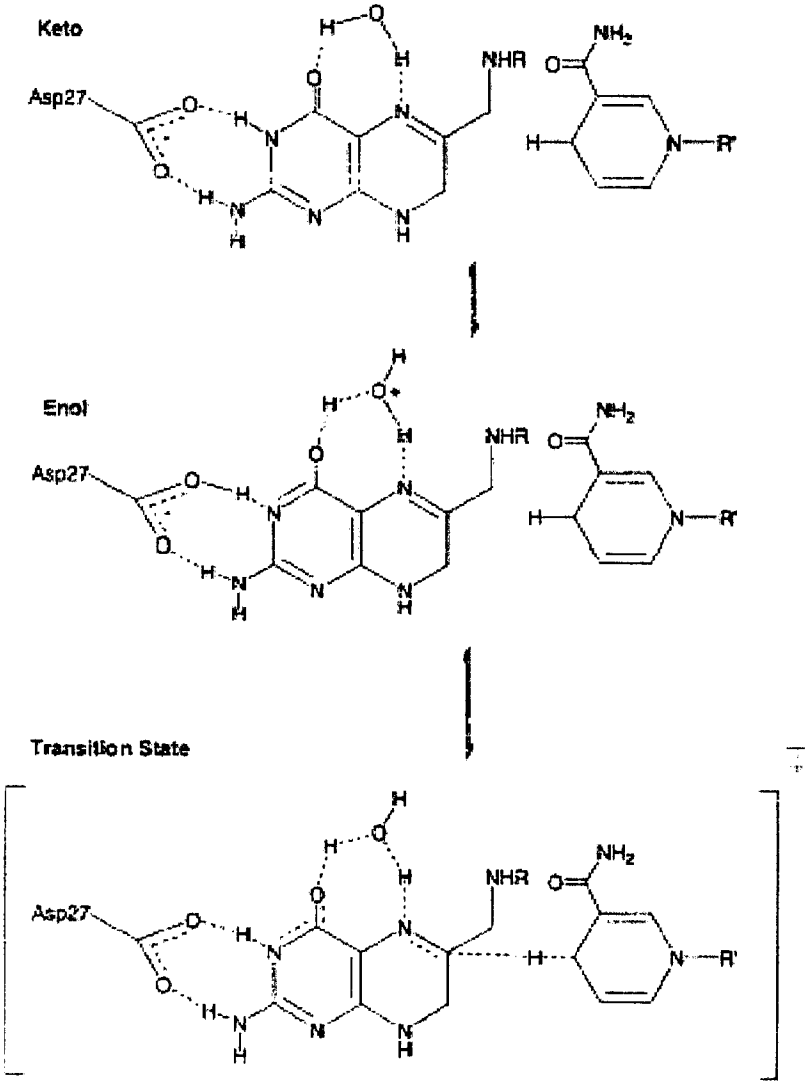


NOTE: H⁻ is transferred directly. If labeled with ^3H, Never see a label in H_2O. Hallmark of H⁻ Transfer RXNs.

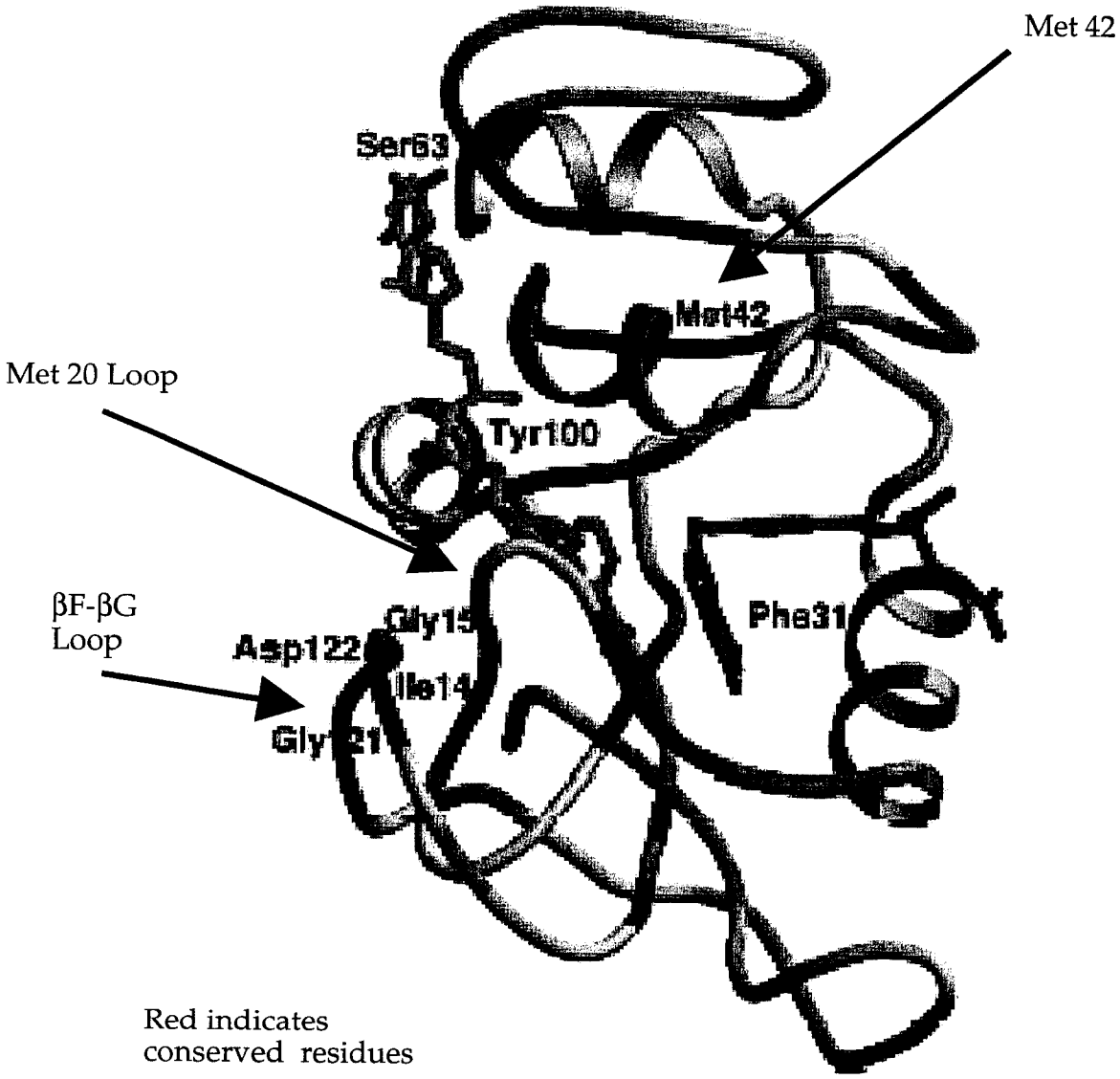
Dihydrofolate Reductase (DHRF)

The Proposed Mechanism-

The mechanism shown below is based on ketone-enol tautomerization driven by the dielectric constant of the active site. This is proposed to raise the pKa of Asp27 to 6.5, however the enzyme is very pH sensitive and this proposed pKa is controversial. The mechanism also depends on a structurally conserved water in the active site.

