

Proteomics and Mass Spectrometry (BMG 744)

Mitochondrial Proteomics

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Mitochondria function

“Classical”

- ATP
- Heme & porphyrin synthesis
- Urea cycle
- β -oxidation of fatty acids
- $\text{H}_2\text{O}_2 \rightarrow$ Oxidative Damage

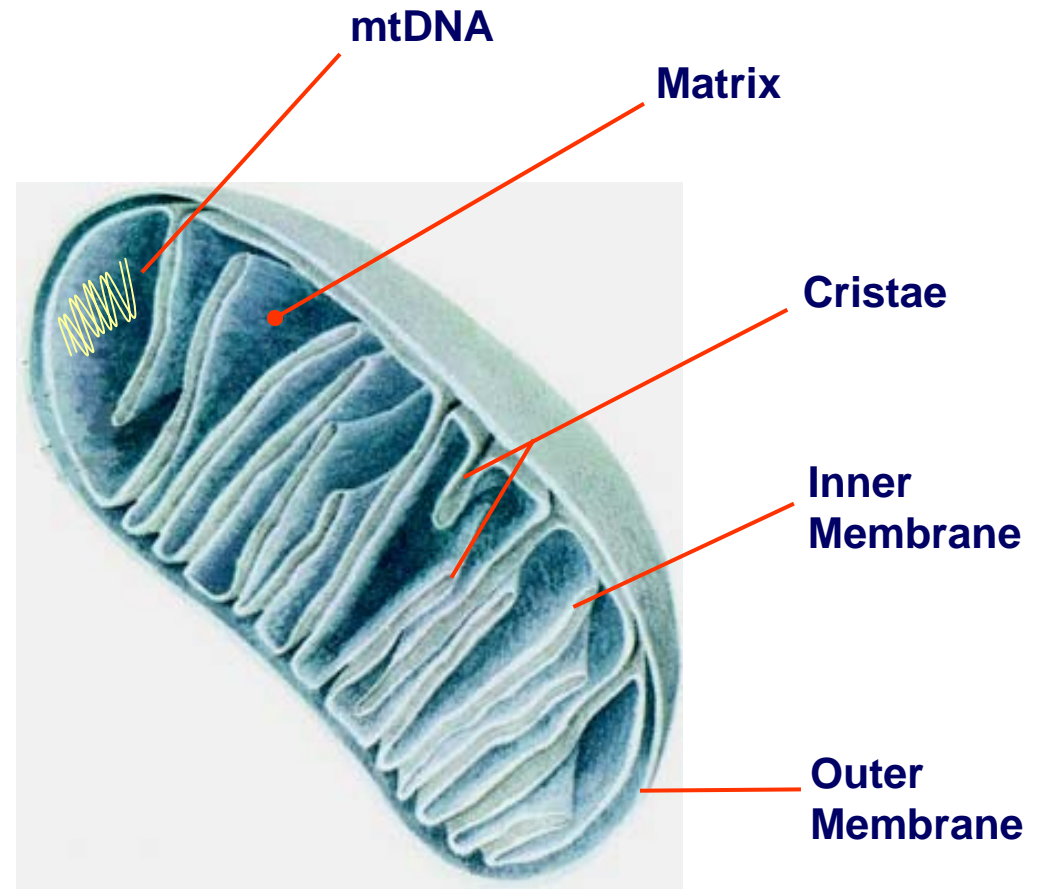
“Novel”

- Generation of $\text{H}_2\text{O}_2 \rightarrow$ redox cell signaling
- NO-cytochrome c oxidase signaling pathway
- Necrosis & Apoptosis

Mitochondria Dysfunction Leads to Disease

- Diabetes
- Ischemia/Reperfusion Injury
- Major neurodegenerative diseases – Parkinson's, Alzheimer's, ALS, Multiple Sclerosis, Huntington's
- Cardiomyopathy
- Sepsis
- Cancer
- Alcohol-induced liver disease & other tissues
- Aging

Mammalian mitochondrion



Outer membrane - quite permeable; contains pores, which allow diffusion of molecules <1000 molecular weight

Inner membrane - invaginations called cristae; site of oxidative phosphorylation system
- metabolite transport across membrane - specific carriers

Matrix - contains enzymes of tricarboxylic (Kreb's) cycle, enzymes for fatty acid oxidation, some enzymes for amino acid oxidation; these enzymes - involved in energy metabolism; mtDNA and mtRibosomes

Nature. 1981 Apr 9;290(5806):457-65.

Sequence and organization of the human mitochondrial genome.

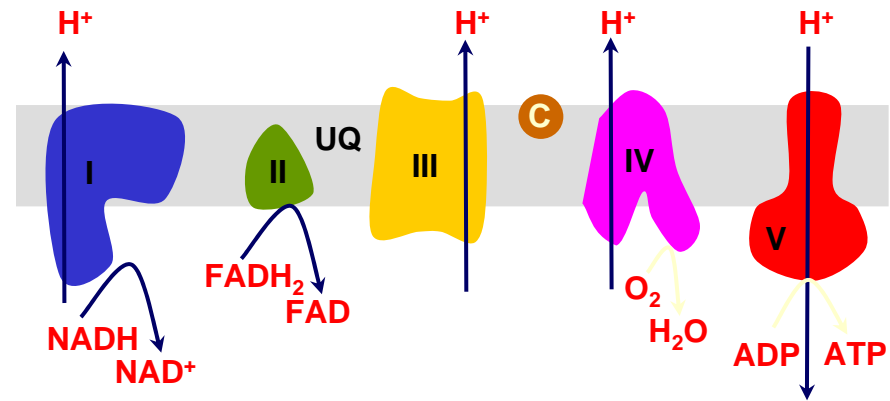
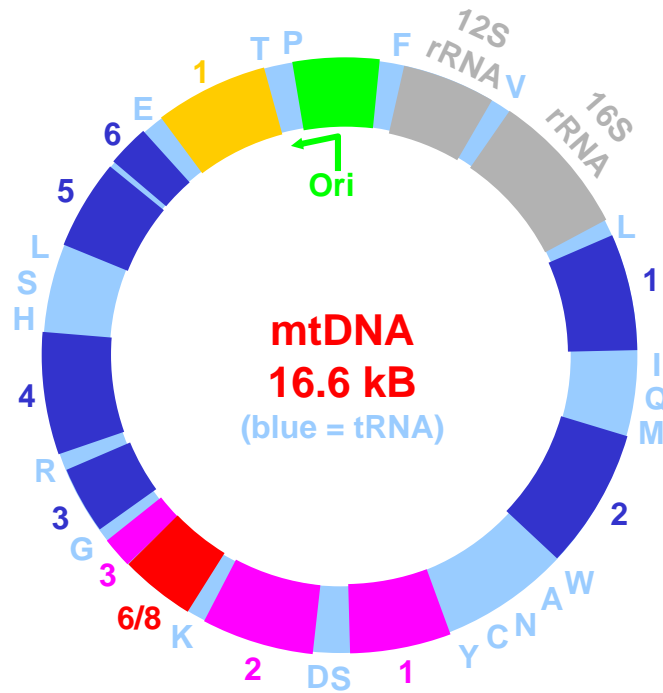
Anderson S, Bankier AT, Barrell BG, de Bruijn MH, Coulson AR, Drouin J, Eperon IC, Nierlich DP, Roe BA, Sanger F, Schreier PH, Smith AJ, Staden R, Young IG.



**12S, 16S rRNAs
22 tRNAs
COX I, II, and III
ATPase 6 and 8
Cytochrome b
7 complex I subunits**

Respiratory Complexes are Coded by Nuclear and Mitochondrial DNA

www.mitomap.org



- 10-12 copies per mitochondrion
- In the matrix (bound to the IMM)
- Maternally inherited
- Virtually “intron-less”, 93% coding

13 polypeptides:
 7 subunits of Complex I
 3 subunits of Complex IV
 2 subunits F₀ ATP synthase
 Cytochrome b

mtDNA Mutations → Disease

Point mutations of tRNAs or OXPHOS genes

Maternally inherited

Decreased OXPHOS activity, pyruvate & fatty acids accumulate,
leading to lactate acidosis and accumulation of TGs

Rate of ATP synthesis is decreased – muscle weakness and exercise intolerance

- **Leber's Hereditary Optic Neuropathy (LHON)**

Single base change in genes encoding 3 complex I subunits (ND1, 4, 6) resulting in decreased complex I activity

- **Mitochondrial Encephalomyopathy, Lactic Acidosis, Stroke (MELAS)** – mutation tRNA for leucine

- **Myoclonic Epilepsy and Ragged Red Fibers (MERRF)**
mutation in tRNA for lysine

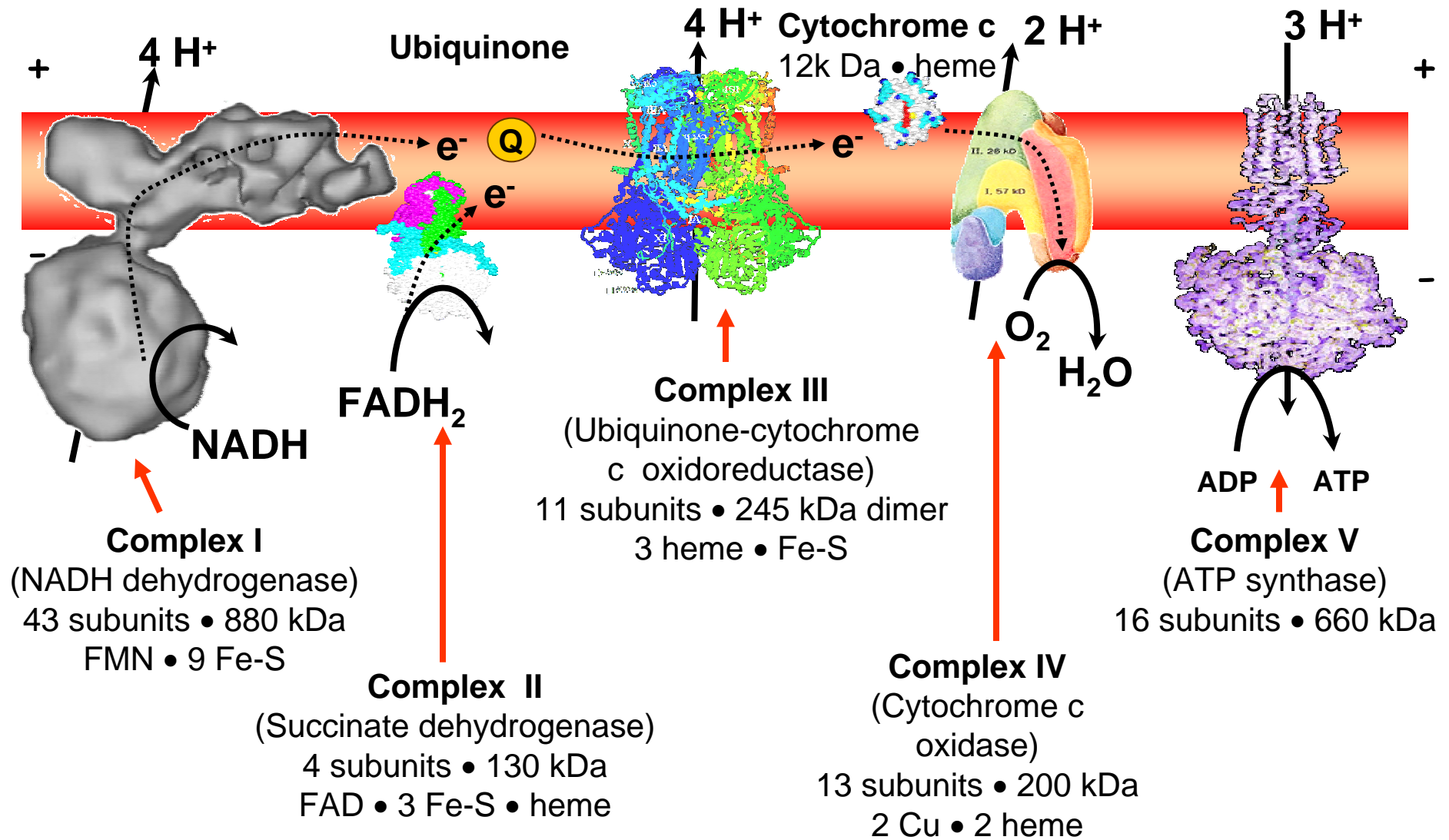
- **Kearn's-Sayre Syndrome (KSS)**

- **Neuropathy Ataxia Retinitis Pigmentosa (NARP)**

- **Hypertrophic Cardiomyopathy**

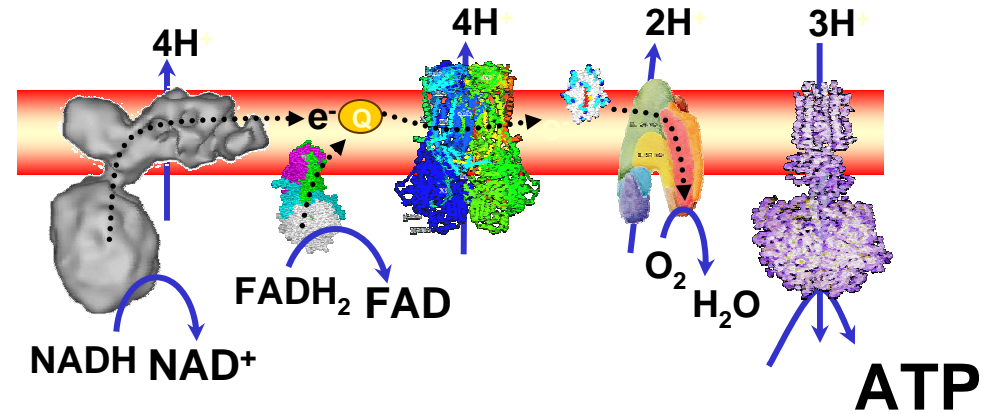
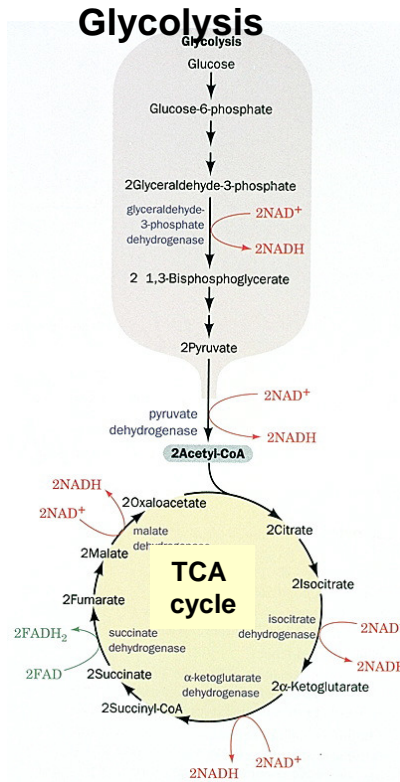
- **Leigh's Syndrome**

