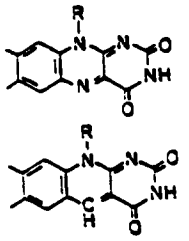
5-Deazaflavin Coenzymes : Darker and Lighter Sides



riboflavin

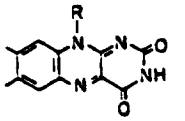
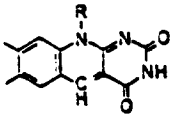
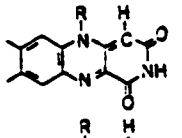
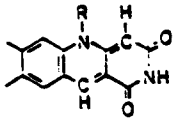
5-deazariboflavin

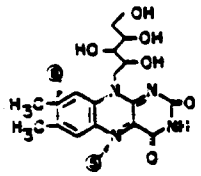
(A) Dark Reactions: Thermodynamically Activated (Lower Redox Potential) Hydride Donor to drive cosubstrate reductions for Biosynthetic transformations)

(B) Light Reactions λmax : 400-430nM (depending on substituents). Absorb photon of visible light ——> photo excited state, act either as photosensitizing pigment (to generate excited state of a second chromophore) or as strong one electron reducing agent

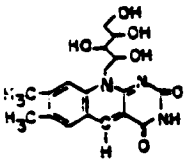
Both ground state chemistry (2e, hydride transfer) and excited state chemistry photoexcitation and/or 1e transfer) of 8-hydroxy-5-deazaflavin are exploited in specific biological contexts

(Carba) Deaza Analogs of Riboflavin

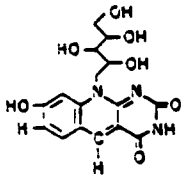
Structure	Species	$E^{\circ\prime}$	Coenzymatic competence
	riboflavin	-210 mV	$1e^{\ominus}$ and $2e^{\ominus}$
	5-deazariboflavin	-310mV	$2e^{\ominus}$ only
	1-deazariboflavin	-290mV	$1e^{\ominus}$ and $2e^{\ominus}$
	1,5-dideazariboflavin	-370mV	incompetent



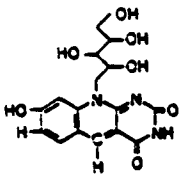
Riboflavin



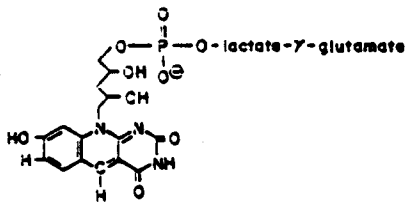
5-Deazariboflavin
(5-carba)



8-Hydroxy-7-demethyl-
5-deazariboflavin
(F_0)

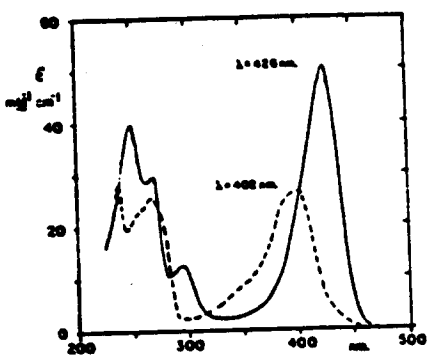


F_0



F_{420}

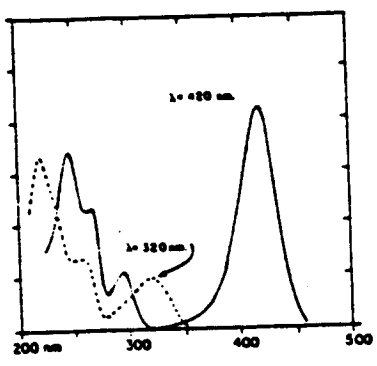
(up to 100mg/kg of methanogenic bacteria).