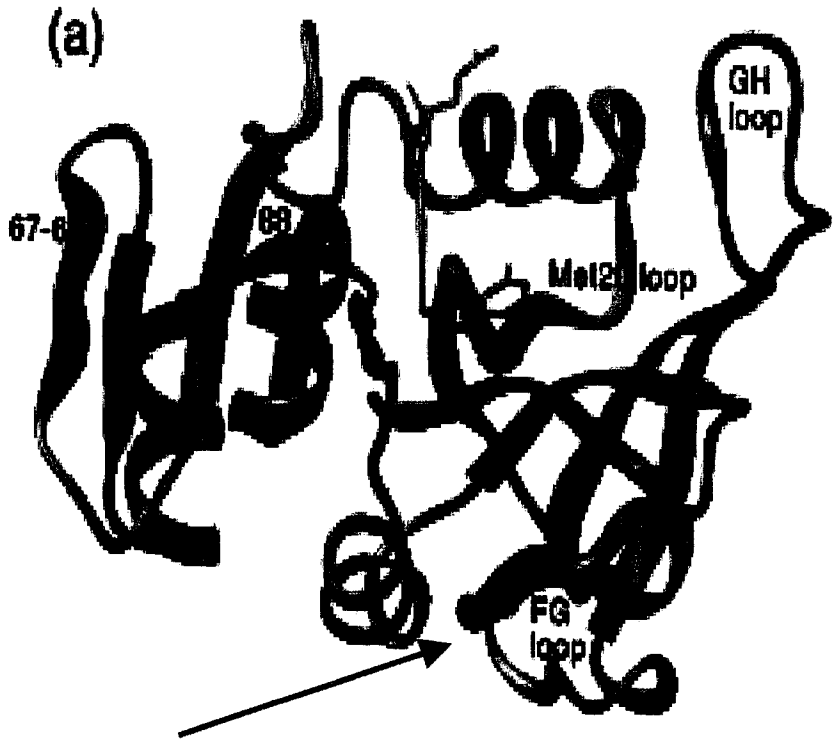


The Topology of DHFR

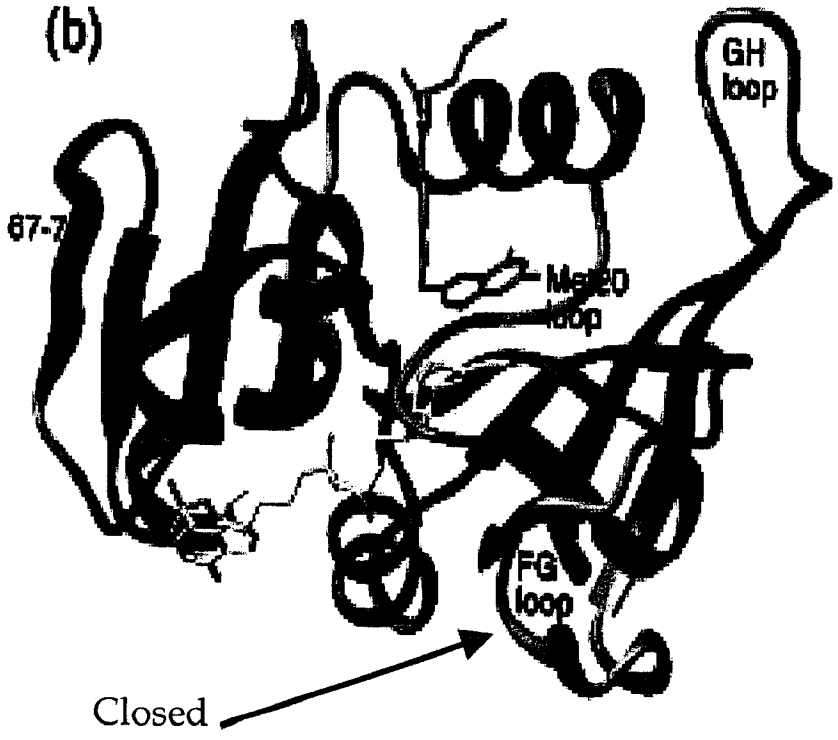


Amide NH of G15 and E17 located in the Met20 loop form hydrogen bonds with D122 of the βF-βG loop. This loop interaction actively controls NADPH affinity and hydride transfer rate.

Models for fast motions in DHFR

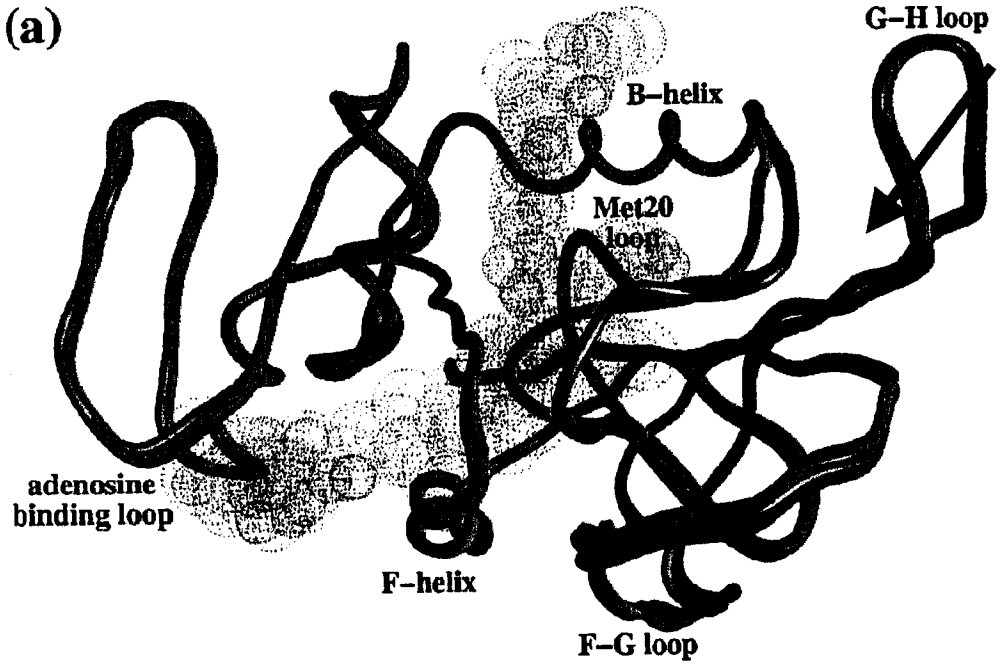


Occluded



Closed

What has been learned from the many structures of DHFR



X-ray data from many structures and from different space groups were compared and 3 enzyme states were defines.

1) Open – Black ribbon, no substrate bound
2) Closed – green ribbon, Michaelis complex
3) Occluded – red ribbon, products or THF only complex

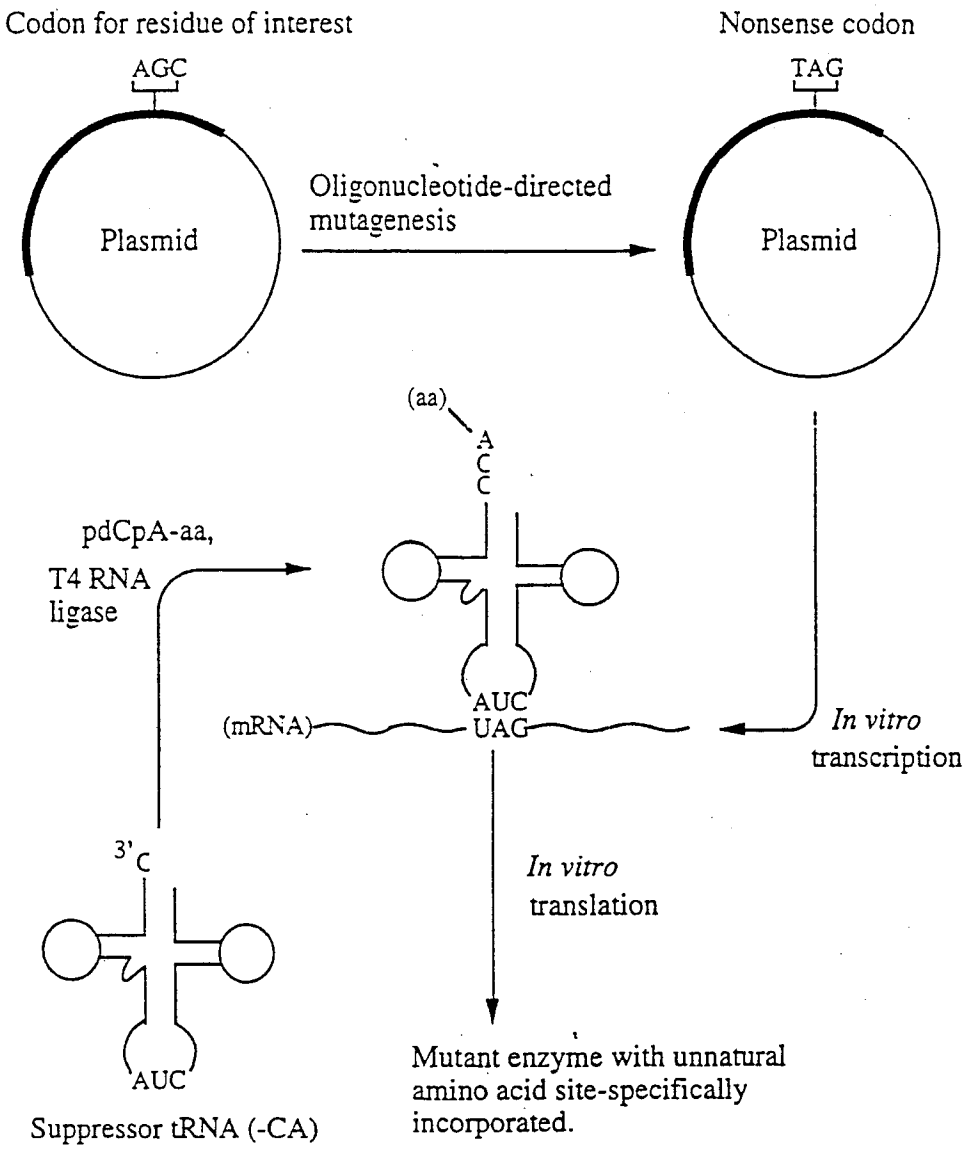
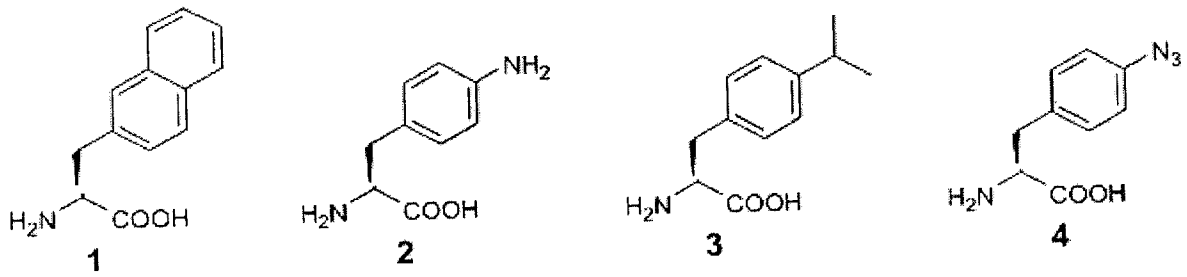


Figure 1.1 A new methodology for the site-specific incorporation of unnatural amino acids into proteins.



some of available un-natural aa in vitro.