Organization of Oxidative Phosphorylation Complexes



Courtesy of PS Brookes

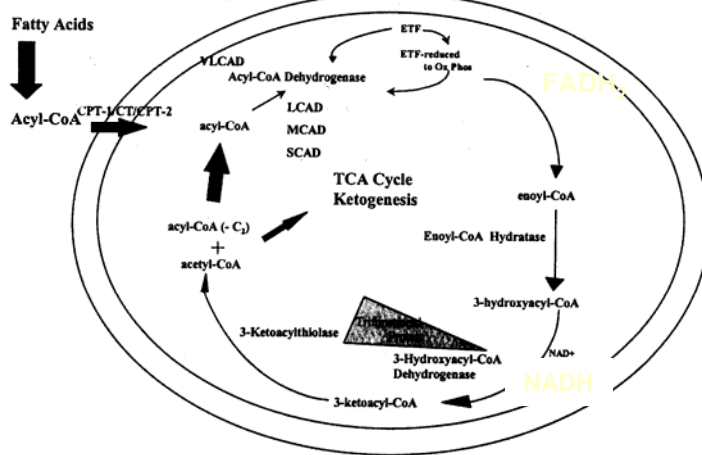
Glycolysis, TCA cycle, and β -oxidation of fatty acids make NADH & FADH₂ (reducing equivalents)



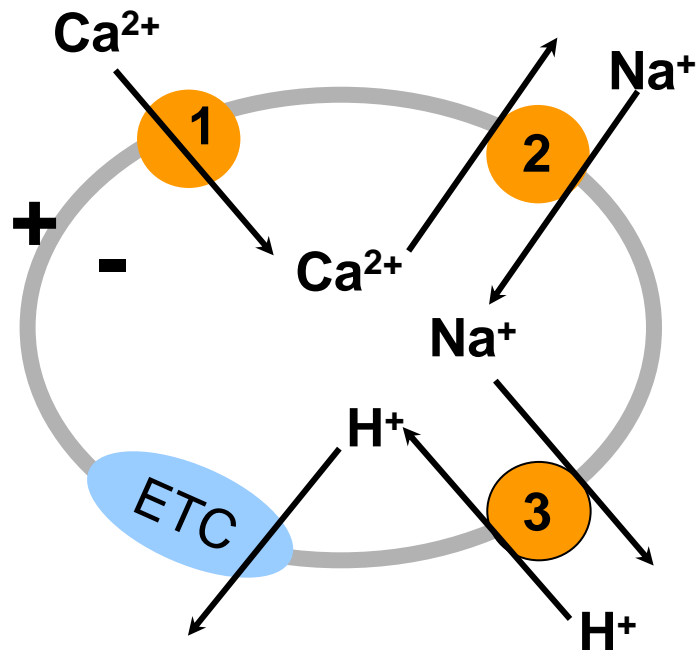
“Oxidative Phosphorylation”

1. Electrons from dehydrogenase's are transported to NADH and FADH₂
2. These electrons are then passed into the electron transport chain, where through the reoxidation of NADH and FADH₂, they participate in several sequential oxidation-reduction reactions before reducing O₂ to H₂O.
3. In this process H⁺ are pumped across the inner mitochondrial membrane
4. The free energy stored in the resulting electrochemical gradient that drives the synthesis of ATP from ADP + P_i via Oxidative Phosphorylation.

Mitochondrial Fatty Acid Oxidation



Mitochondrial Ca^{2+} handling



1. Ca^{2+} uptake transporter
Sensitive to cytosolic $[\text{Ca}^{2+}]$
Essentially non-saturable
2. $\text{Na}^{+}/\text{Ca}^{2+}$ exchanger
Always works at V_{max}
3. $\text{Na}^{+}/\text{H}^{+}$ exchanger
Always works at V_{max}

- Δ cytosolic $[\text{Ca}^{2+}]$ mirrored by Δ matrix $[\text{Ca}^{2+}]$
- Control of ATP synthesis – Ca^{2+} activates Krebs' cycle
Increase NADH supply to respiratory chain
Increase ATP generation

Mitochondria and Calcium Toxicity

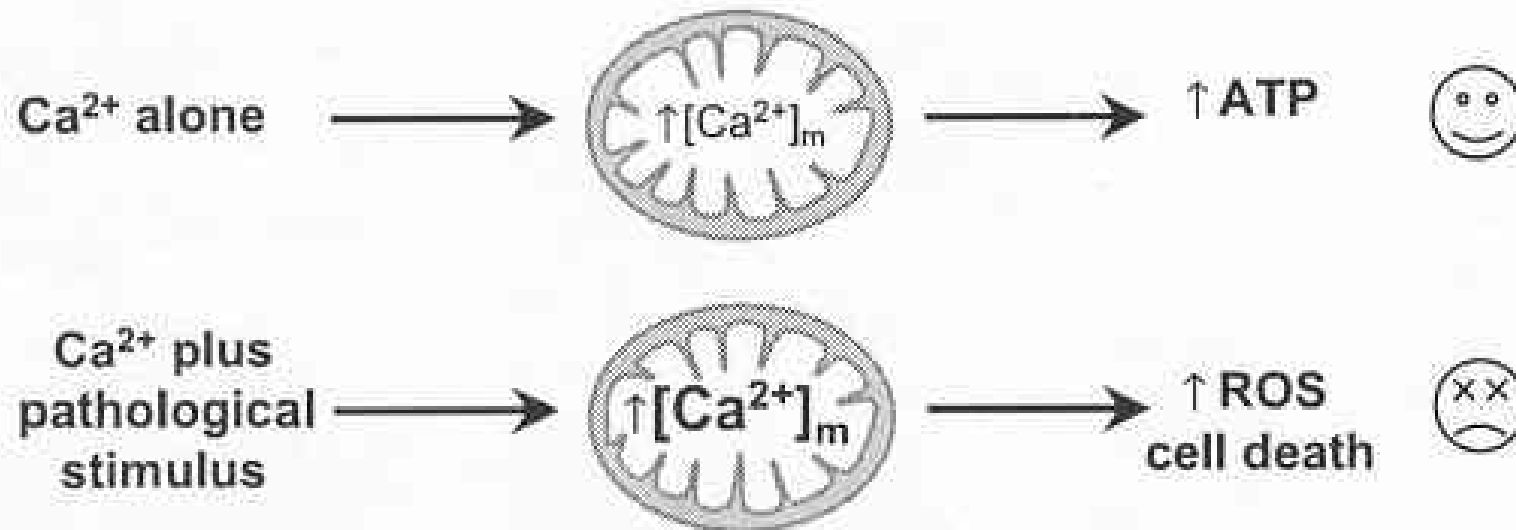
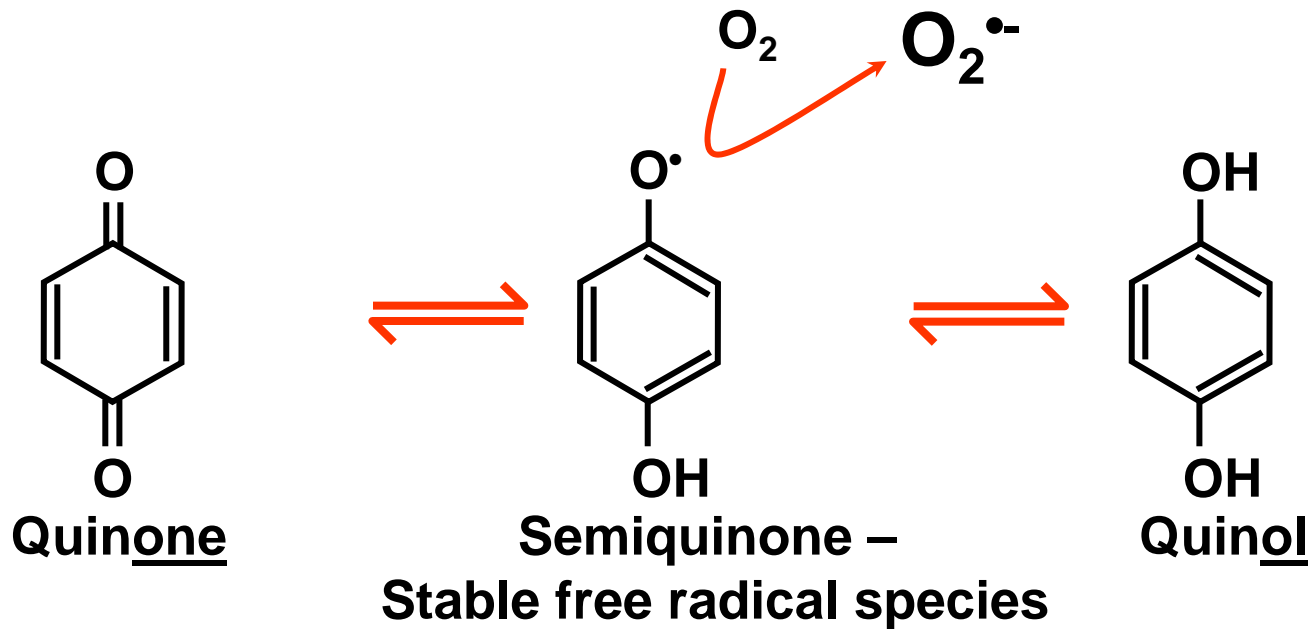
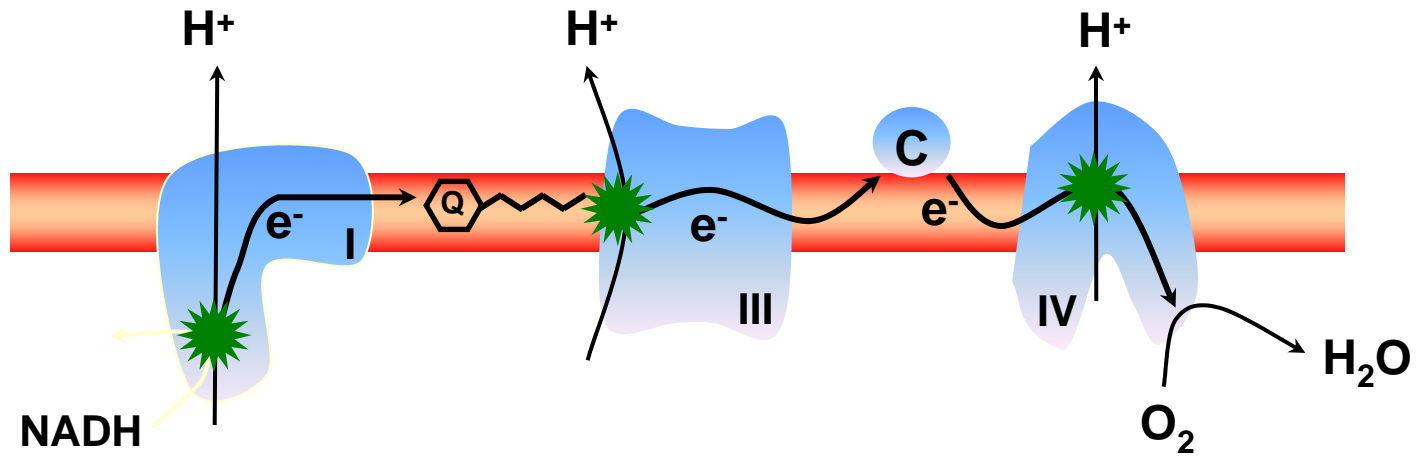


Fig. 1. Two-hit hypothesis for mitochondrial Ca^{2+} in physiology and pathology. Under physiological conditions, Ca^{2+} is beneficial for mitochondrial function. However, in the presence of an overriding pathological stimulus, Ca^{2+} is detrimental. Similarly, Ca^{2+} can potentiate a subthreshold pathological stimulus, resulting in pathogenic consequences. See text for full explanation. $[\text{Ca}^{2+}]_m$, mitochondrial matrix Ca^{2+} concentration; ROS, reactive oxygen species.

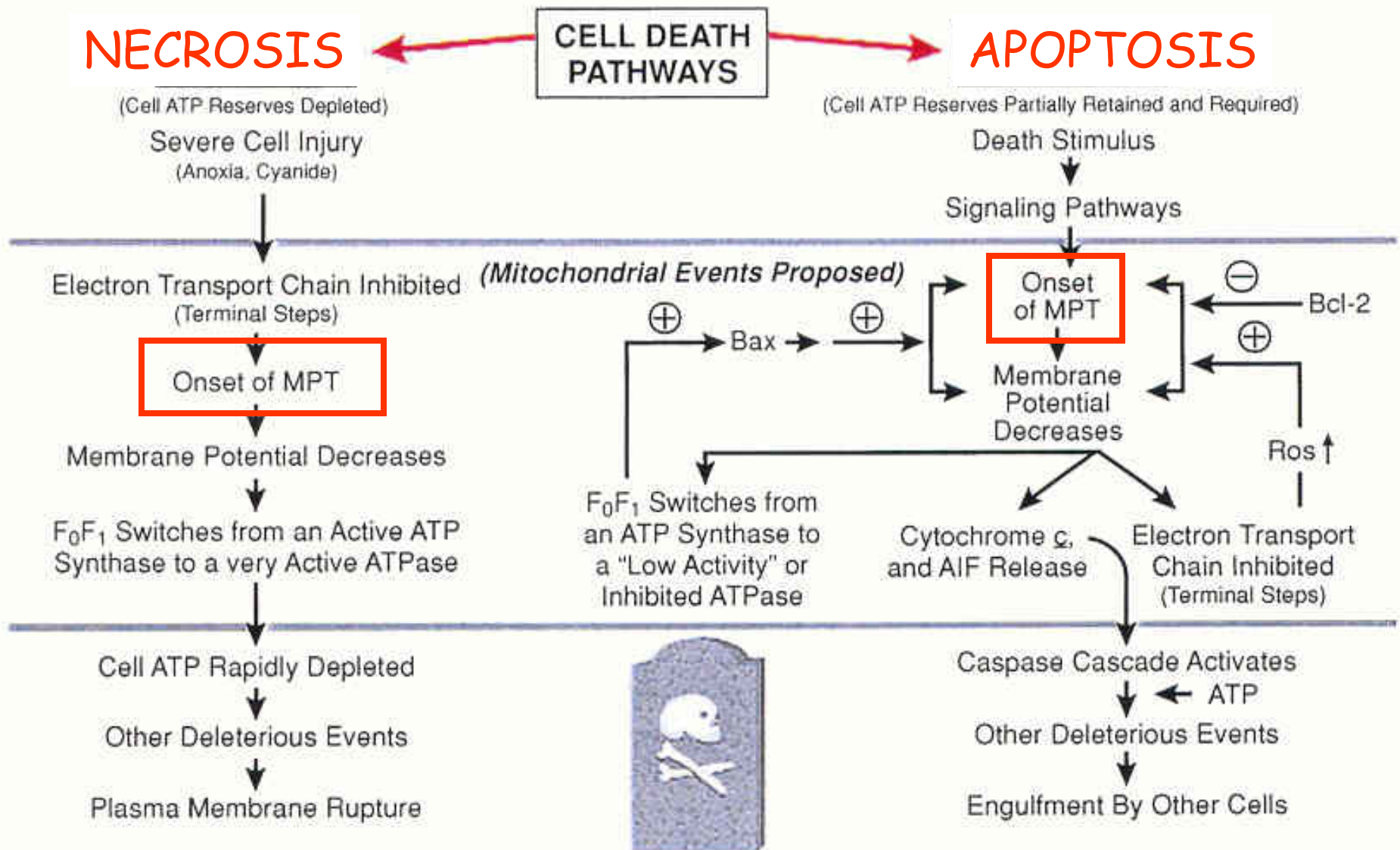
Mitochondrial sites of reactive oxygen species production