7-methyl-8-OH-5-deazariboflavin

Acid (pH=4.5)

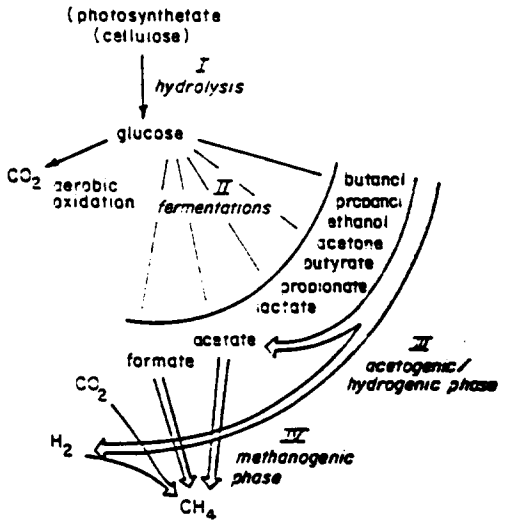
Base (pH=8.3)



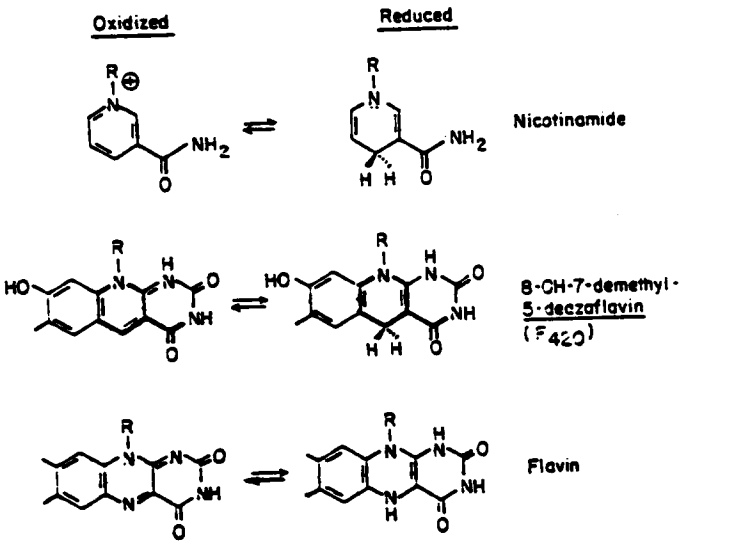
7-demethyl-8-OH-5-deazariboflavin

Oxidized (pH=8.3)

Reduced (BH$_4^-$)



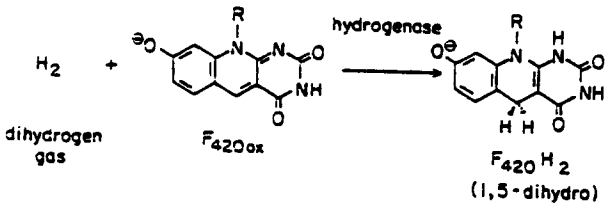
Anaerobic degradation of organic material in four consecutive phases.
(Vogels, 1979)



5-Deazaflavin - a hybrid between nicotinamide and flavin - an early progenitor ?

The Lighter Side of 3-Hydroxy-5-Deazaflavin Coenzymes

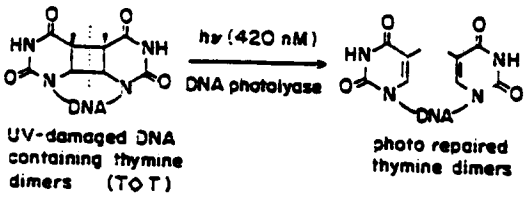
1) A Light Substrate: Hydrogenase (F_{420}-reducing)



Hydrogenase cofactors, tightly bound : Ni
Fe_4S_4
FAD

(nickel-based hydrogenation catalyst)

2) Light as Substrate: DNA Photolyase (F_{420}-containing)



UV-damaged DNA containing thymine dimers (T◇T)

photo repaired thymine dimers

DNA photolyase cofactors, tightly bound : F_{420}
$FADH_2$

(net retro 2+2 cyclobutane fragmentation)