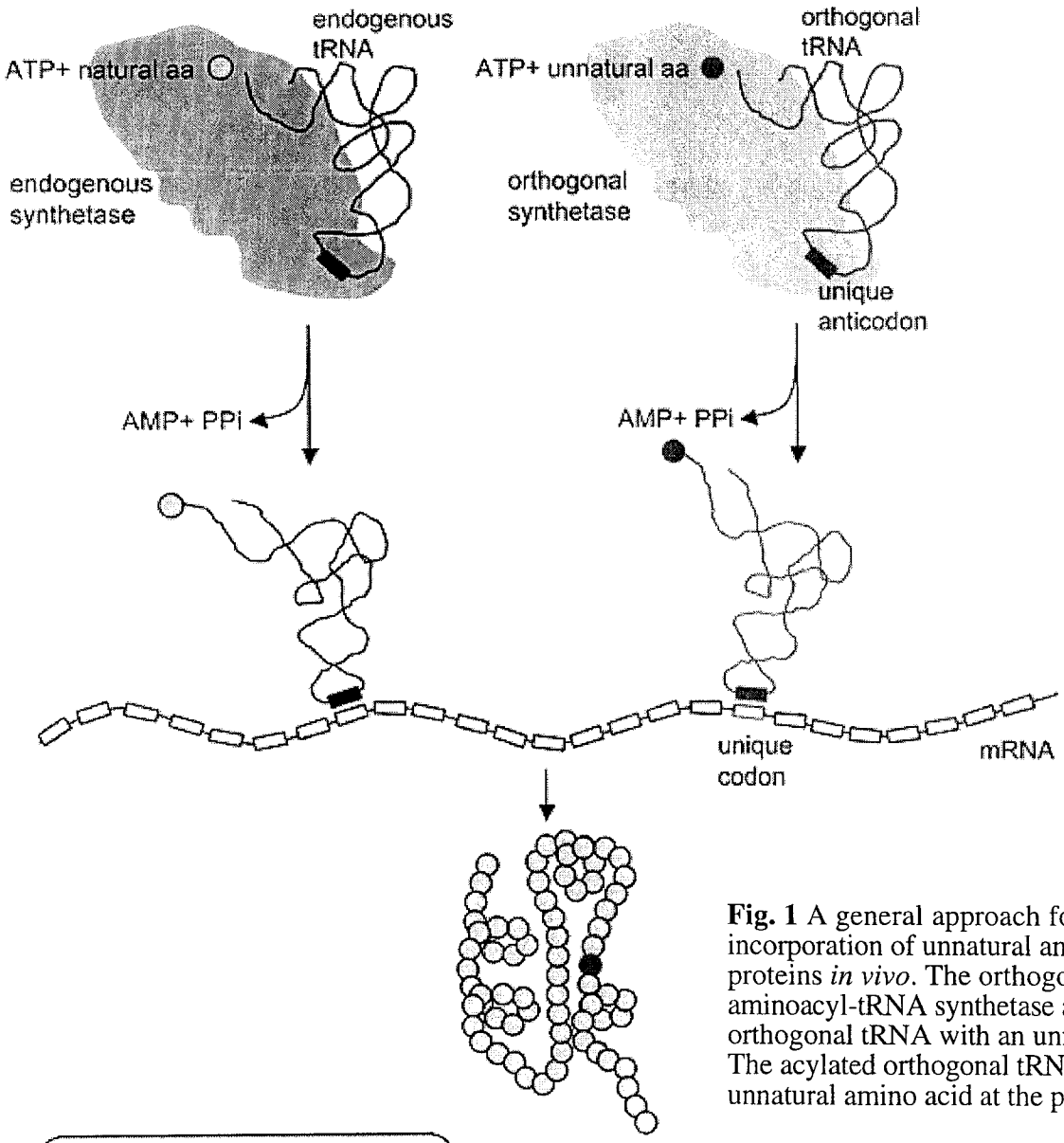


Fig. 1 A general approach for the site-specific incorporation of unnatural amino acids into proteins *in vivo*. The orthogonal aminoacyl-tRNA synthetase acylates the orthogonal tRNA with an unnatural amino acid. The acylated orthogonal tRNA inserts the unnatural amino acid at the position specified by

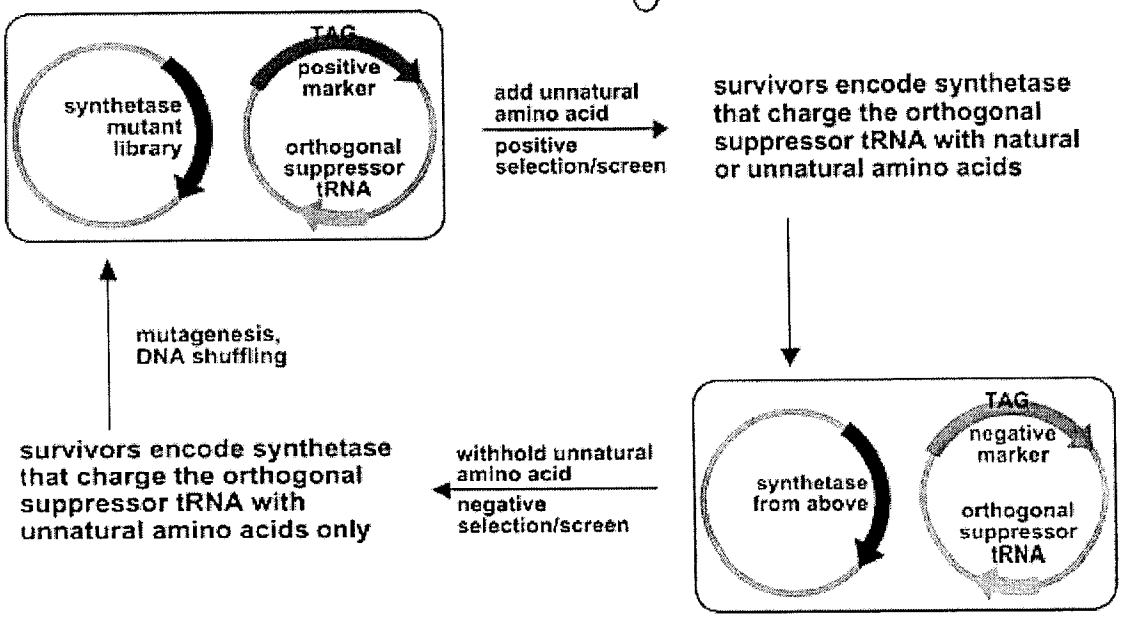
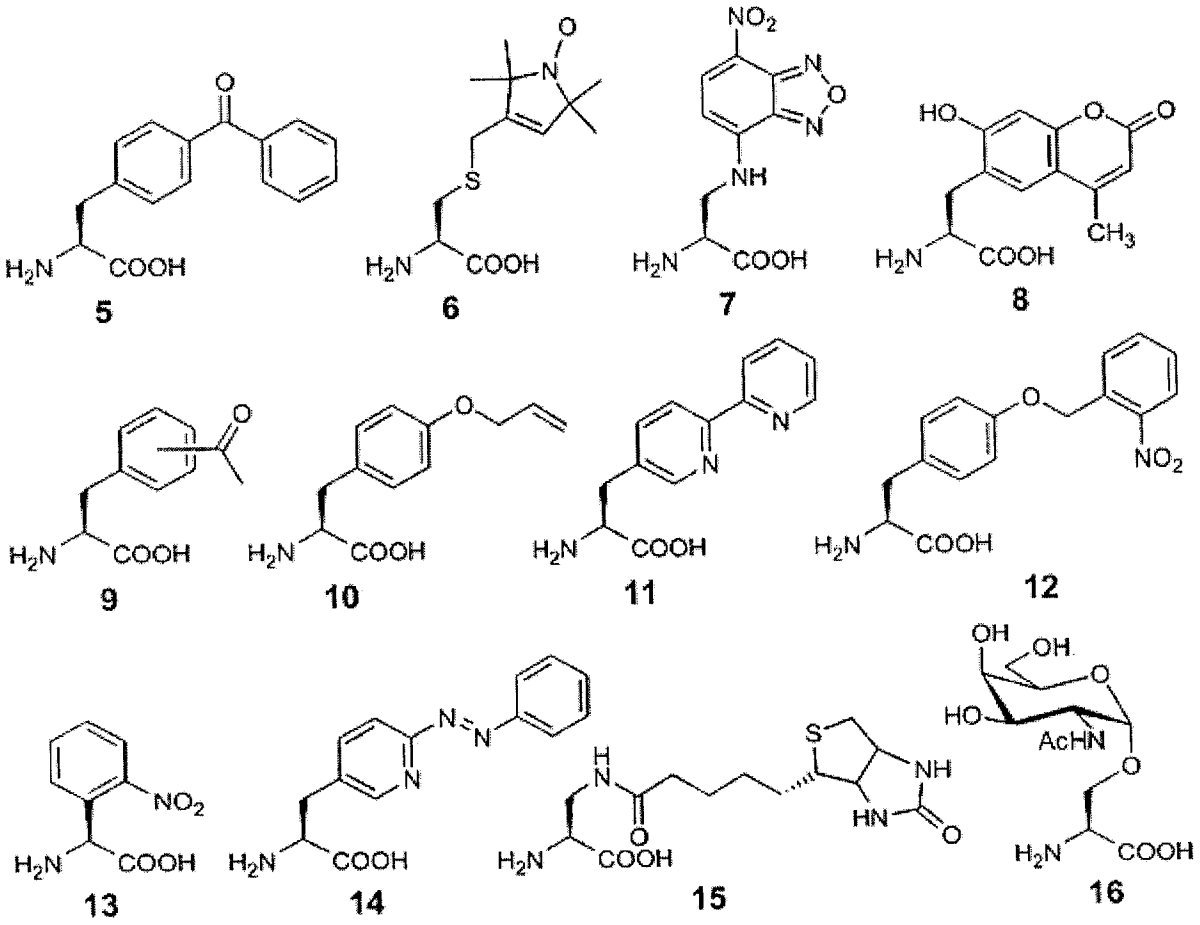


Fig. 2 Schematic illustration of the general selection/screen for aminoacyl-tRNA synthetases with unnatural amino acid



Scheme 3 Structures of unnatural amino acids discussed in the text.