- < 5% electrons "leak" from etc to form $O_2^{\bullet-}$
- ROS damage proteins, lipids, and mtDNA
- Complex I - NADH dehydrogenase
- Complex III - Ubiquinone cytochrome c reductase
- Presence of stable **semiquinone intermediate** in enzyme complex

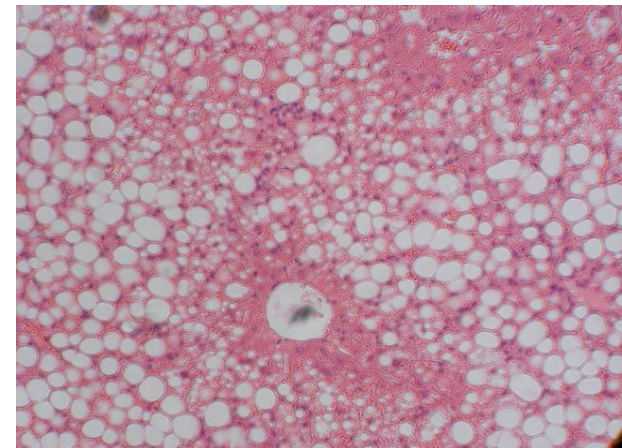
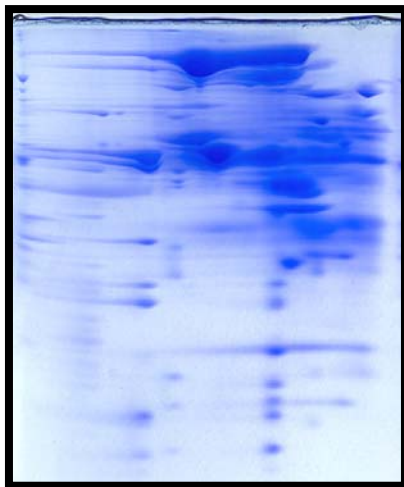
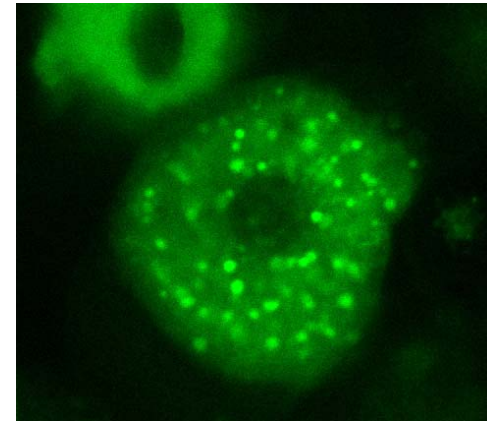
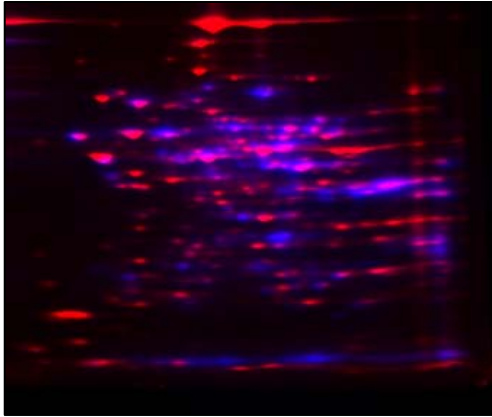
Mitochondrial $O_2^{\bullet-}$ Generation – Complex I and III



B) Mitochondrial Events During Cell Death



Mitochondria Function & Cell Viability & Pathology: Linked to alterations in Mitochondria Proteome



How to study the mitochondrial proteome?

Separation Techniques

2D IEF/SDS-PAGE – hydrophilic (matrix) proteins

Affinity fractionation (Lopez and Kristal)

- calcium binding proteins, glycosylated proteins, hydrophobic proteins

Sucrose density gradient centrifugation (Capaldi)

- separate intact protein complexes via sucrose gradient fractionation

Gel filtration (Mootha)

- size separation using gel filtration chromatography into 15-20 fraction, digested, and analyzed via LC-MS/MS

Immunocapture (Capaldi)

- monoclonal antibodies against complexes (complex I, ATP synthase, PDH)

BN-PAGE (Schagger & von Jagow)

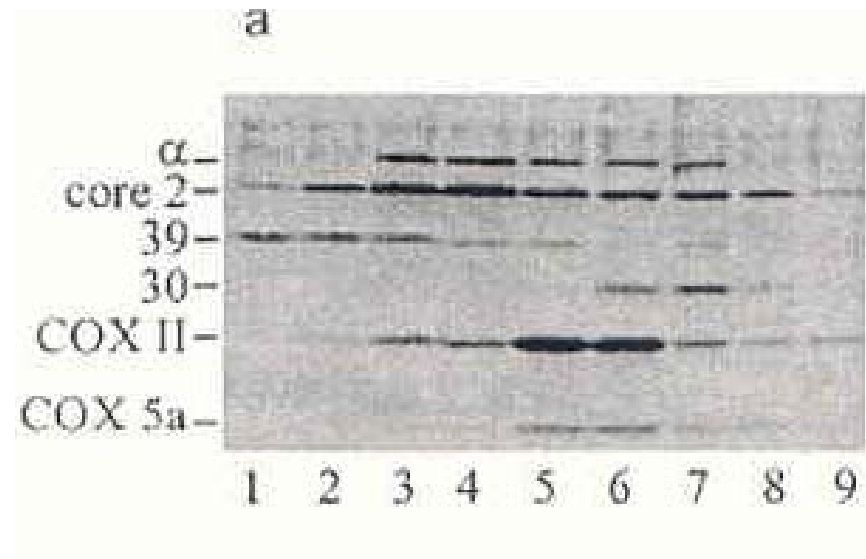
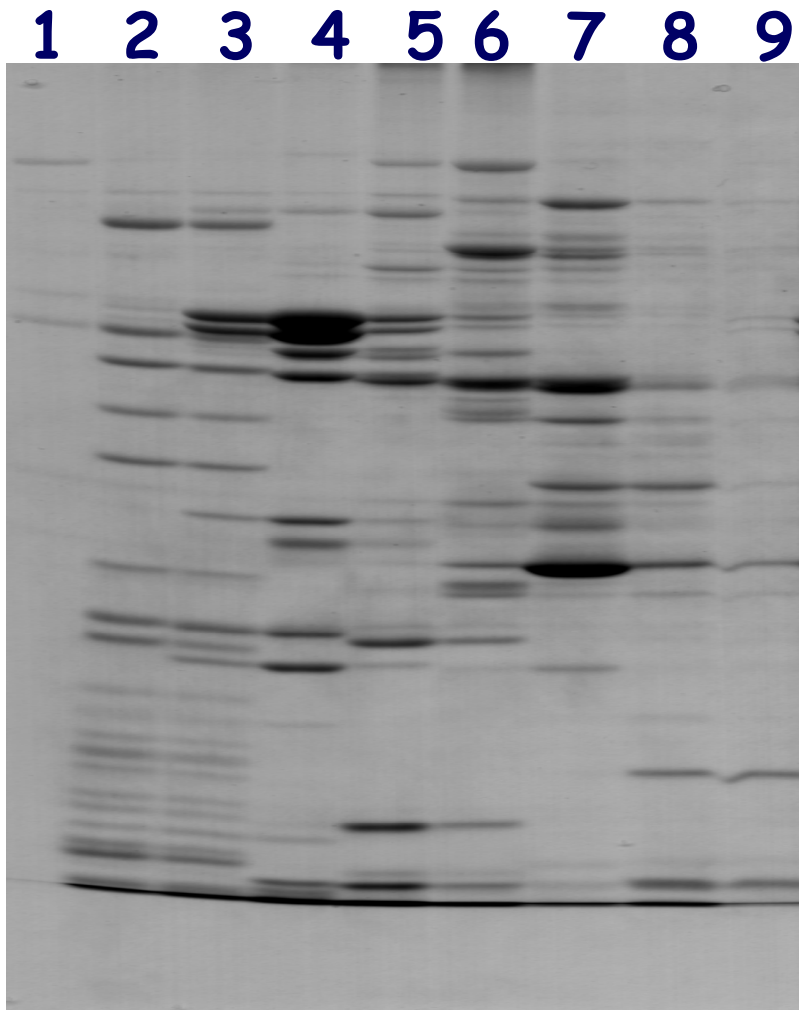
- separate OXPHOS complexes intact under non-denaturing conditions (1-D) followed by denaturing conditions to separate individual polypeptides (2-D)

Sucrose density gradient centrifugation (Capaldi)

- Mitochondrial extracts (1% LM) loaded onto 10-35% step fraction sucrose gradient, centrifuged overnight, fractions collected from bottom of tube
- Large protein complexes (complex I) found in higher density sucrose fractions, whereas free proteins found in lighter sucrose fractions
- Protein fractions analyzed via 1-D or 2-D electrophoresis
- MALDI and LC/MS/MS identified 615 distinct proteins, 19% previously undefined.

Taylor et al Nature Biotech 21. 281-6 (2003)

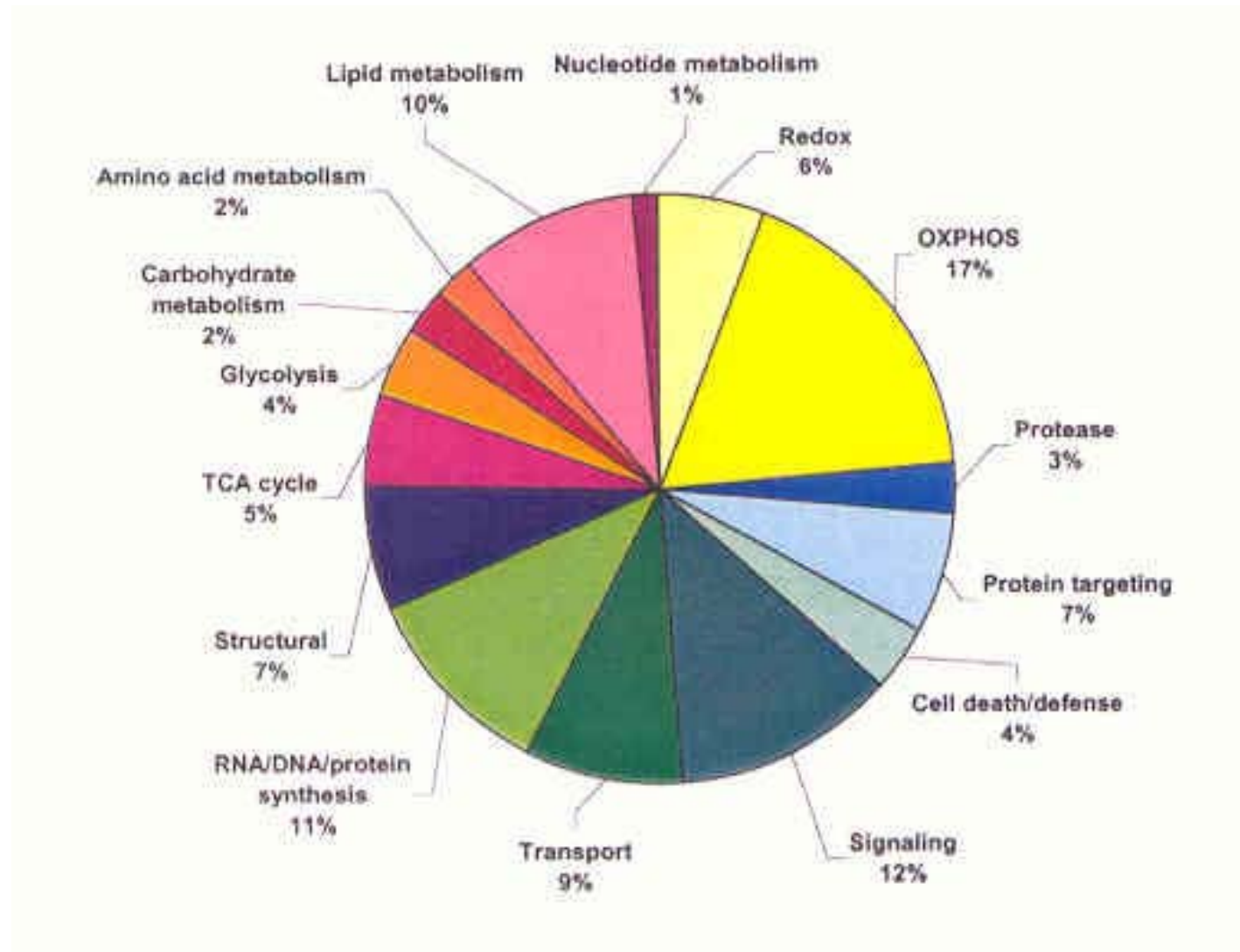
Size dependent fractionation of mitochondrial complexes by sucrose gradient



**Bovine heart
9 fractions**

Mitochondria Proteome

Taylor et al Nature Biotech 21. 281-6 (2003)



Immunocapture (Capaldi) - rapid, small amts. tissue

Mitochondria treated with 1% LM

Extract incubated (10 mg) with monoclonal ND6 antibody-crosslinked to protein G-agarose beads, beads washed, immunocaptured complex I eluted, and run on 1-D and 2-D gels
MALDI and LC MS/MS to identify proteins