F_{420}-reducing Hydrogenase (M. thermo)

α (47 KD)
β (31 KD)
γ (26 KD)

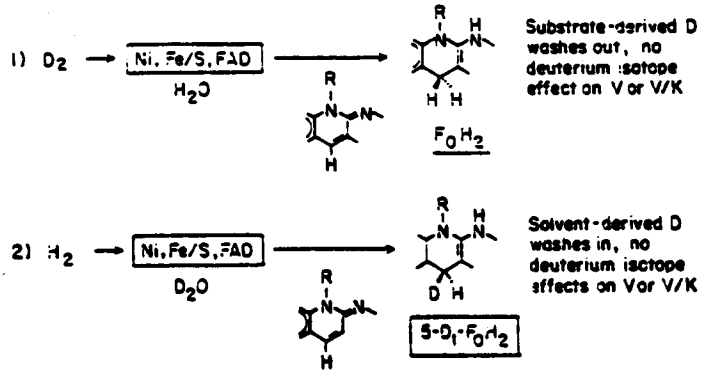
$\alpha_1\beta_1\gamma_1$ (104 KD) ⇌ $\alpha_8\beta_8\gamma_8$ (832 KD)

major active species, in vitro and in vivo (?)

per 104 KD

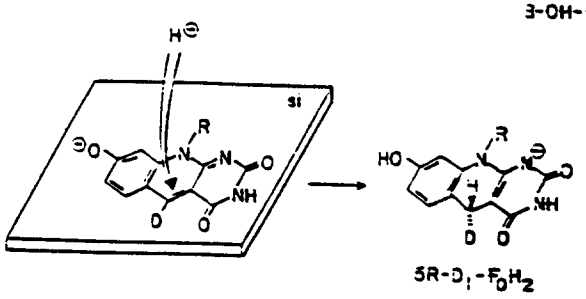
Nickel (1)
Iron/Sulfur (3 4Fe/4S clusters)
FAD (1)

Substrate and Solvent Hydrogen Transfers by the F_o-Reducing Hydrogenase



Yamazaki et al, PNAS 1985

M. Vannielli Hydrogenase $5\text{-}0\text{-}F_0 + H_2 \xrightarrow[H_2O]{} 5R\text{-}D_1\text{-}F_0H_2$ incoming $H^{\ominus}$ equivalent added to si face of 3-OH-5-deazaflavin



Redox Roles for Coenzyme F_{420} in Nonmethanogenic Organisms

A. Streptomycetes : Chlortetracycline Biosynthesis

→ 5a, 11a-dehydrochlortetracycline + $F_{420}H_2$

↓

Chlortetracycline + F_{420}

B. Streptomycetes / Cyanobacteria : Photoreversion of Cyclobutane-Containing Dimers in DNA

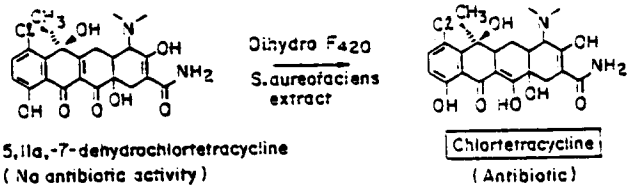
UV-damaged-DNA + bluegreen hν $\xrightarrow[\text{enzyme-}F_{420}]{\text{photorepair}}$ Native DNA

Non Methanogen Roles for 8-Hydroxy 5-Deazaflavins

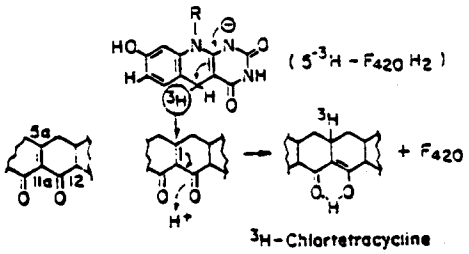
Streptomycetes:

Obligate Redox Cofactor in the Last Step of Chlortetracycline Biosynthesis

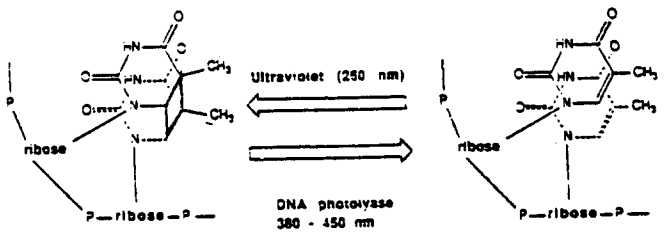
McCormick et al., JACS, 1960
1982



Possibility:



Photoreactivation by DNA Photolyase



cis-syn-intrachain thymine dimer

DNA photolyase: Photorepair of Pyrimidine-Dimer Lesions in DNA

Issues:

1. Availability

Low concentration in cells makes molecular characterization of enzyme difficult ⇒ clone, sequence, overexpress, purify to homogeneity in quantity.

2. Assay

Need sensitive assay for cyclobutane cleavage

(a) assay regain of ability of plasmid DNA to provide ampicillin resistance by photorepair of lesions in β-lactamase gene.

(b) Use gel shift assay with purified enzyme to monitor complexation with a T ◇ T-containing oligonucleotide of specific length and sequence.

Fold Purification Required to Homogeneity

	Fold	Cloning/Overpopulation
Anacystis DNA Photolyase	70,000X	Walsh; Yasui
Streptomycetes DNA Photolyase	20,000X	
Methanobacterial DNA Photolyase	6,000X	
Eco Photolyase	15 molecules/cell	Sancars

⇒ need to clone gene, over produce enzyme

E. coli DNA Photolyase

MW ~50,000

Catalytic turnover number: 2.4 min^{-1}

Substrate: T◇T-containing DNA, minimal size a 4 mer

λ_{max} for action spectrum: 380nM

Quantum yield: 0.5 → 1.0

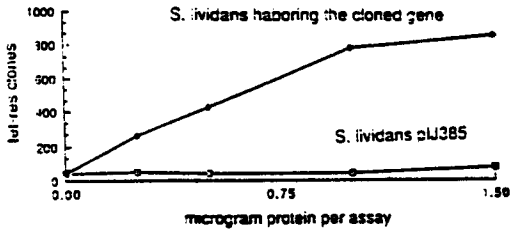
Discrimination ratio: $10^6/1$

To Study the F420 (deazaflavin)-containing DNA Photolyases for Catalyst Structure and Function

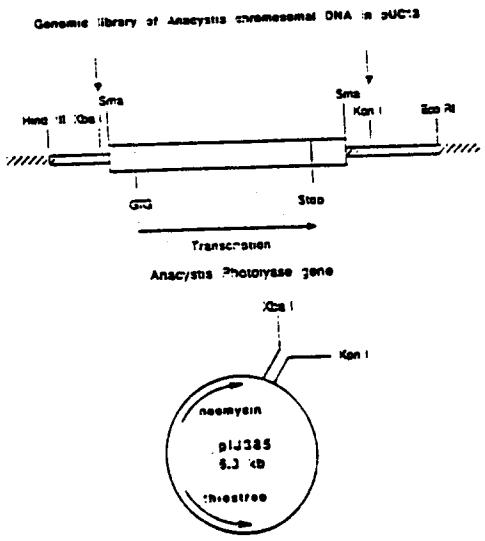
1. Purify a trace amount of photolyase (e.g. 20,000 fold from Anacystis nidulans) and determine N-terminal sequence.
2. Make complementary oligonucleotide, clone from a genomic DNA library, determine DNA sequence.
3. Express gene and overproduce enzyme in a usable heterologous host (e.g. Anacystic gene in Streptomyces lividans).
4. Purify to homogeneity (heparin-sepharose, T◇T-DNA-cellulose) and characterize.