Flavin References:

Flavins have been divided into four families based on structure and sequence homology of short motifs: Protein Science 2001, 1712-1728.

Mechanism of amino acid oxidases and a good overview of flavin chemistry: The Chemical Record 1 183-194 (2001)

Old Review articles that are excellent as the chemical possibilities have been worked out from model studies many years ago: Accounts of Chemical Research (1980) 13, 256-262 (Walsh) and 13, 148-155 (Bruice); J. Biol Chem 269, 2249-2262 (1994).

Structure Planning Diagrams for Four Classes of Flavoproteins

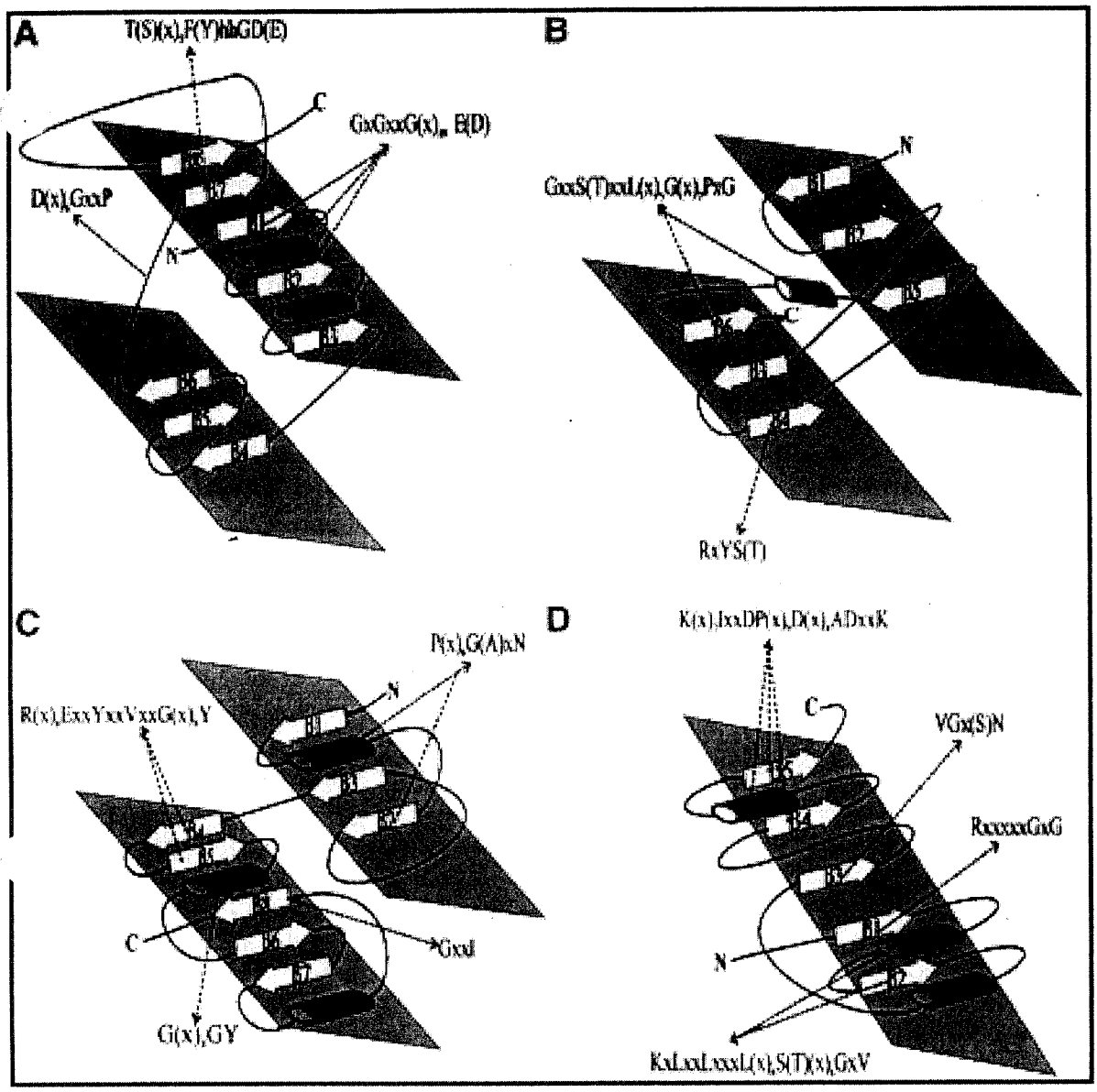
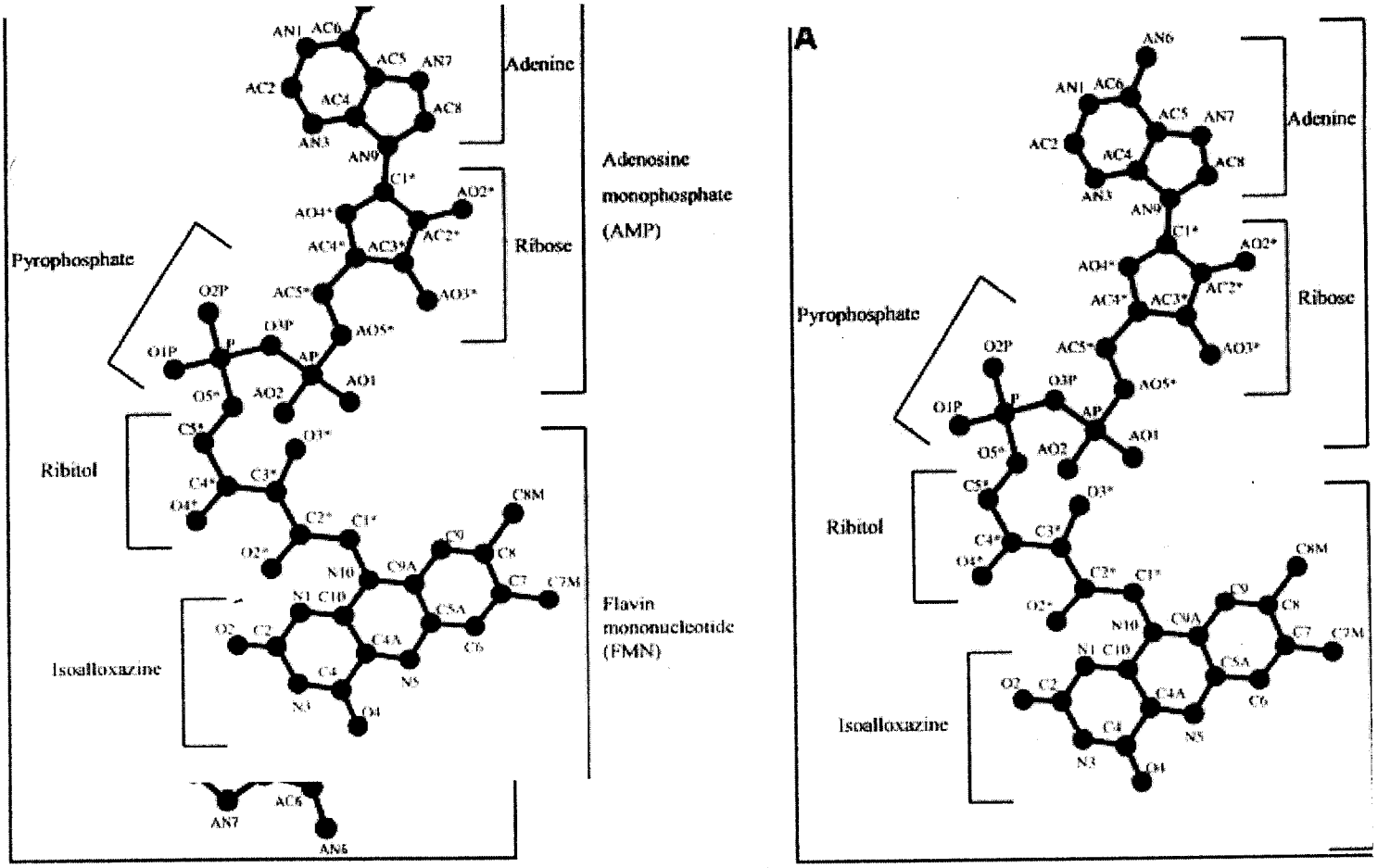
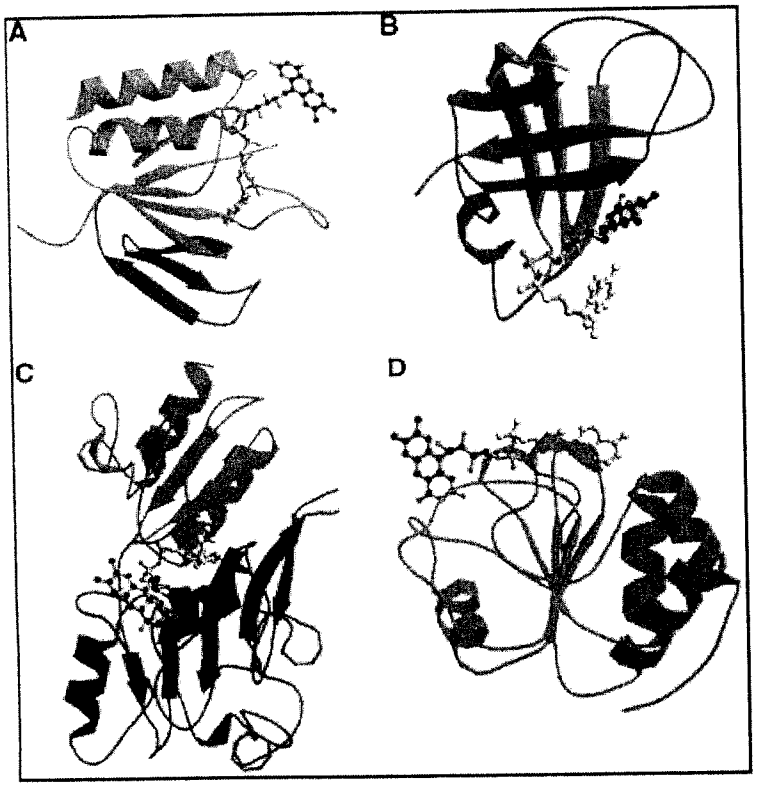