29 protein bands

Using MALDI
& LC MS/MS -
Identified
42 of 45 proteins

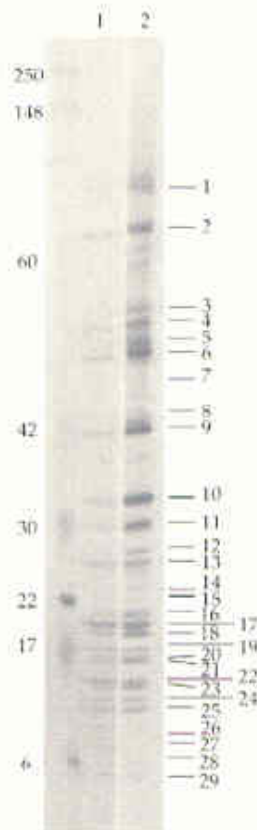
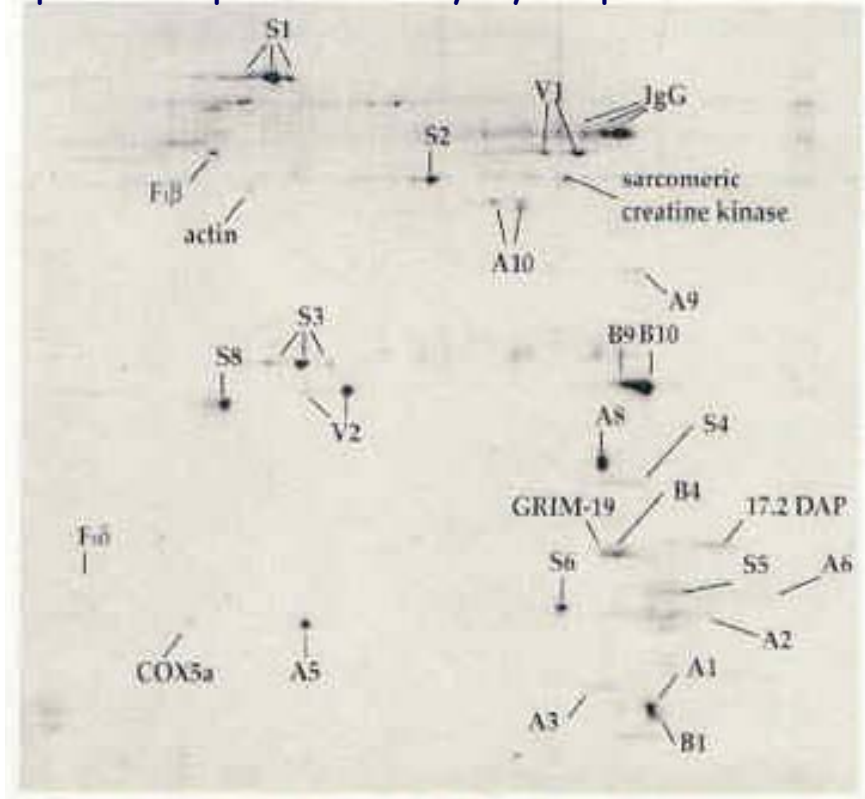


FIG. 1. 10 μ g of immunopurified human heart complex I separated by 10–20% acrylamide SDS-PAGE. Lanes were stained with Coomassie Brilliant Blue (lane 1) or a mass spectrometry compatible silver nitrate staining procedure (lane 2).

Murray et al JBC 278:13619, 2003

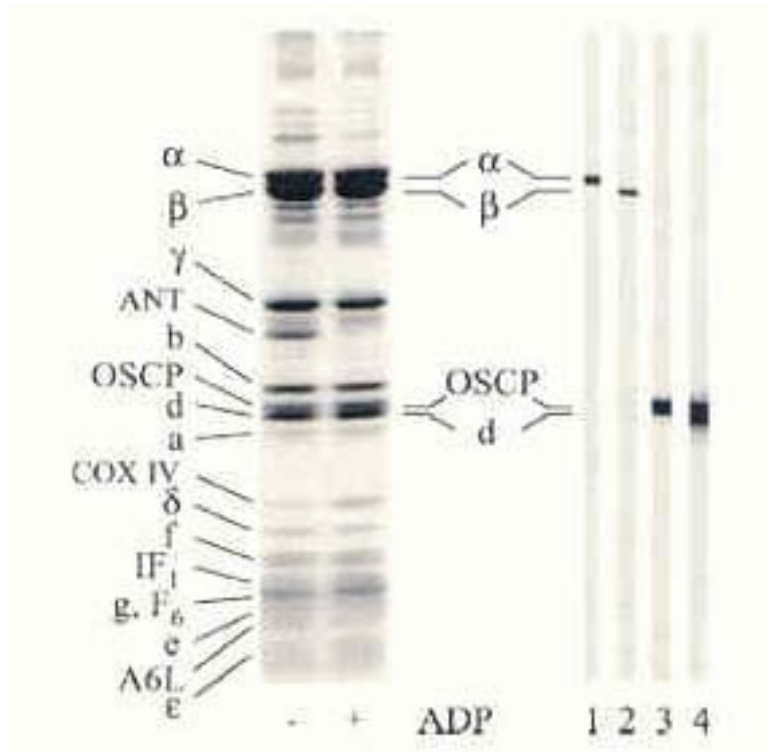
21 protein spots - mostly hydrophilic subunits



Immunopurified human heart complex I
By 2-D IEF/SDS-PAGE - 10 ug, 1-D linear
pH 3-10 strip, 2-D 15% gel (Sypro ruby),
MALDI

Immunocapture - ATP synthase

Human Heart - 16 subunits



Immunocaptured ATP synthase is active

Aggeler et al. JBC 277:33906, 2002

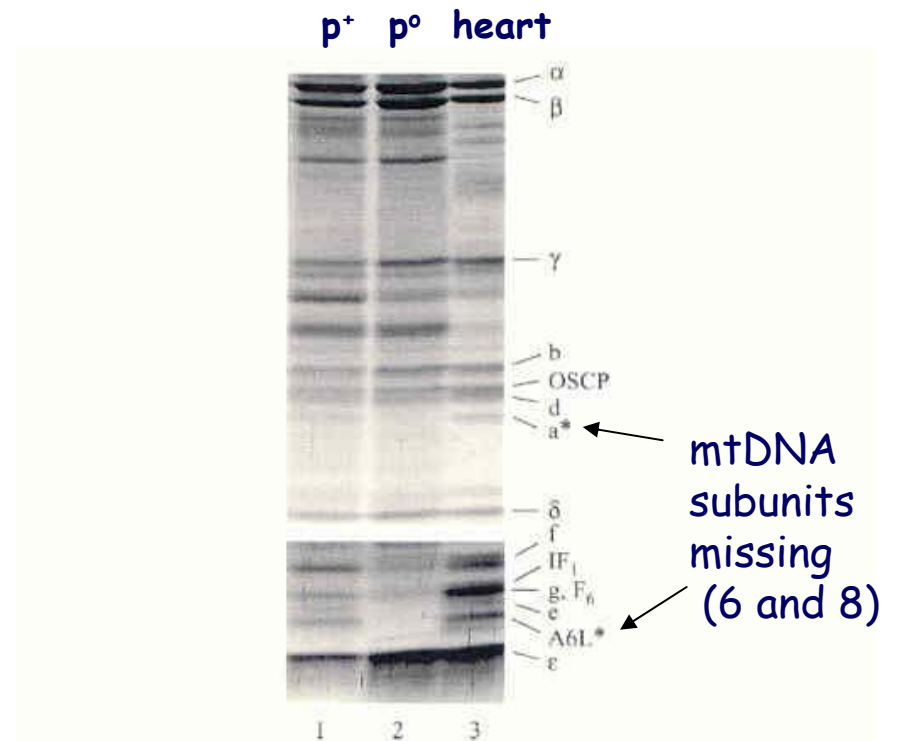


FIG. 5. Immunoprecipitation of F₁F₀ from mitochondria of fibroblast MRC5. 1 mg of fibroblast mitochondria from p⁺ (lane 1) and

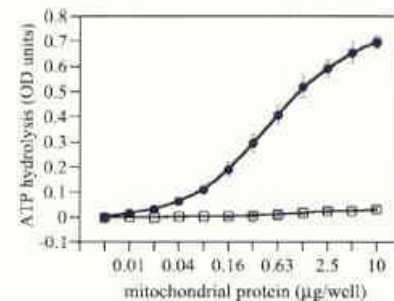
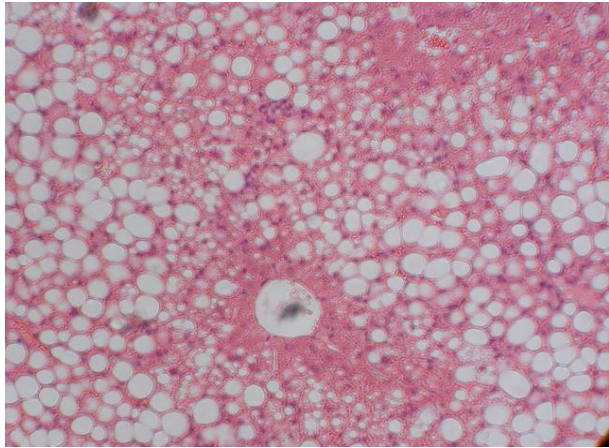


FIG. 2. Human F₁F₀ ATPase immunocaptured by the anti-F₁F₀ mAb 12F4AD8AF8 is active as an ATPase and is sensitive to oligomycin inhibition. Human heart mitochondria were solubilized

Application of mitochondrial proteomics:

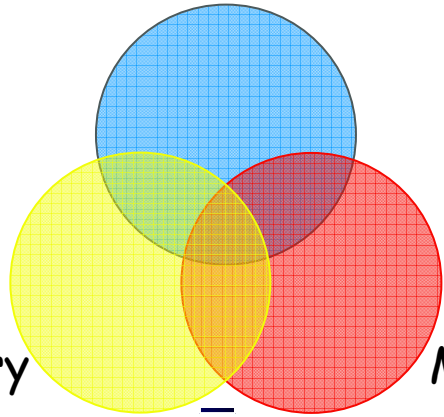
Chronic alcohol-induced mitochondrial dysfunction & liver disease