A.M. Eker (Holland); A. Yasui (Japan);
J. Piret (Boston); A. Kiener, C. Walsh (Boston)

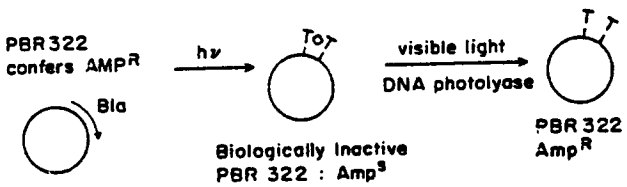
Expression of *Anacystis* DNA Photolyase gene in *Streptomyces lividans*



Cloning of Anacystis DNA Photolyase in Streptomyces



Assay



Assay regain of transformation activity: score for Amp^R colonies.

Assay sensitivity: nanograms DNA photolyase activity, suitable for detection of low levels of enzyme in crude extracts

Assay for Photolyase activity

A) **Transformation assay:**

Based on transformation efficiency of UV irradiated plasmid DNA carrying antibiotic resistance gene. (number of tet^r clones per µg plasmid DNA)

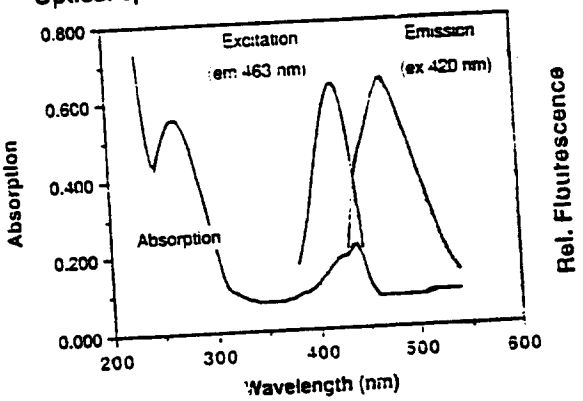
0.5 µg pBR322 in 50 µl buffer
↓
add cell extract
↓
illuminate (dark control)
↓
transform *E.coli* CSR 603 (recA, phl⁻)
↓
score for tet^r clones

Sensitivity: 1 ng enzyme in crude extract

Purification of *Mb. thermoautotrophicum* DNA Photolyase

Fraction	Volume ml	Protein mg	Number of tet^r clones formed by 1µg protein	Total number of transformants	Yield %	Purification factor
cell free extract after 100.000*g spin	200	2400	160	$3.8*10^8$	100	1
Hecann agarose	160	40	8300	$3.3*10^8$	87	52
oligo-dT cellulose	150	35	8300	$3.0*10^3$	69	52
UV irradiated ss-DNA agarose	2	50 µg	$1*10^6$	$5.0*10^7$	17	6250