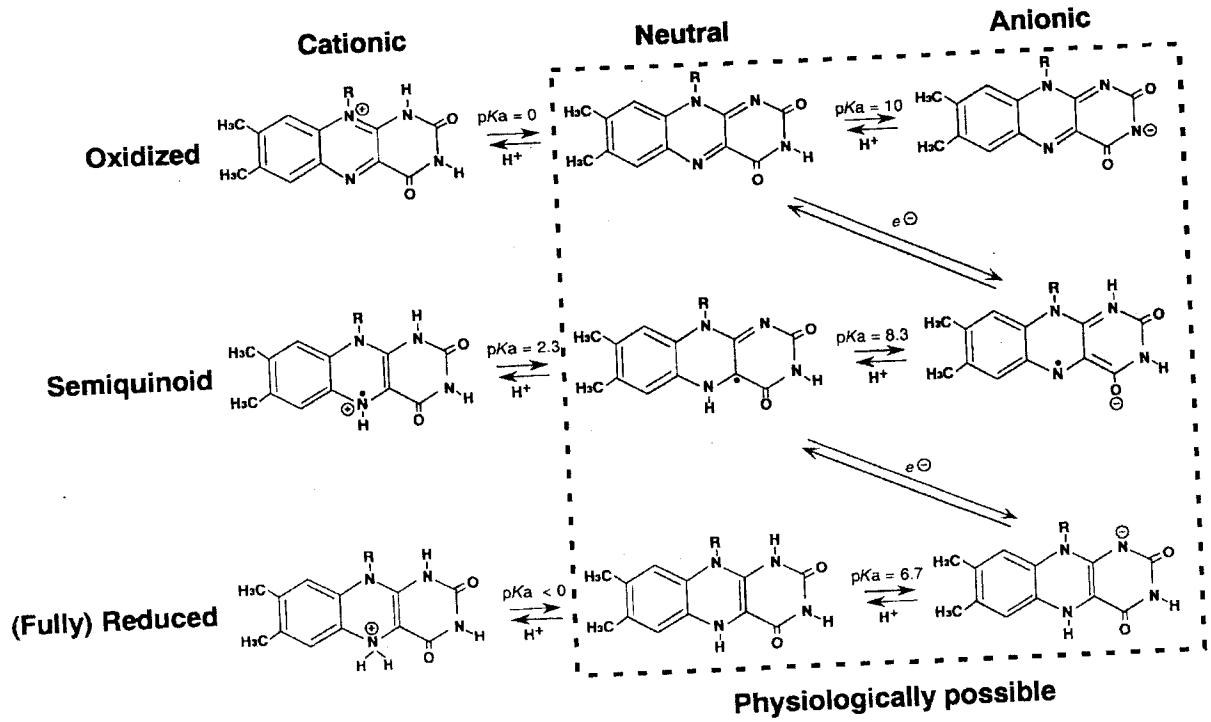
Fig. 3. Topological diagram of FAD-binding domain of the four FAD-family folds. (*A*) Rossmann fold ($ß_1\alpha_1ß_2\alpha_2ß_3$) adopted by the glutathione reductase (GR) family members. For a full description of this fold, it must be noted that there are two subfamilies, GR_1 and GR_2 (see text), and that there are exceptions to the generalizations described here. For example, D-amino acid oxidase of the GR_2 subfamily is an exception to the rule that the FAD-binding fold in the GR family contains a 3-strand ß-meander connecting ß3 and ß4; instead, it has a crossover α-helix. (*B*) Ferredoxin reductase (FR) family fold adopting a cylindrical ß-domain organized into two orthogonal sheets, $ß_1ß_2ß_5$ and $ß_3ß_4ß_6$. (*C*) The *p* -cresol methylhydroxylase (PCMH) family fold consists of two α + ß subdomains; one is composed of three parallel ß-strands ($ß_{1-3}$) and the second contains five antiparallel ß-strands ($ß_{\ \ 8}$) surrounded by α-helices. (*D*) The pyruvate oxidase (PO) family fold consists of five parallel ß-strands ($ß_{1-5}$) interspersed by α-helices similar to the double Rossmann fold found in dehydrogenases. Cylinders represent α-helices and arrows denote ß-strands. The location, indicated in dashed lines , of the conserved sequence motifs in each of the FAD-family folds is listed in Table

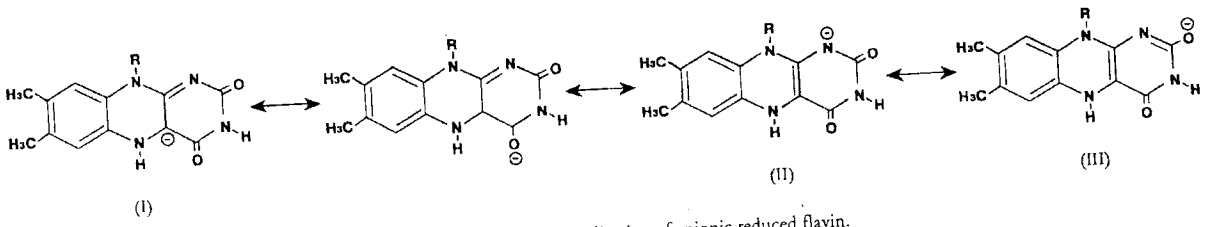


ig. 2. The FAD cofactor conformations. (*A*) An elongated conformation where the adenine ring is distal from the isoalloxazine ng. The division between the component parts FMN and AMP composing the FAD is shown. The AMP is composed of an adenine ng connected to a ribose that is connected to a phosphate group. The FMN moiety is composed of the isoalloxazine-flavin ring nked to a ribitol, which is connected to a phosphate group. (*B*) Bent conformation where the AMP portion is folded back, placing ıe adenine and isoalloxazine rings in close proximity. Variation in the proximity of the two rings determines the degree of cofactor exibility. All atoms composing the FAD cofactor are labeled.



Actual Structures: FAD and Protein Families
Protein Science 2001 10 1712
A. Glutathione reductase family
B. Ferredoxin reductase family
C. p-Cresol Methylhydroxylase
D. Pyruvate Oxidase





Scheme 4. Resonance hybridization of anionic reduced flavin.