Chronic Alcohol

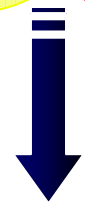


ROS/RNS
stress

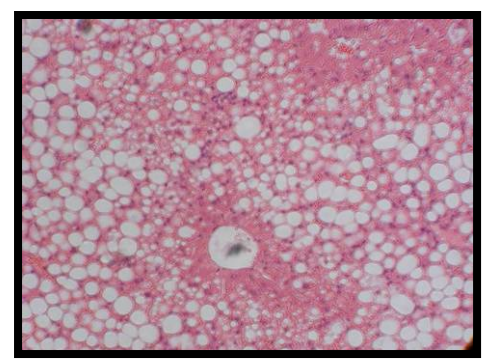


Inflammatory
Response

Mitochondria
Dysfunction

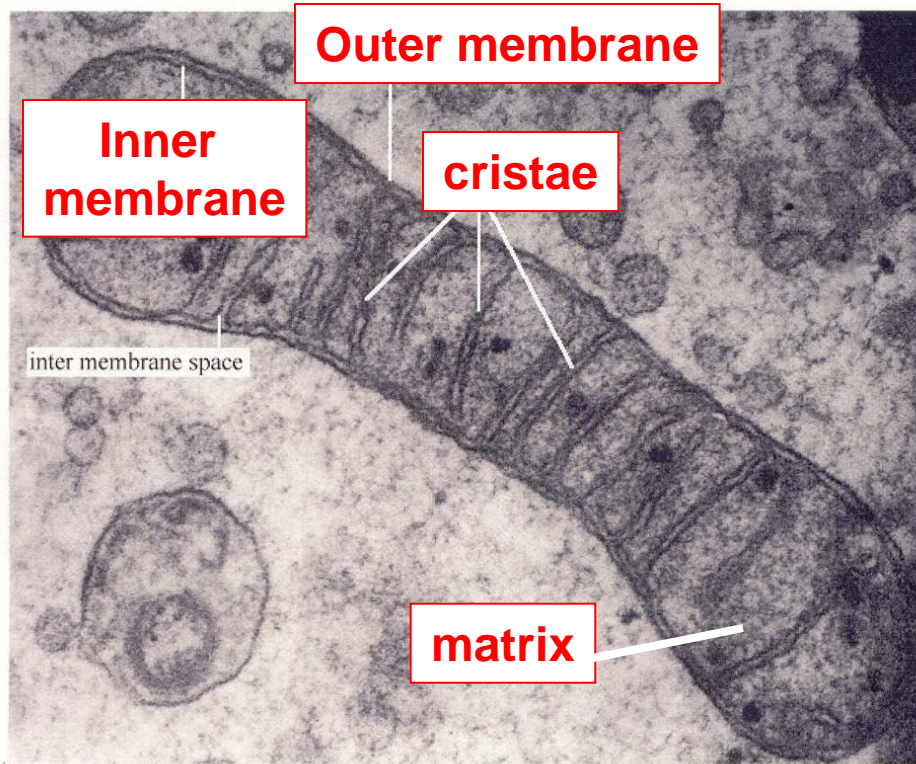


Pathology -
Steatosis
Hepatitis
Fibrosis

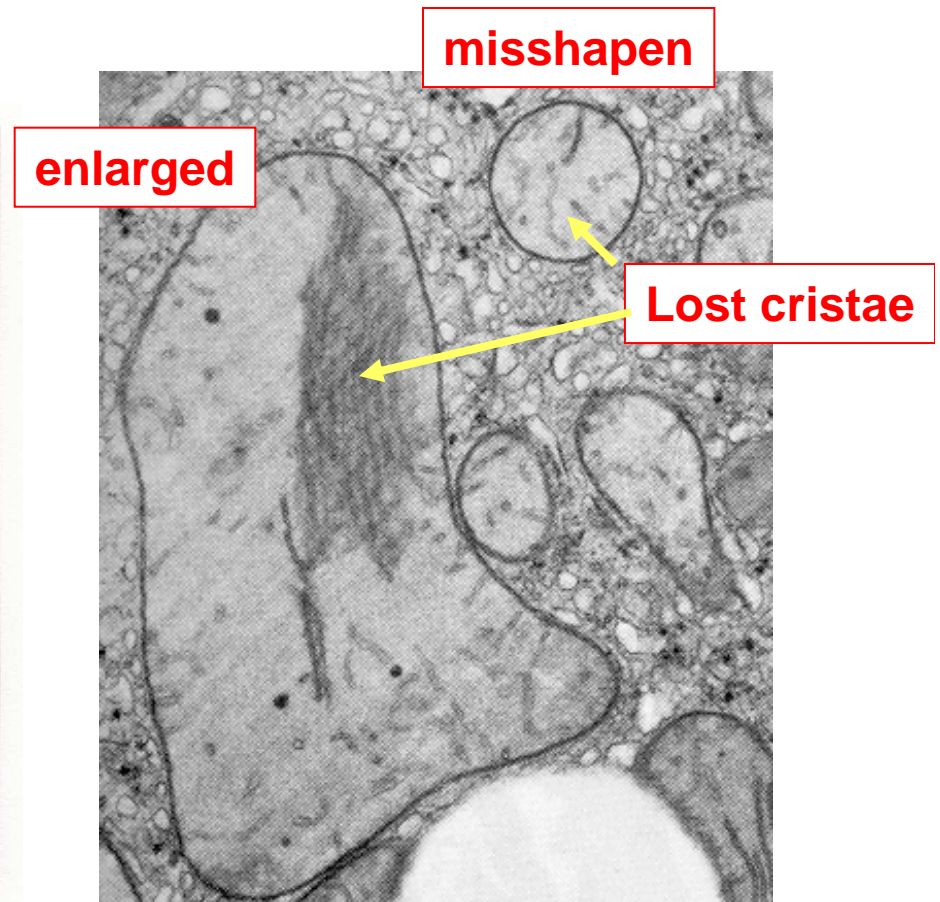


Effects of Chronic Ethanol Consumption on Mitochondrial Energy Metabolism

Structural abnormalities

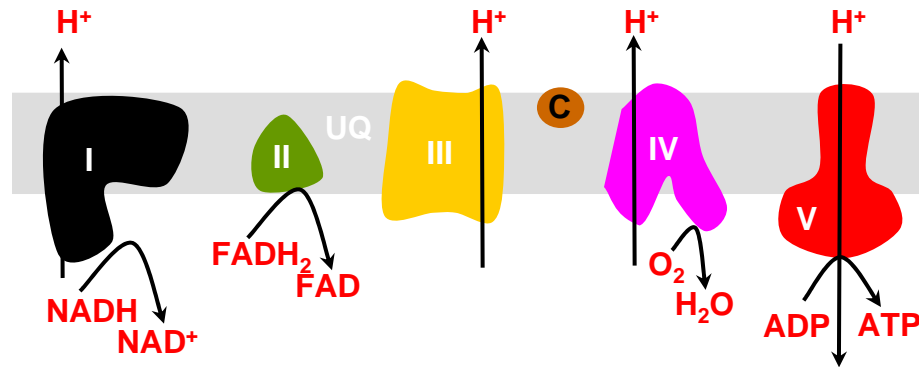


Normal mitochondria



Alcoholic mitochondria

Effects of Chronic Ethanol Consumption on Mitochondrial Energy Metabolism - **Functional abnormalities**



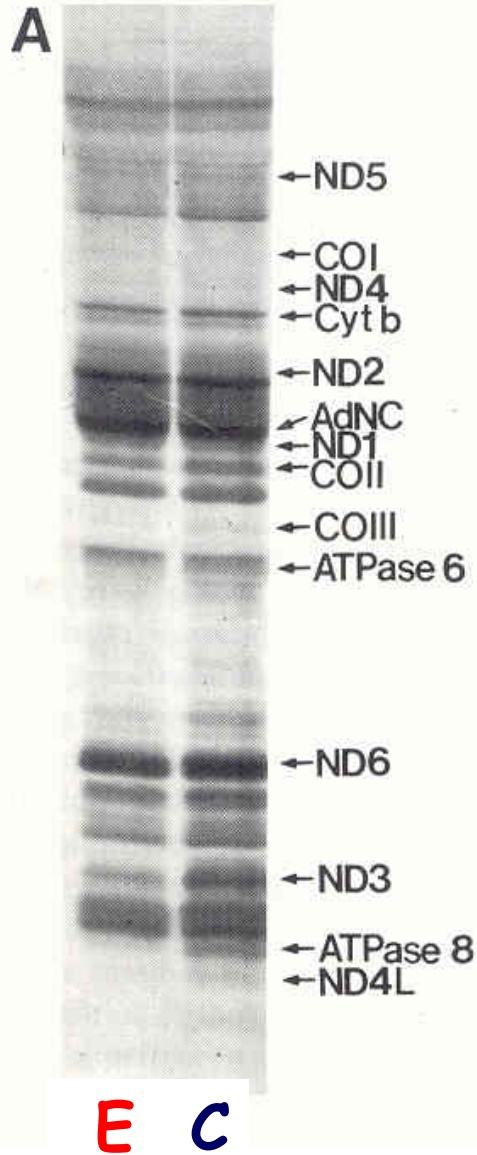
Decrease in state 3 respiration by 25-40%

- ↓ NADH-linked substrates & fatty acids
- ↓ succinate-driven
- ↓ cytochrome oxidase

Decrease in the rate of ATP synthesis

Defects in complexes I, III, IV, and ATP synthase

SMP - gels



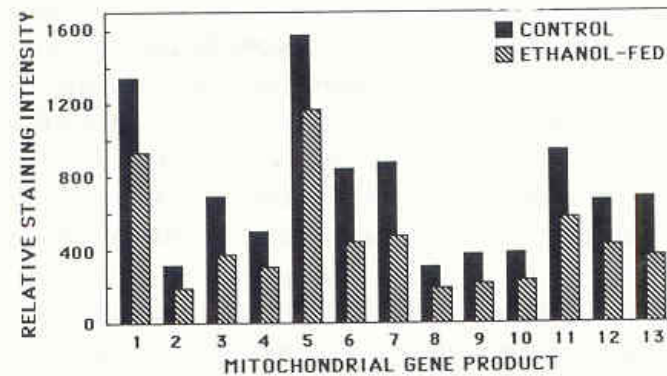
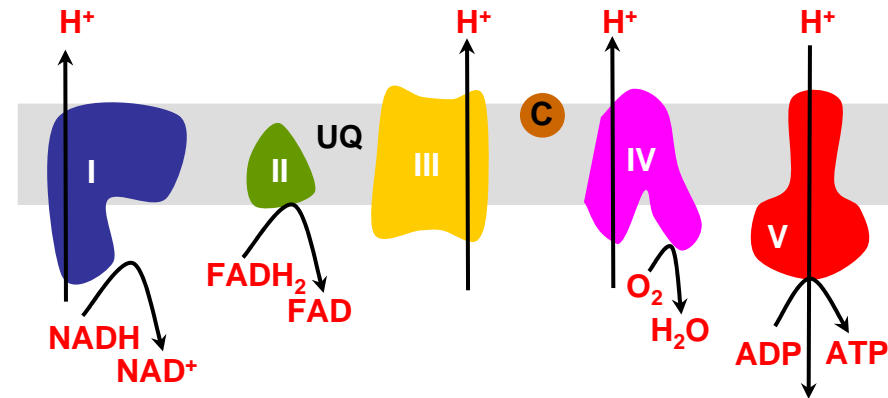
13 mtDNA encoded polypeptides:

7 subunits of Complex I

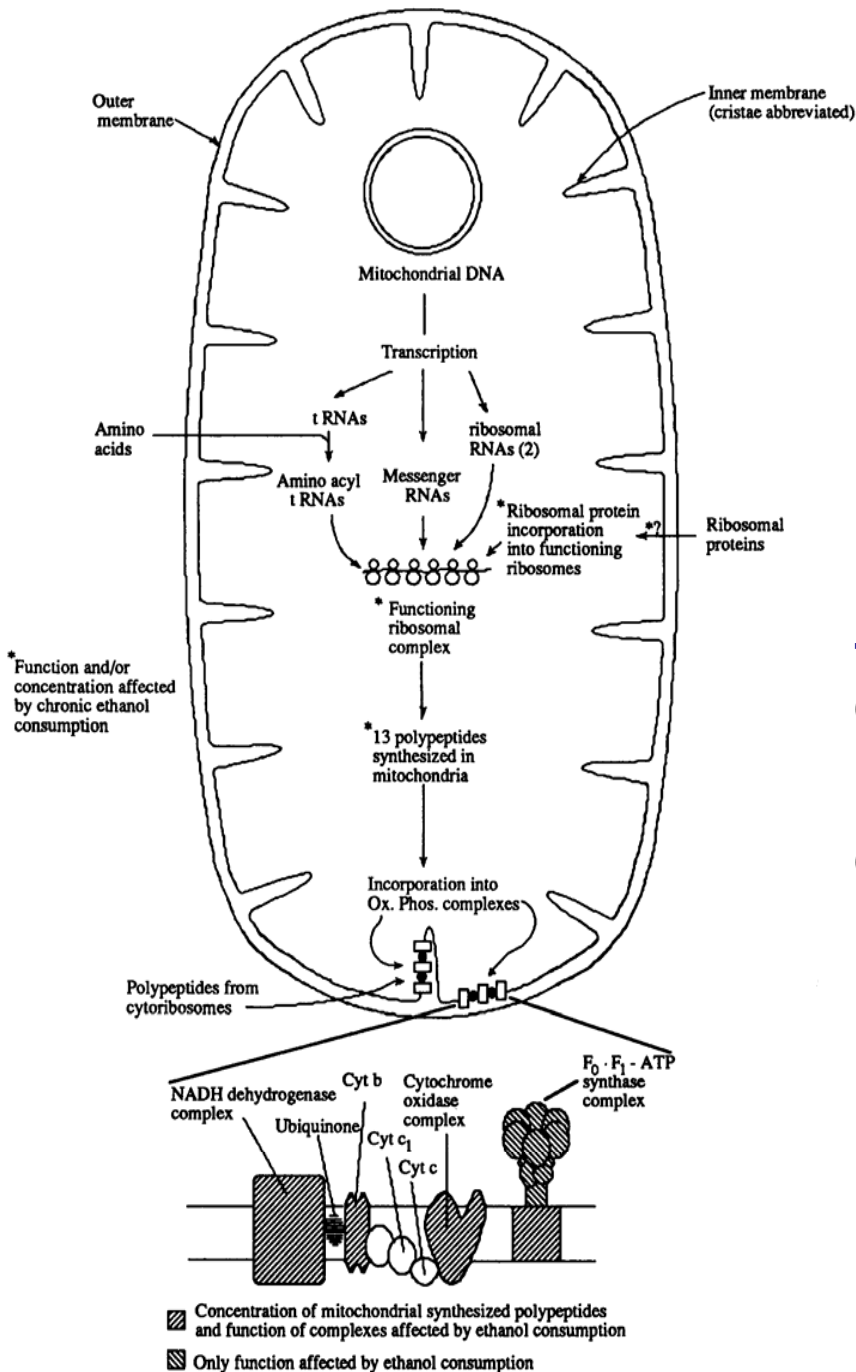
3 subunits of Complex IV

2 subunits F₀ ATP synthase

Cytochrome b

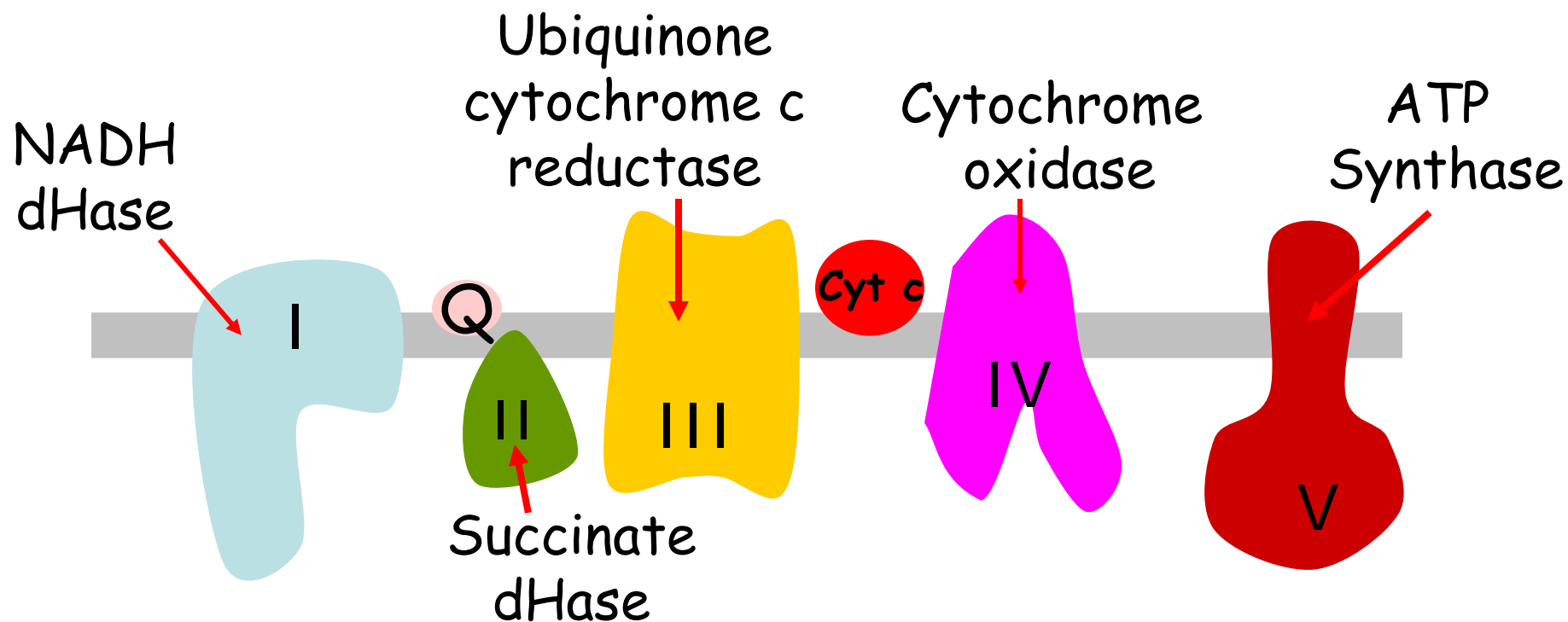


In ethanol mitochondria, there is a decrease in the concentration of all 13 mitochondrial-encoded polypeptides



Decreased translation capacity of mitochondrial ribosomes due to chronic alcohol-associated modifications in the structure and function of mitochondrial ribosomes
(Arch Biochem Biophys 398:41, 2002)

Damage to mtDNA by chronic alcohol
(JBC 279: 22092, 2004)