Optical spectra of methanogen DNA photolyase



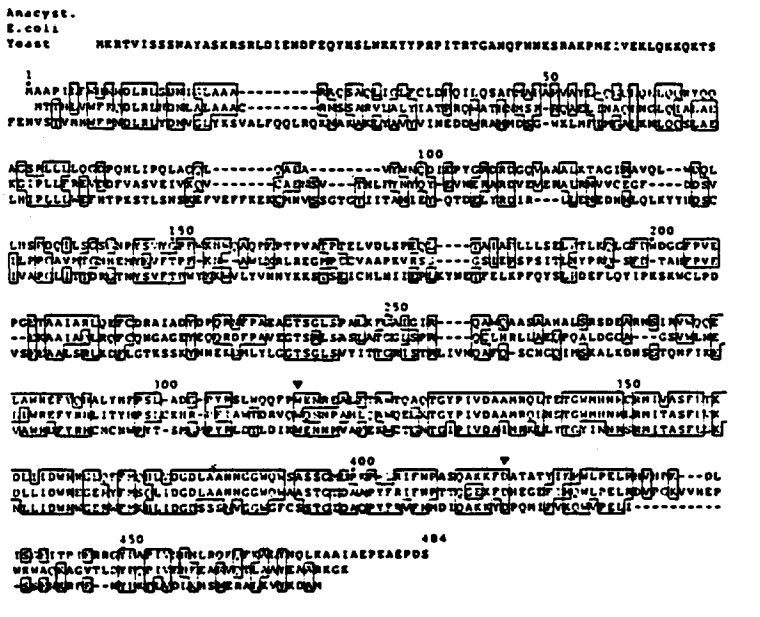
Two Types of DNA Photolyases

- Based on λmax of photoreversion action spectrum
- Based on cofactor content

Type 1	Type 2
E. coli, yeast	cyanobacteria, streptomycetes, green alga, methanobacteria
λmax for photorepair: 380 nM	λmax for photorepair: 430 nM
cofactor content:	cofactor content:
(a) one 5,10-CH—tetrahydrofolate (λmax = 380 nM)	(a) one 8-hydroxy-5-deazaflavin (λmax = 420 nM)
(b) one $FADH_2$	(b) one $FADH_2$
(A. Sancar, G. Sancar)	(A. Eker, A. Yasui, C. Walsh)

Note $FADH_2$ is in autoxidizable dihydro oxidation state, leuko form, unlikely to be light harvesting chromophore

Anacystis nidulans DNA Photolyase Protein sequence



Some Mechanistic Questions

What features of T◇T-containing DNA substrate are recognized?

What is mechanism of DNA photolyase (photosensitized cleavage?)

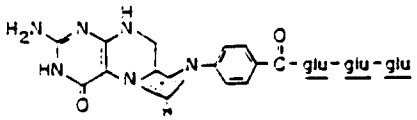
and What are the roles of the bound cofactors?

Nature of Interaction of DNA Photolyase with T◇T-containing DNA

- specific for cys-syn T◇T, does not repair trans-syn T◇T
- recognizes the bent or kinked shape of T◇T adducts (ca 29° bend / dimer)
- footprinting (by DNAse) of photolyase - T◇T DNA complexes ⇒ ~4bp recognition site

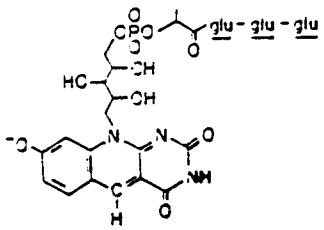
Light Harvesting Chromophores in DNA Photolyases

Type 1 (380 nM)



N-5,10-methenyl H_4 pteroyltriglutamate

Type 2 (430 nM)



F_{420} triglutamate

Hypothesis - a major binding determinant provided by the oligoglutamyl side chain

Observation (A.Yasui, Tohoku, Japan)

express type 2 gene (Anacystis nidulans) in type 1 host (E.coli)

find the A.nidulans DNA photolyase now has a 380 nM action spectrum

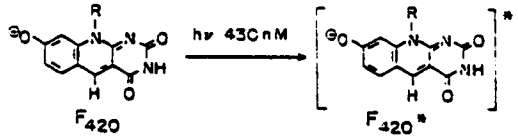
⇒ replacement of F_{420} cofactor (not made in E.coli) by folate cofactor to reconstitute an active DNA photolyase

Mechanistic Proposal for DNA Photolyases (Type 1 and Type 2)

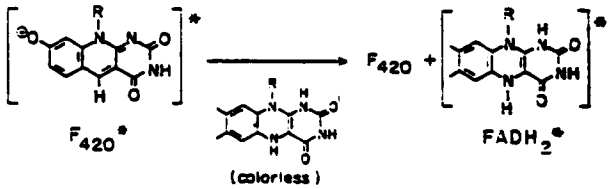
1. Absorption of visible light by bound cofactor (Pterin or deazaflavin) acting as a light harvesting, photosensitizing pigment.
2. Energy Transfer From Excited State of Chromophore to $FADH_2$
3. Reaction of $FADH_2^*$ as one electron transfer agent, directly or via a tryptophanyl side chain, to yield $FADH^\bullet$ and Thymine Dimer radical anion
4. Cleavage of Pyrimidine dimer radical anion to T, T• and $FADH^\bullet$, followed by radical recombination to T.T. $FADH_2$-enz.