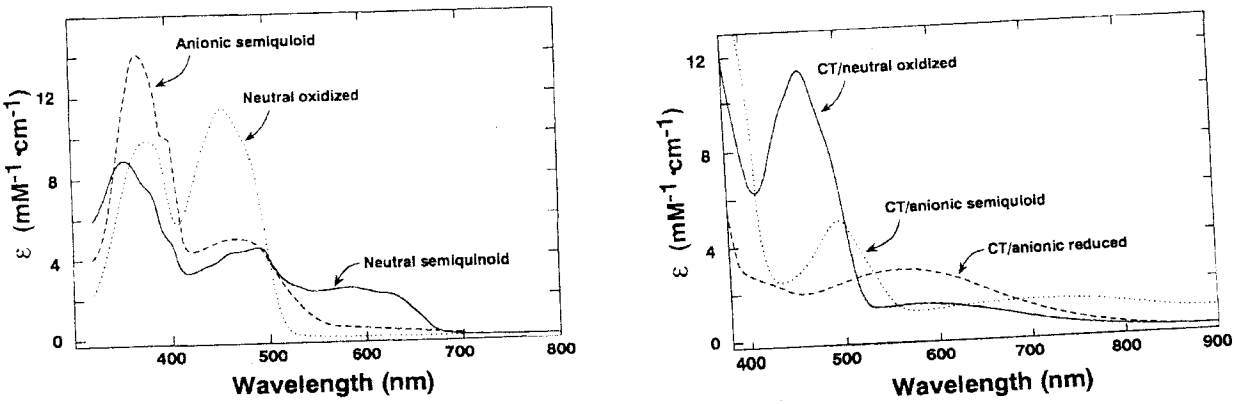


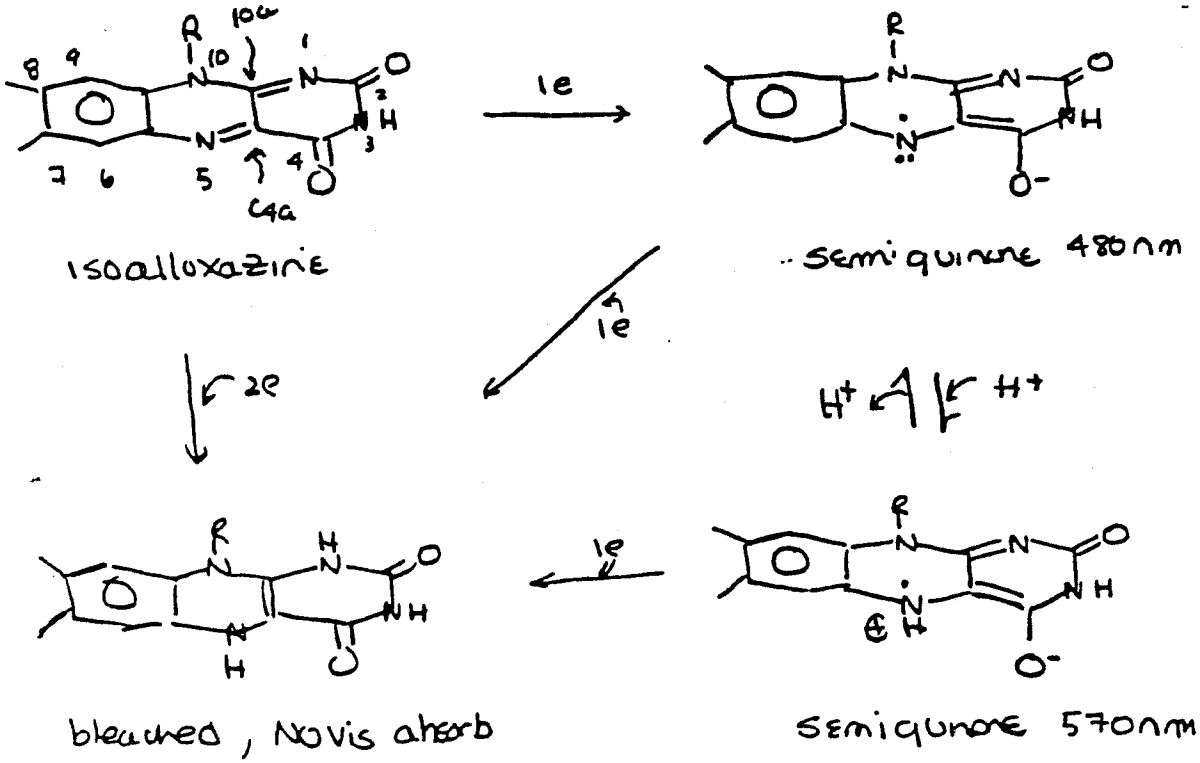
Fig. 2. Absorption spectra of d-amino acid oxidase at different states of flavin (courtesy of Dr. Y. Nishina). Top: neutral oxidized (dotted line), anionic semiquinoid (dashed line), and neutral semiquinoid (solid line) states of flavin. Bottom: charge-transfer states of neutral oxidized flavin (solid line), anionic semiquinoid flavin (dotted line), and anionic reduced flavin (dashed line).

Example of Rich spectroscopy associated with flavins

stopped flow Hsieh

A. Chemistry

(1) Flavins can undergo either one electron or two electron reduction reactions. They thus play a pivotal role in accepting electrons from 2e⁻ reductants (organic compounds) and donate e⁻s to one e⁻ oxidants (metal centers).



(2) While flavins are chemically reactive at multiple positions, enzymatically all reactions thus far examined appear to involve either the C4a position or the N-5 position.

(3) Flavins are tightly bound (covalently (through C-8 to H, C, or Y) or non-covalently, Kds are 10^{-7} to 10^{-11}) and thus be re-oxidized on the enzyme by either oxygen or an ET protein.

(4)FADH2 is rapidly reoxidized by O_2 regenerating H_2O_2. The non-enzymatic reaction is almost as rapid as the enzymatic reaction. $T_{1/2}$ is less than 1 sec.

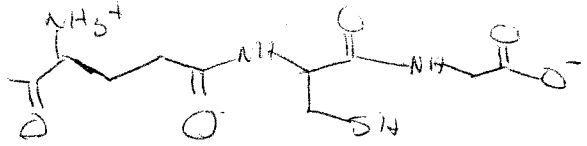
(5) Flavins rapidly disproportionate in solution making kinetic studies complex:
FAD + FADH 2FADH•

(6) pKa of N-1 is 6 to 7.

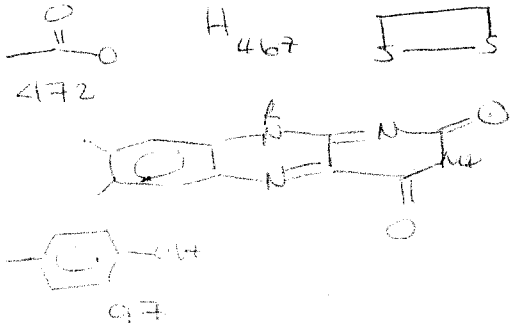
(7)The reduction potentials vary from –450 to +110 mv. The redox potential is modulated by the protein environment. This contrasts with NAD where the reduction potential is –320 mv. The reduction potentials use the biochemical reference state at pH 7 and microM concentrations.

Glutathione Reductase :: reoxidn of enzyme uses a S–S within the active site.

$GS\text{-}SG + NADPH \rightleftharpoons 2\ GSH + NADP^+$ irrev

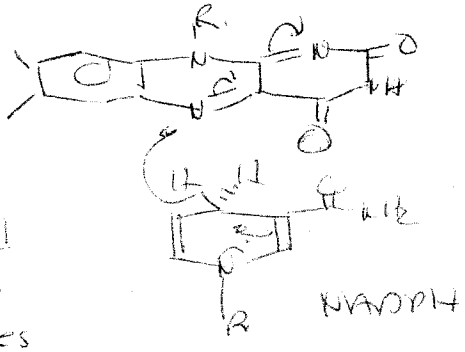
GSH =  glutathione

Proposed mechanism:

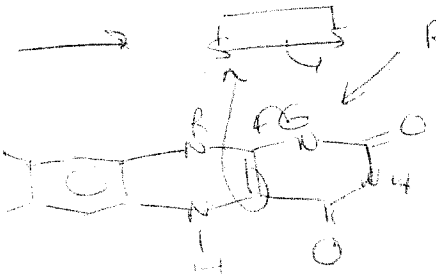


NADPH ⇌

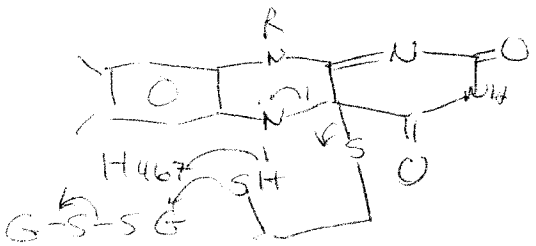
N-5 Chemistry



Tyr 197 moves on NADPH binding

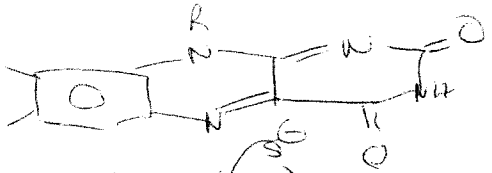


C-4a Chemistry



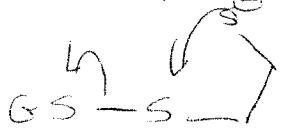
proposed intermediate based on model systems