Chronic alcohol-induced lesions

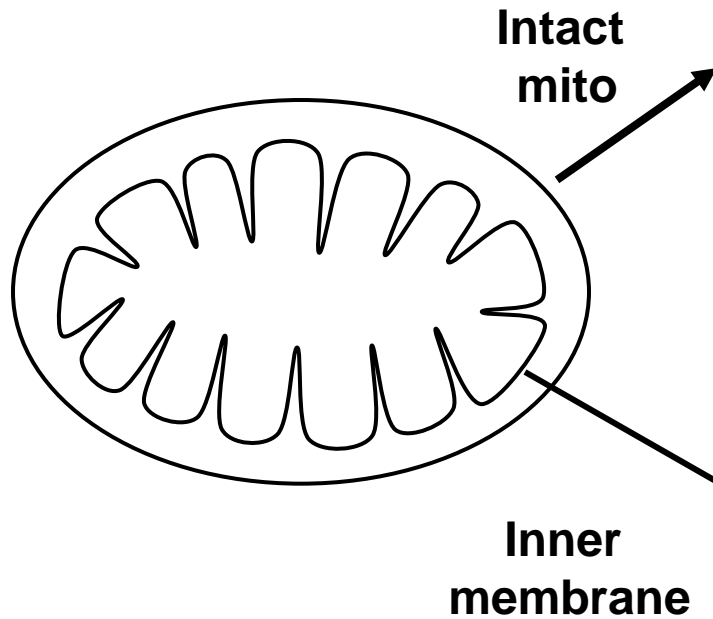
Decreased

Activity & heme of IV
 cytochrome b
 Fe-S centers of I
 ATPase and ATP-P_i (F₀)

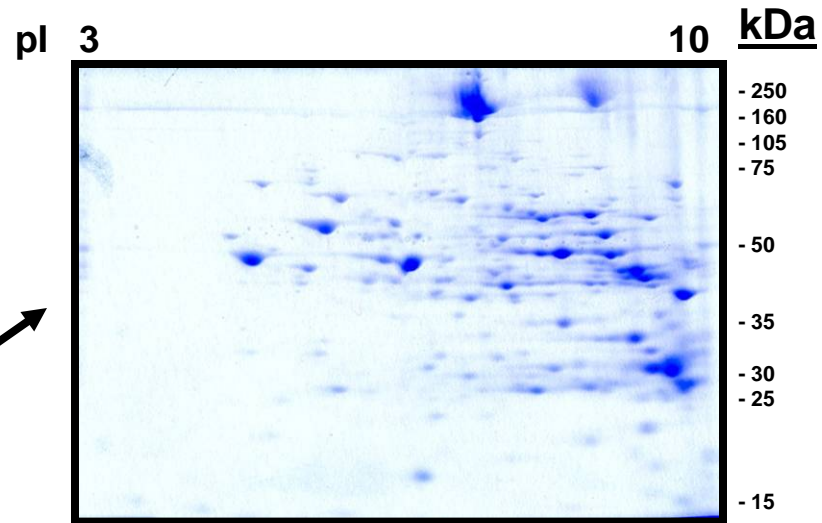
No change

Cytochrome c and c₁
 Ubiquinone
 Succinate dHase
 Catalytic F₁ portion
 ANT & carriers

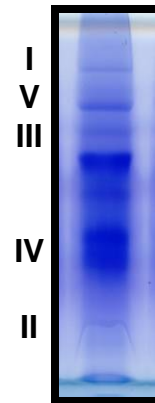
Strategy to reveal chronic alcohol-related alterations to mitochondria proteome



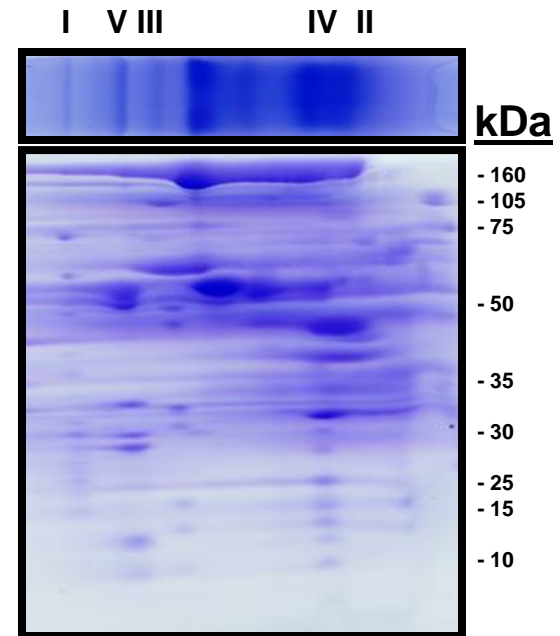
A. 2-D IEF/SDS-PAGE



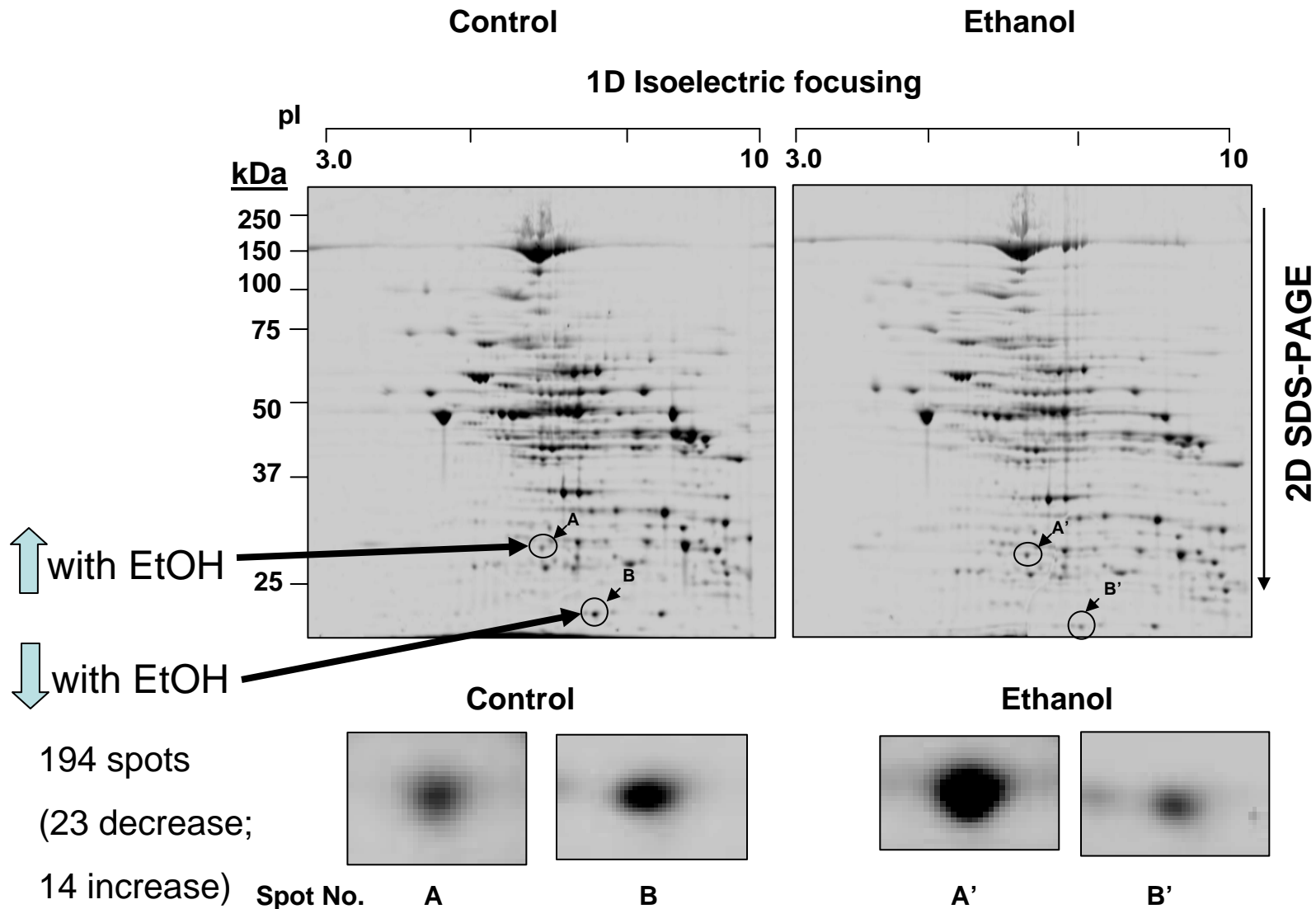
B. 1-D BN-PAGE



C. 2-D BN-PAGE



Protein Changes with Chronic Ethanol Consumption

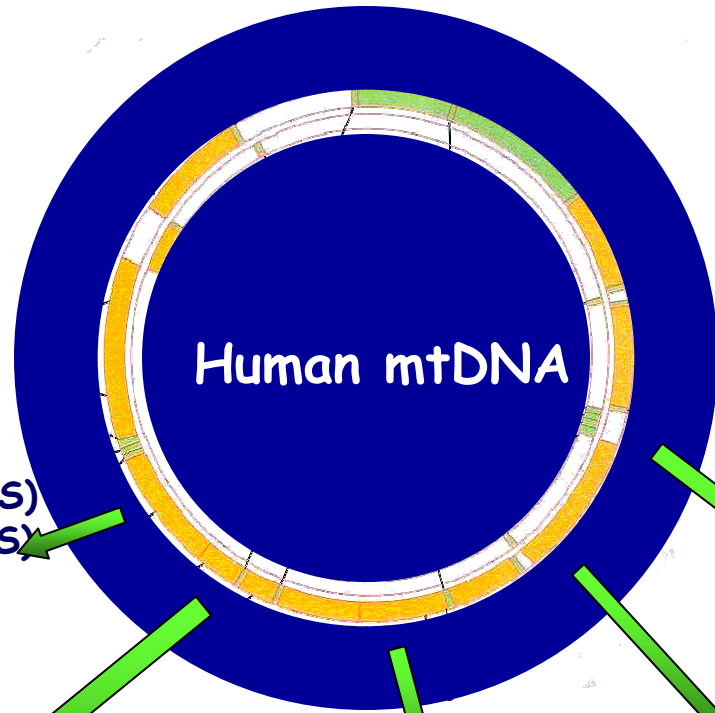


Proteins with different abundances in liver mitochondria following chronic ethanol consumption – identification of proteins from 2-D IEF/SDS-PAGE gels.

Protein	Mass calc. (kDa)	MOWSE score	Mean fold change ^a	
acyl-Coenzyme A dehydrogenase, very long chain	70.7	72	1.31	β-oxidation
acyl-Coenzyme A dehydrogenase, medium chain	46.5	94	0.78	
acyl-CoA dehydrogenase, short-chain specific	44.9	67	0.81	
β-ketoacyl CoA thiolase	41.8	84	0.12	
Δ ³ , Δ ² -enoyl-CoA isomerase	32.2	100	1.58	
2,4-dienoyl-CoA reductase (NADPH)	36.1	78	1.70	
oxoglutarate dehydrogenase (lipoamide); α-ketoglutarate dehydrogenase	116.0	64	2.87	TCA cycle-related
Glutamate dehydrogenase	61.4	101	0.56	
Respiratory Complexes I,III,IV,V				Ox-phos
ATP synthase beta subunit	51.1	144	0.65	
Chain A, Rat liver F ₁ -ATPase (alpha subunit)	55.2	156	0.71	
Ubiquinol-cytochrome c reductase iron-sulfur subunit	27.7	108	0.48	
ubiquinol-cytochrome c reductase, core protein II precursor	48.3	67	0.21	
60kDa heat shock protein (Hsp60)	60.9	115	0.84	Chaperones
dnaK-type molecular chaperone grp75 (Hsp70/GRP75)	73.7	121	1.80	
aldehyde dehydrogenase	48.2	75	0.44	EtOH metabolism
3-hydroxyisobutyrate dehydrogenase	35.3	67	1.41	
3-mercaptopyruvate sulfurtransferase	32.9	78	1.82	AA catabolism

^a Mean fold change was determined by averaging the fold change observed from 5 pairs of control and ethanol-fed animals.

^b p values were determined using a two-tailed paired Student's t-test on the normalized protein spot densities obtained using PDQuest.



13 proteins
³⁵S label
 (cycloheximide)

rRNA Large subunit (16S)
 Small subunit (12S)