Mechanistic Proposal for F_{420}- and $FADH_2$-Containing DNA Photolyase

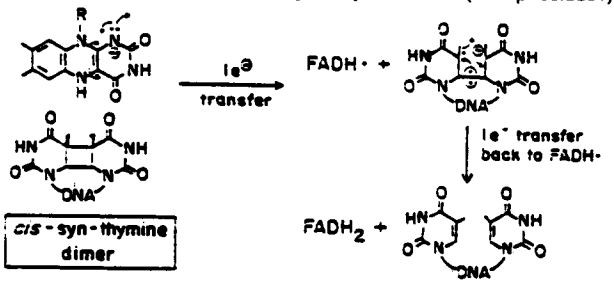
a) Light absorption

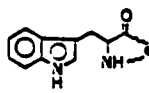


b) Energy transfer from F_{420}* to bound $FADH_2$



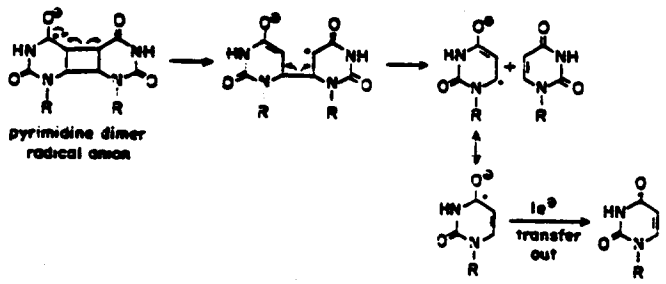
c) One electron transfer from $FADH_2$* to thymine dimer (via trp residue?)



d) $FADH_2$* +  ⇌ FADH· + 

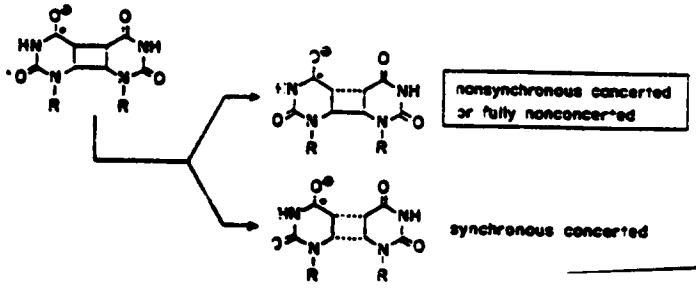
radical fragmentation of TOT dimer

Cleavage of Pyrimidine Dimer Radical Anions



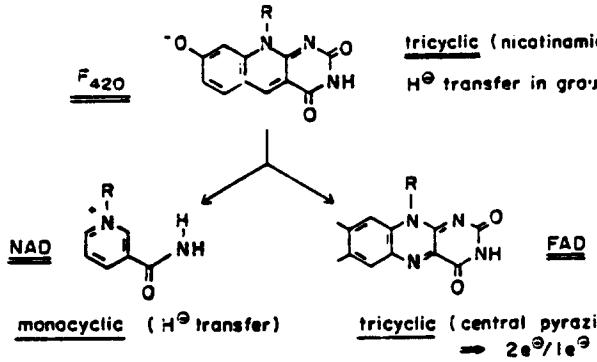
"one electron reduction of pyrimidine dimers significantly decreases the activation energy for splitting via nonsynchronous concerted or fully nonconcerted (i.e. stepwise) pathways but not via a fully concerted pathway"

Hartman et al., J. Org. Chem. 52, 2684, (1987)



Some Divergent Thoughts on Redox Coenzyme Evolution

a) F_{420} (5-deazaflavins) as precursor to NAD and flavins



b) F_{420} as an early light harvesting system (DNA photolyase). Replaced by 5,10-CH -Folate H_4 later in evolution.
Chromophores for DNA photolyase (oligoglutamyl species)