Flavin reoxidized



charge transfer band

GSH



→ products

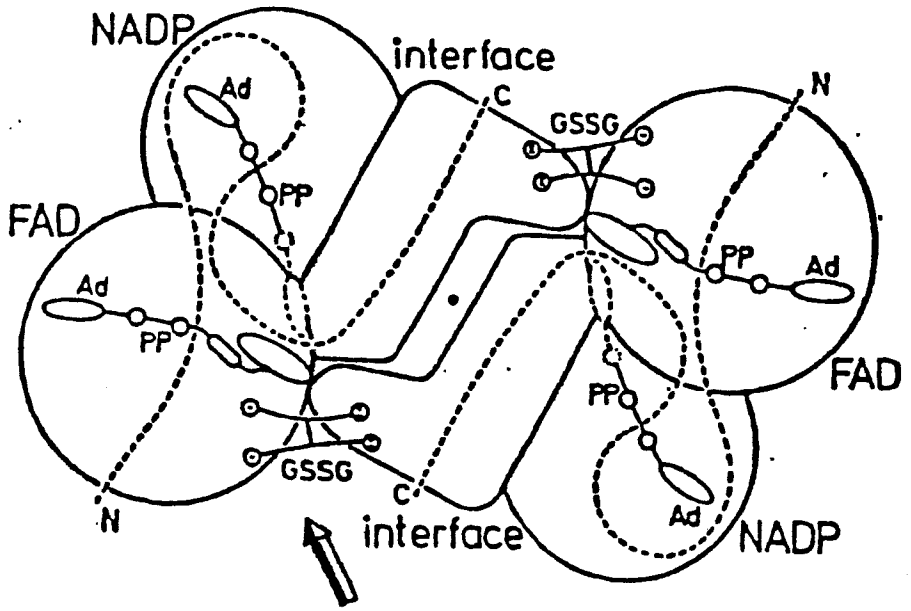
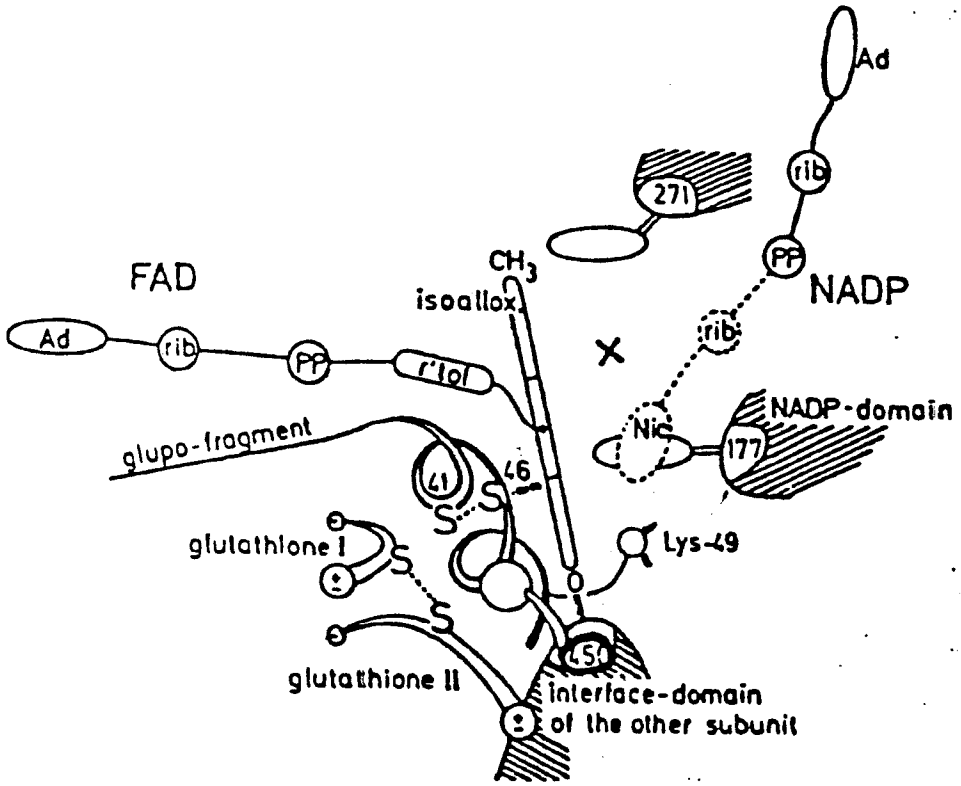
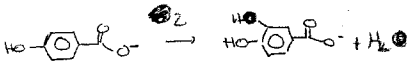


Fig. 2 Sketch of the dimeric enzyme glutathione reductase. The FAD-, NADP- and interface-domains are outlined. The approximate view of Fig. 1 is indicated by an arrow. The general course of the polypeptide chain is given by a dashed line. FAD and NADP bind to their respective domains in an extended conformation. Except for the nicotinamide moiety, NADP can be described as binding at the protein surface. In contrast, FAD binds at the surface of the FAD-domain but at the boundary to the NADP-domain, so that only the adenine moiety extends to the protein surface. Oxidised glutathione (GSSG) binds between subunits. There is a close contact between the flavin ring of one subunit and the interface-domain of the other.



Nature 273 120 (1978) Glutathione Reductase

1. Monooxygenases: One atom from O_2 ends up in product, the other in H_2O.

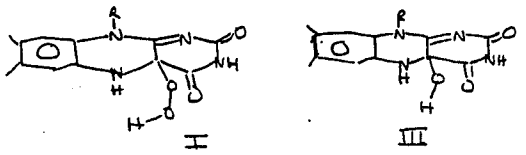
B **Monooxygenases** 

Flavin monooxygenases are rare but serve as an excellent prototype for pteri dependent monooxygenases. The former require activation built into the aromatic substrate (e.g. substrate hydroxylation), while the latter utilize metal cofactors and don't require activated aromatic substrates.

Stopped flow kinetics experiments reveal the presence of three kinetically competent intermediates in the flavin monooxygenease.

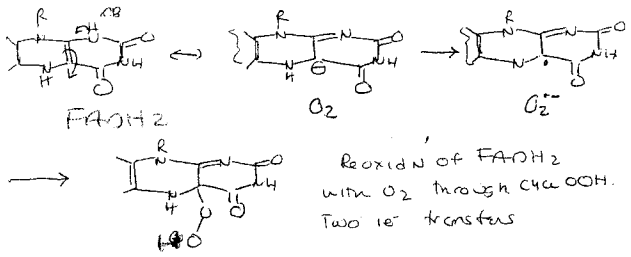
Intermediate	λmax nm	ε $M^{-1}cm^{-1}$
I	380-90	9000
II	390-420	15000
III	380-385	9000

Intermediates I and III are the C4a hydroperoxide and the C4a pseudobase:



I III

(a) E·FAD + NADH ⇌ E·$FADH_2$ + NAD^+

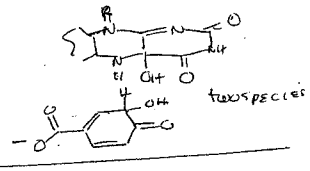
(b) 

$FADH_2$ O_2 $O_2^{\cdot-}$

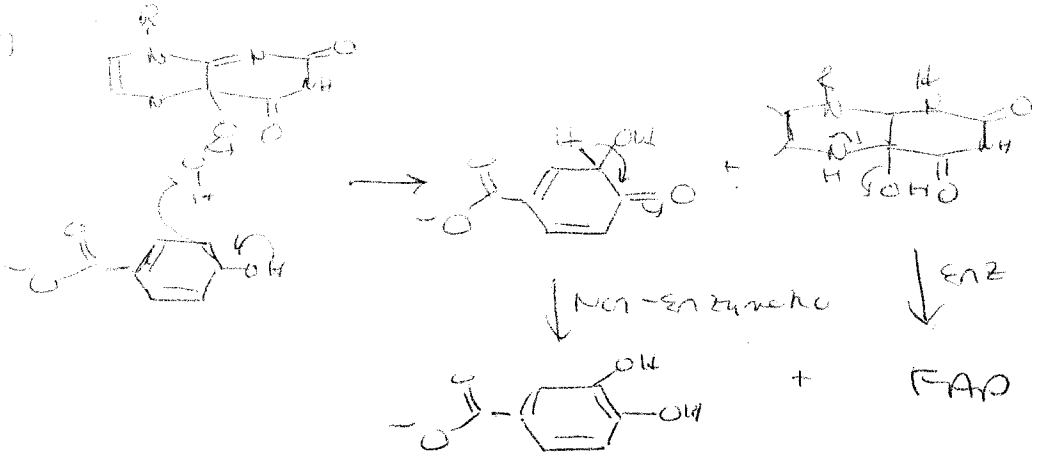
Rxn of 4a-OOH of flavin with substrate yields putative intermed II

Reoxidn of $FADH_2$ with O_2 through C4a-OOH. Two $1e^-$ transfers

Intermediate II has been controversial and involves the interesting chemistry, the actual hydroxylation process.

Possibility for Intermediate II 

two species

(c) 

Non-enzymatic Enz

+ FAD

The flavin moves quite extensively during this set of transformations

Flavin Oxidases: O_2 acts as an e^- acceptor to reoxidize flavin. NO O_2 in product, it is all in H_2O_2

Ex D-amino acid oxidase

Three different mechanisms have been proposed for oxidases. Despite a wealth of information, the mechanism still remains controversial.