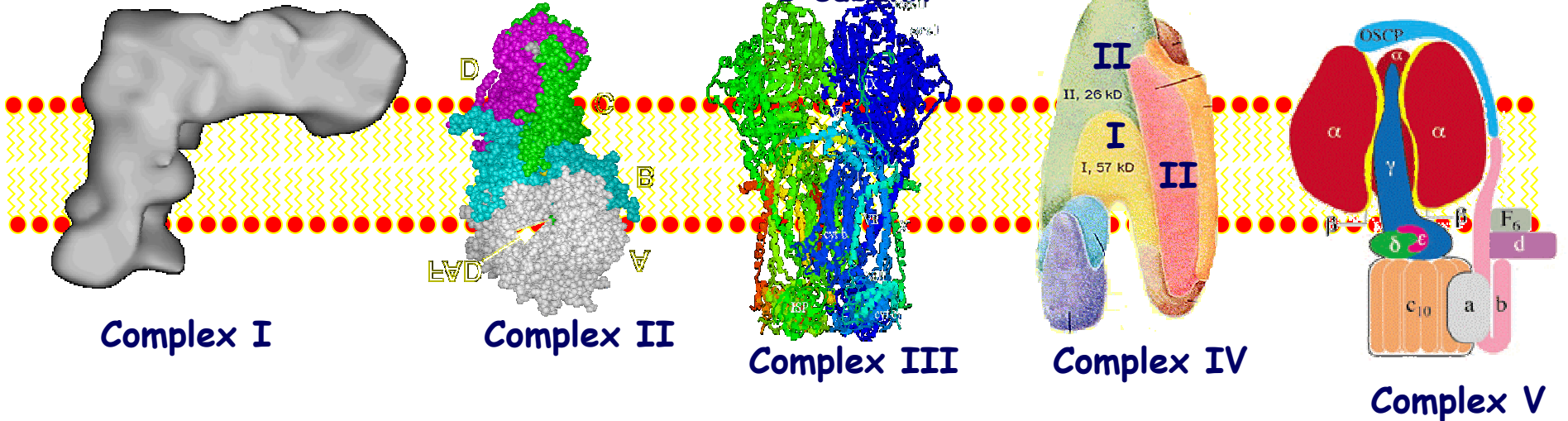
tRNA 22

7 subunits

cytochrome
 b subunit

Subunits
 I, II & III

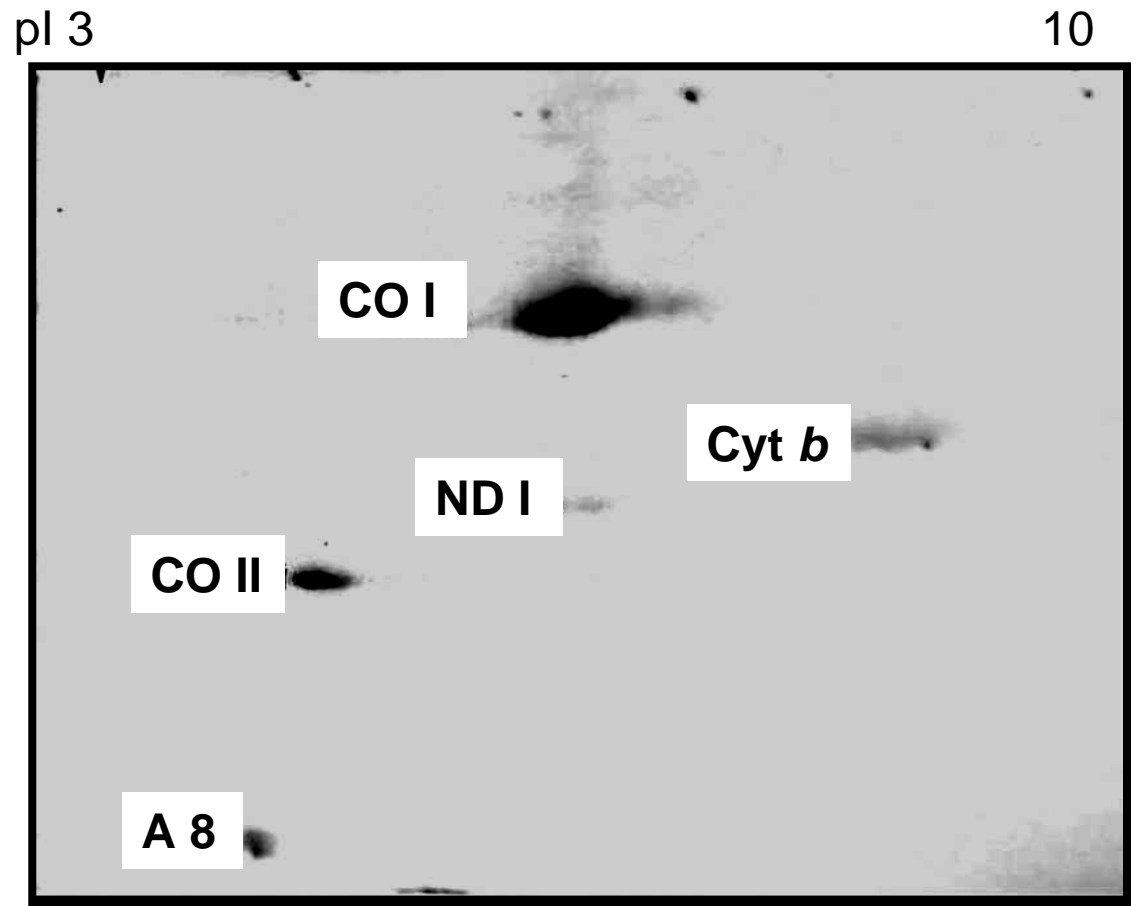
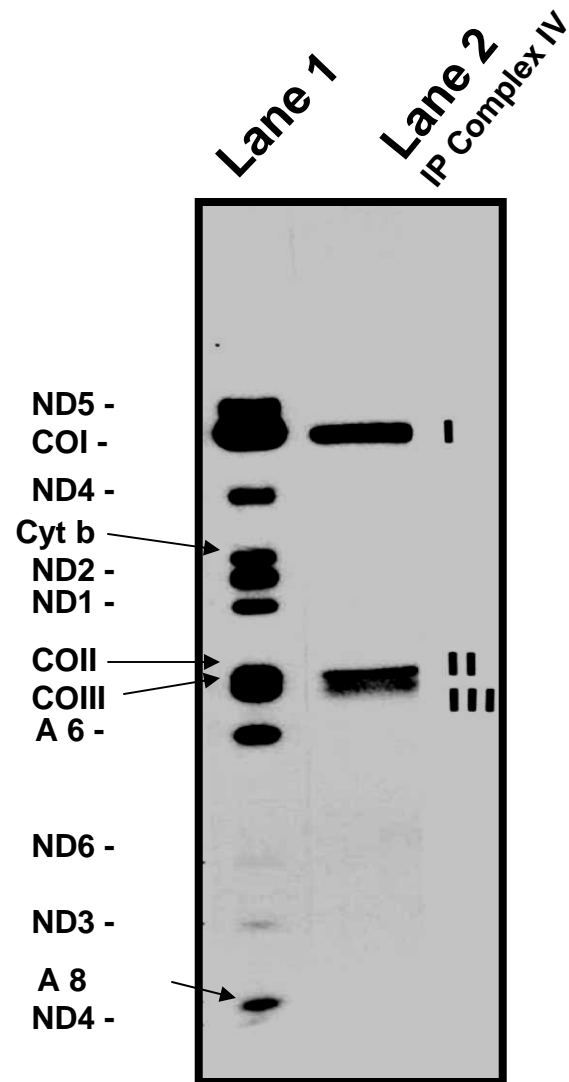
Subunits 6 & 8



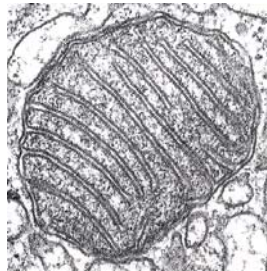
Visualization of ³⁵S-labeled mitochondrial encoded subunits

A. 1-D SDS-PAGE

B. 2-D IEF/SDS-PAGE



Solution... Functional 2D Proteomics: BN-PAGE



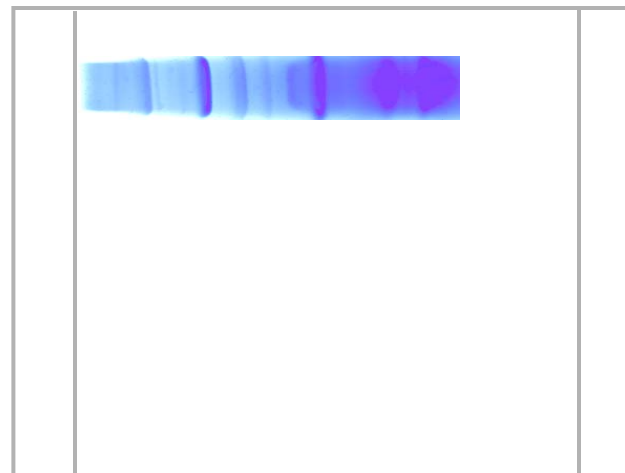
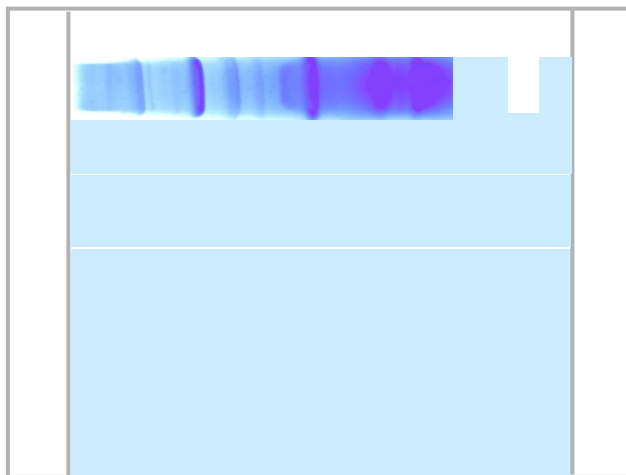
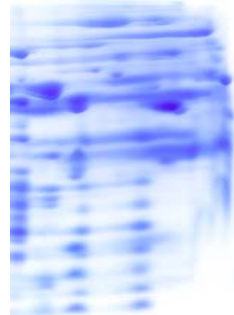
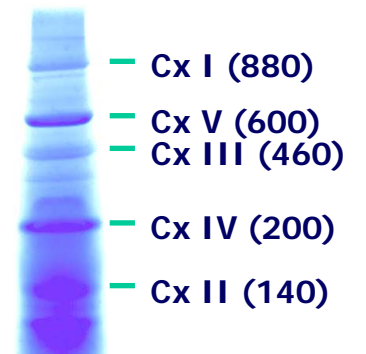
0.25% Coomassie Blue
1.25% Laurylmaltoside

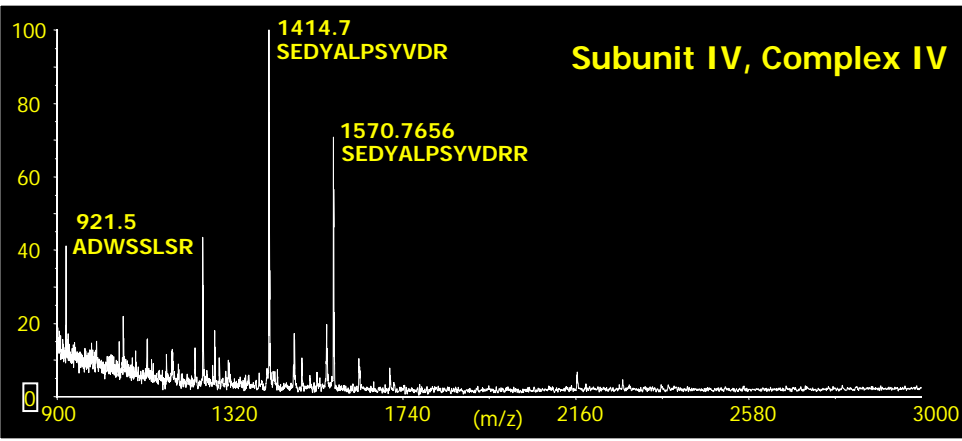
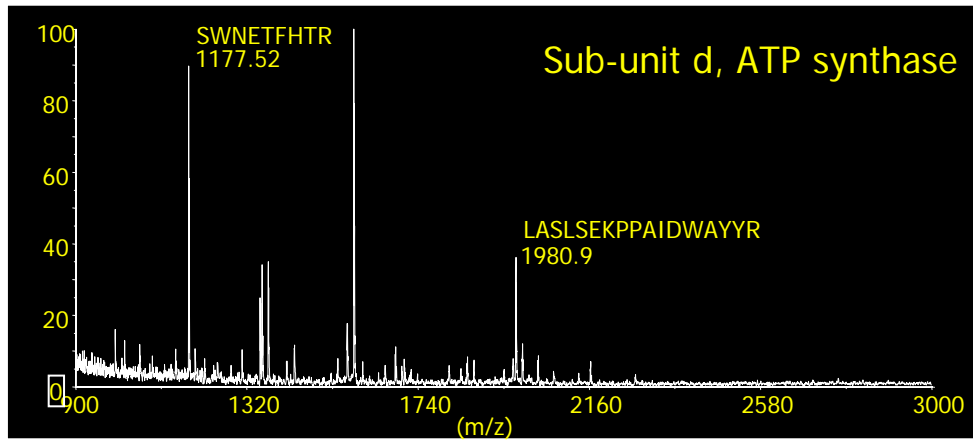
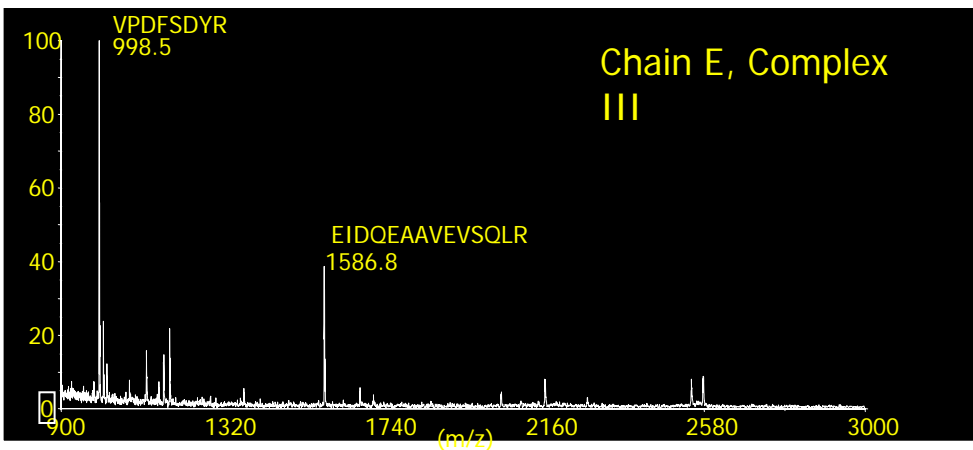
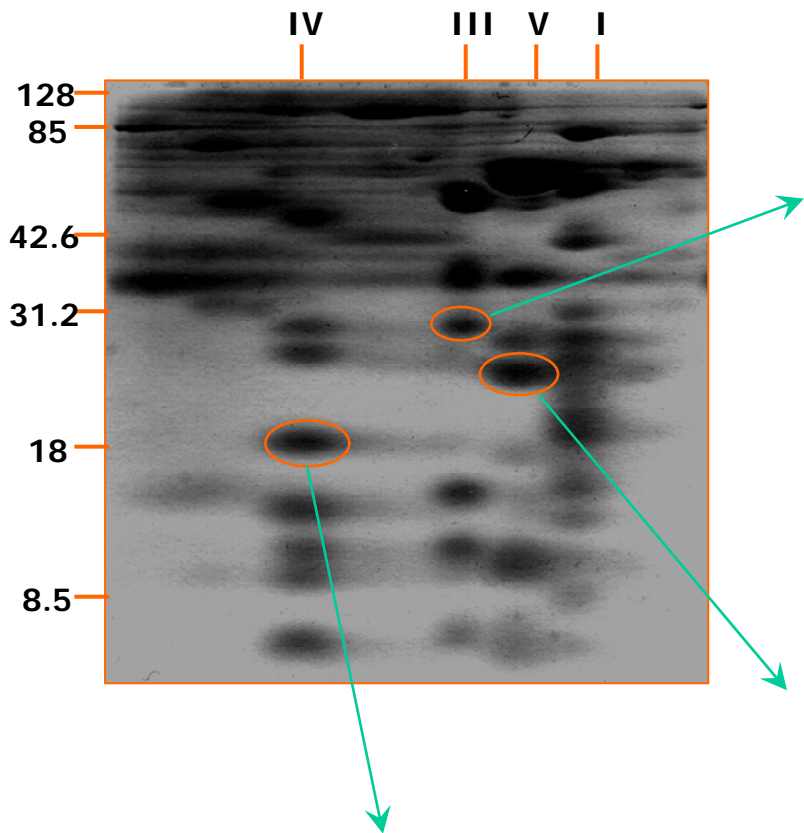