(1) H^- transfer — NO

chemical GBC in active site

(2) $2, 1e^-$ transfers: Based on well characterized model chemistry ~ NO evidence for radical intermediates

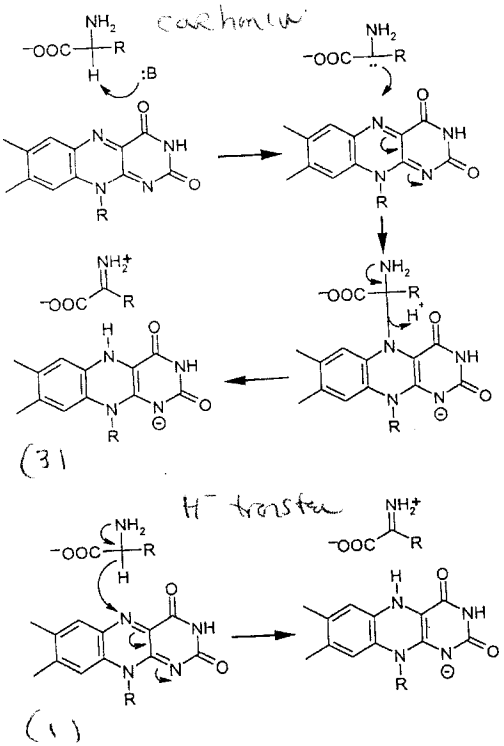
unlikely

(3) Amine substrate attacks at C4a and flavin acts as GBC. (avoids the problem that there is NO GBC in active site)

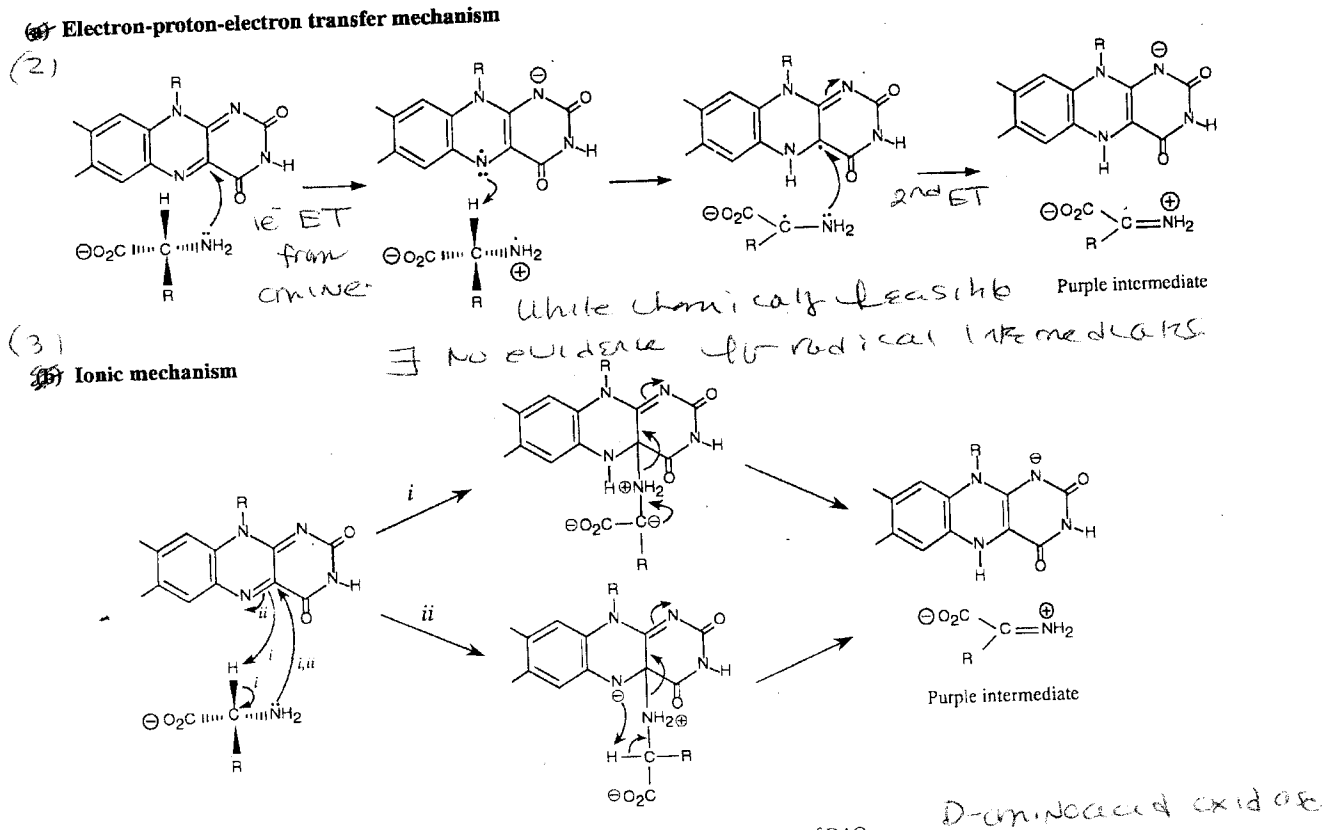
CMLS, Cell. Mol. Life Sci. Vol. 57, 2000

mech (1)

carbanion, H^- transfer



Scheme 2. Reaction mechanisms of DAAOs)(Carbanion mechanism;H) -CH hydride transfer mechanism.



Scheme 3. Mechanisms of reductive half reaction of DAO.

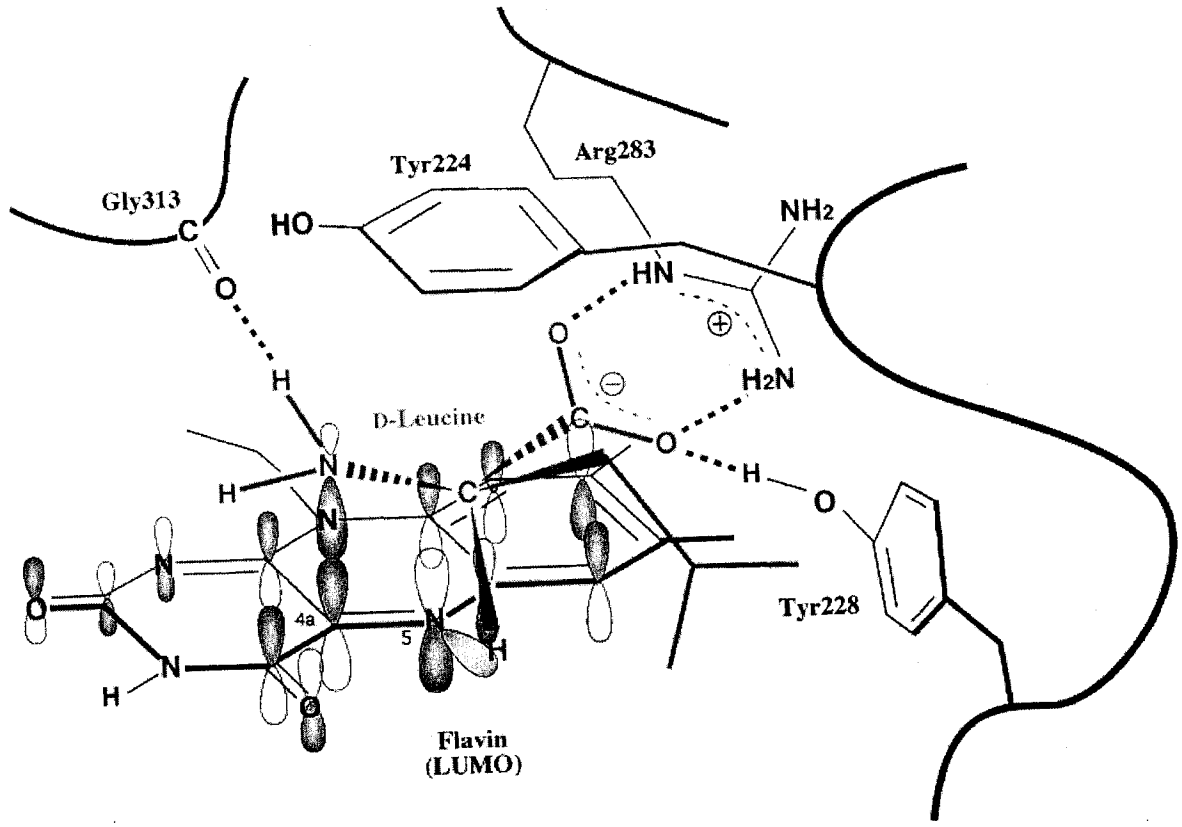


Fig. 6. d-Leucine (red) binding mode in DAO-d-leucine complex model. Note that the lone pair orbital (green) of the amino group of d-leucine overlaps with LUMO of flavin at C(4a) and that the α-hydrogen of d-leucine approaches the lone pair orbital (orange) of flavin N(5).

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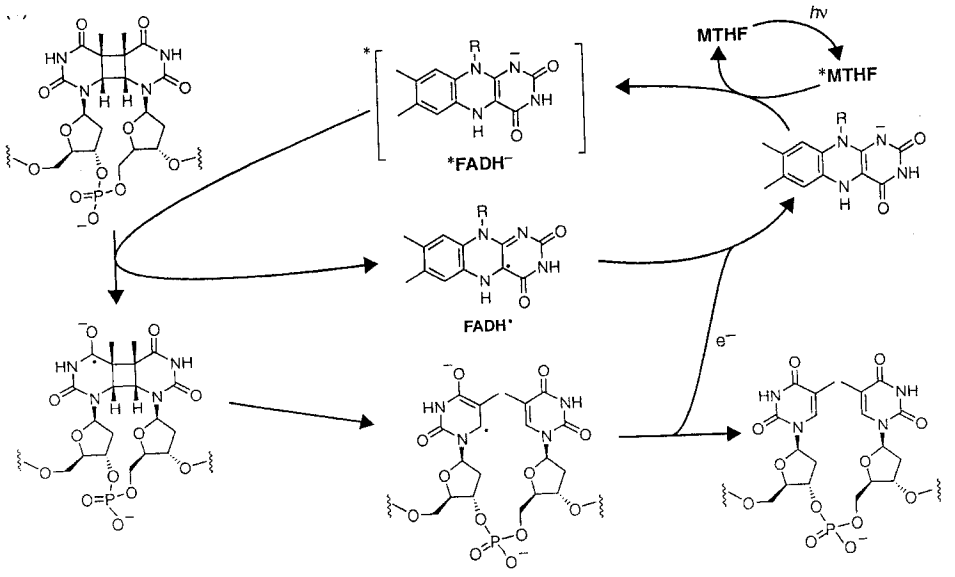

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