Intact protein complexes

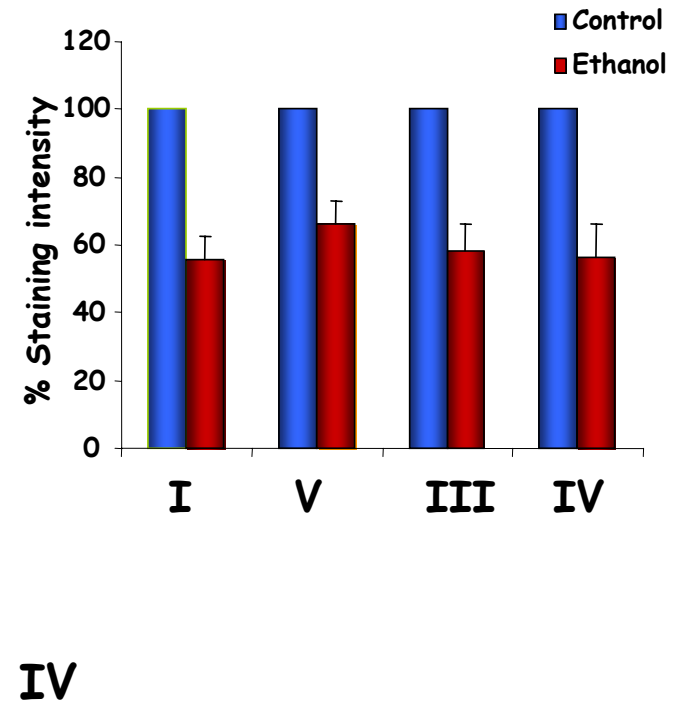
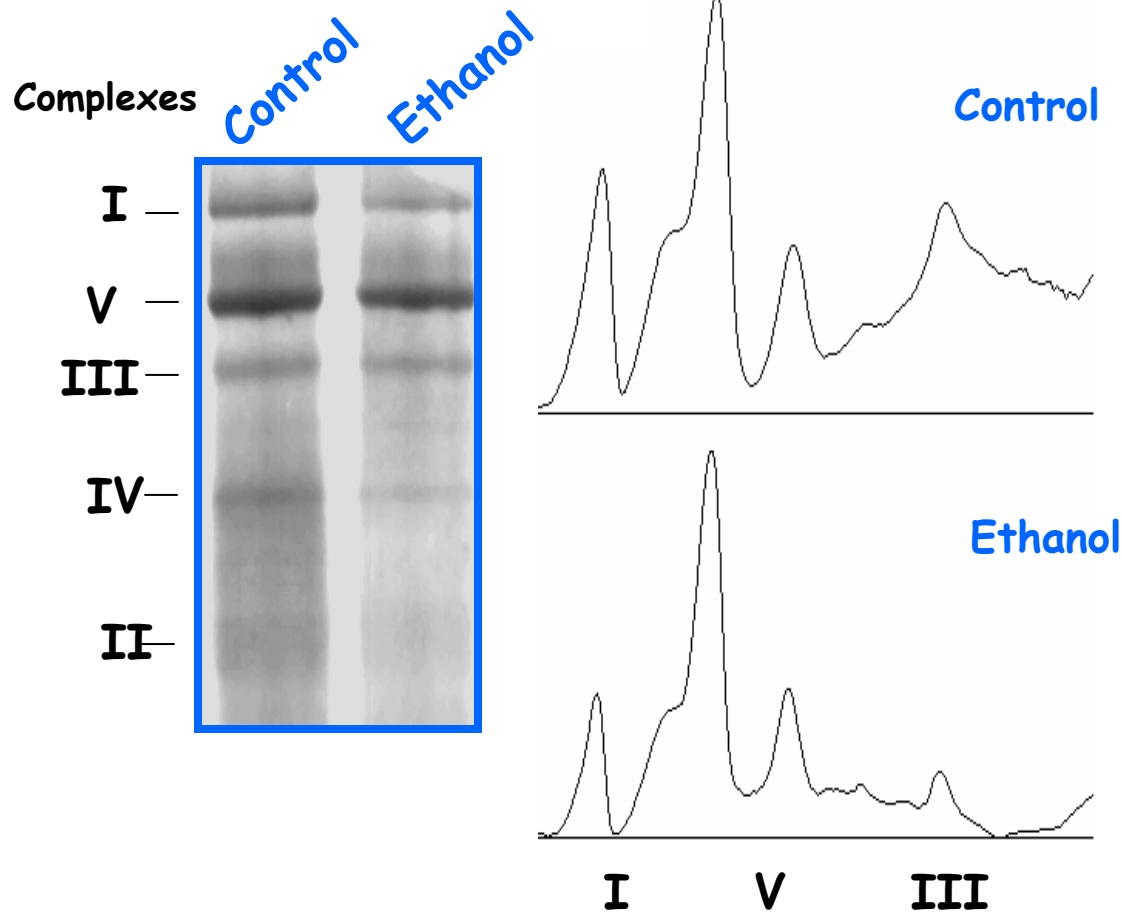


Native gradient
Gel. 20V,
15h, 4°C

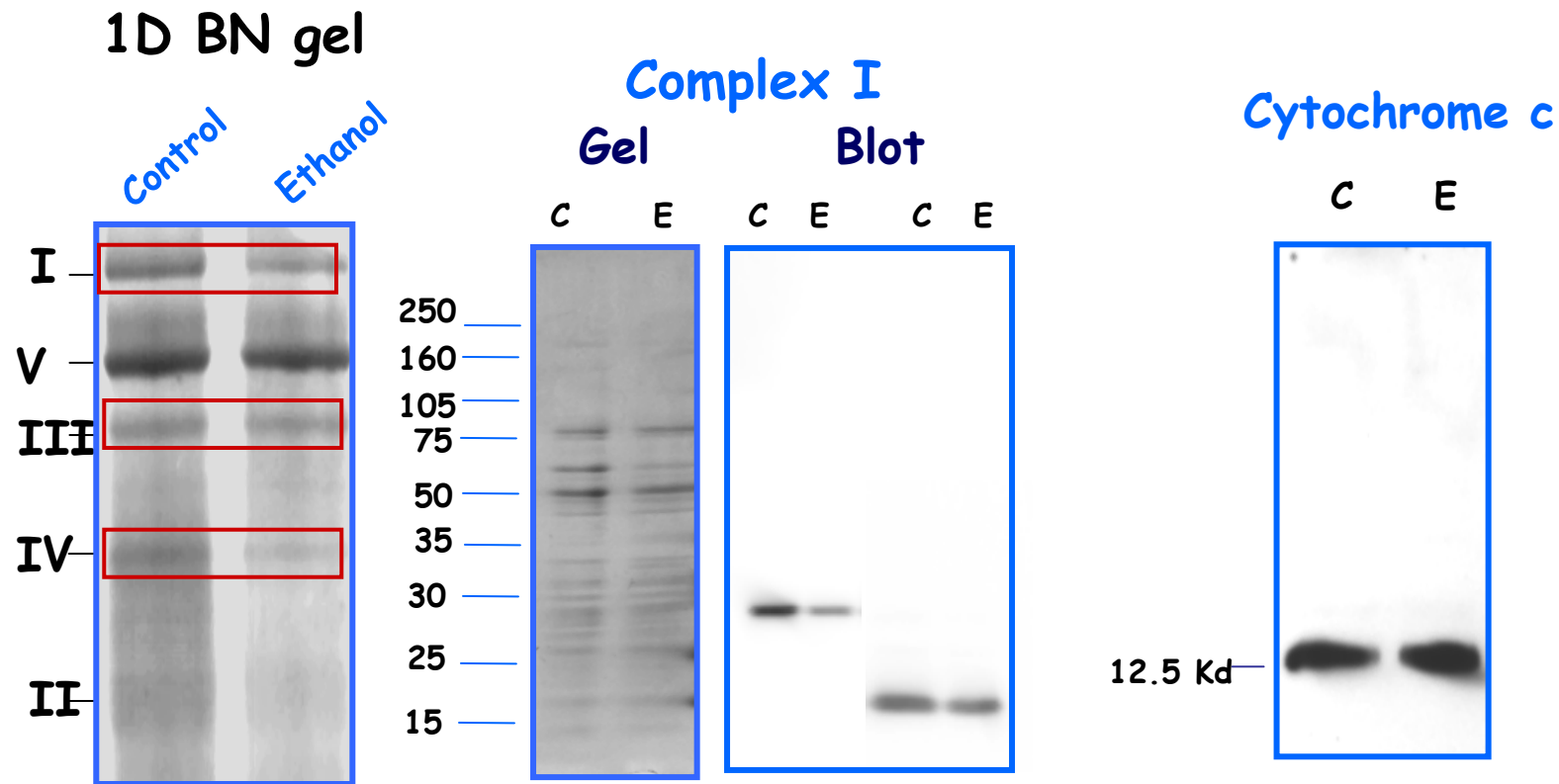




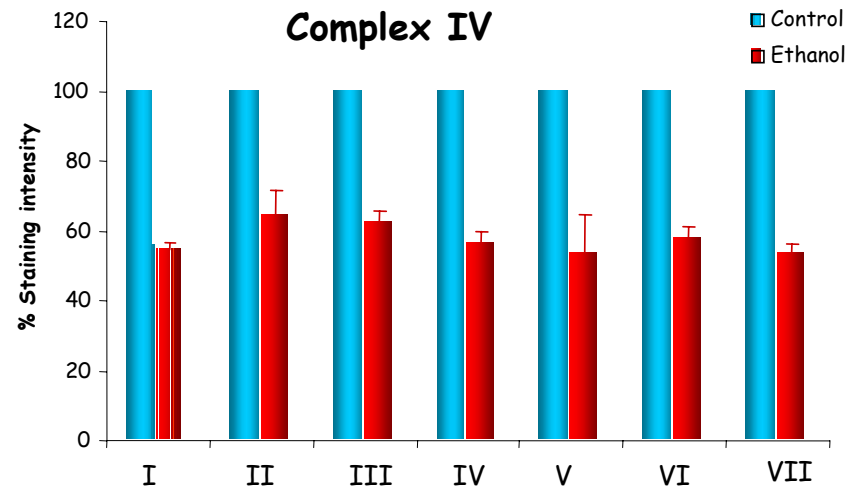
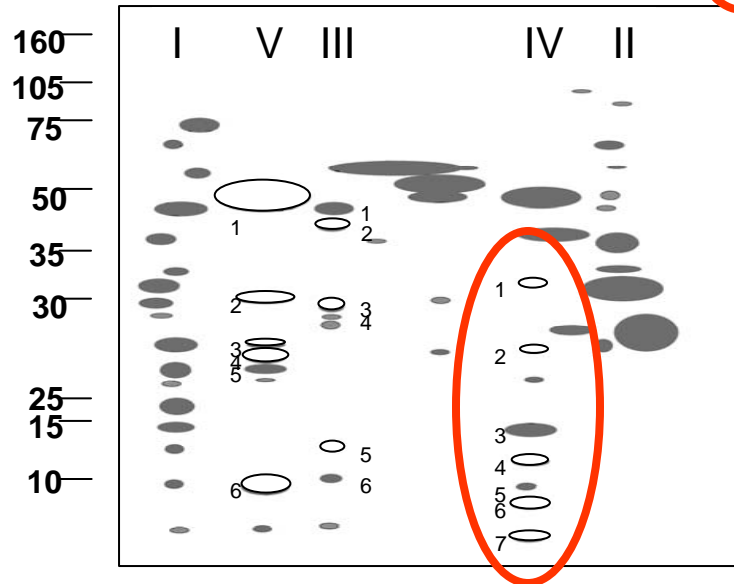
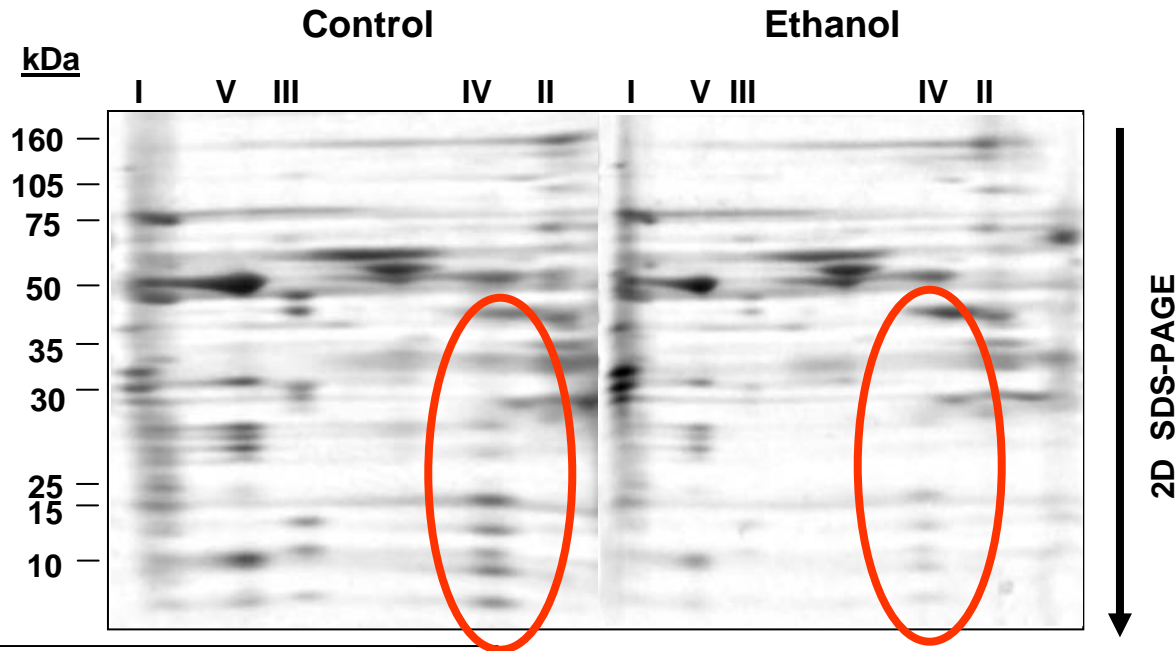
1-D Blue Native gels



Specific Protein Subunits Decreased by Chronic Alcohol



2-DBN-PAGE - Loss of OXPHOS subunits by chronic alcohol



Molecular Chaperones

Hsp60
Hsp70/GRP75

Mitochondria
Ribosome

Ethanol metabolism
Aldehyde dease

**Ethanol-induced
mitochondrial
dysfunction**

Fatty acid metabolism

Acyl-CoA dease, SC
Acyl-CoA dease, MC
Acyl-CoA dease, VLC
2,4-Dienoyl CoA reductase
 β -Ketoacyl-CoA thiolase
 Δ^3, Δ^2 -Enoyl-CoA isomerase

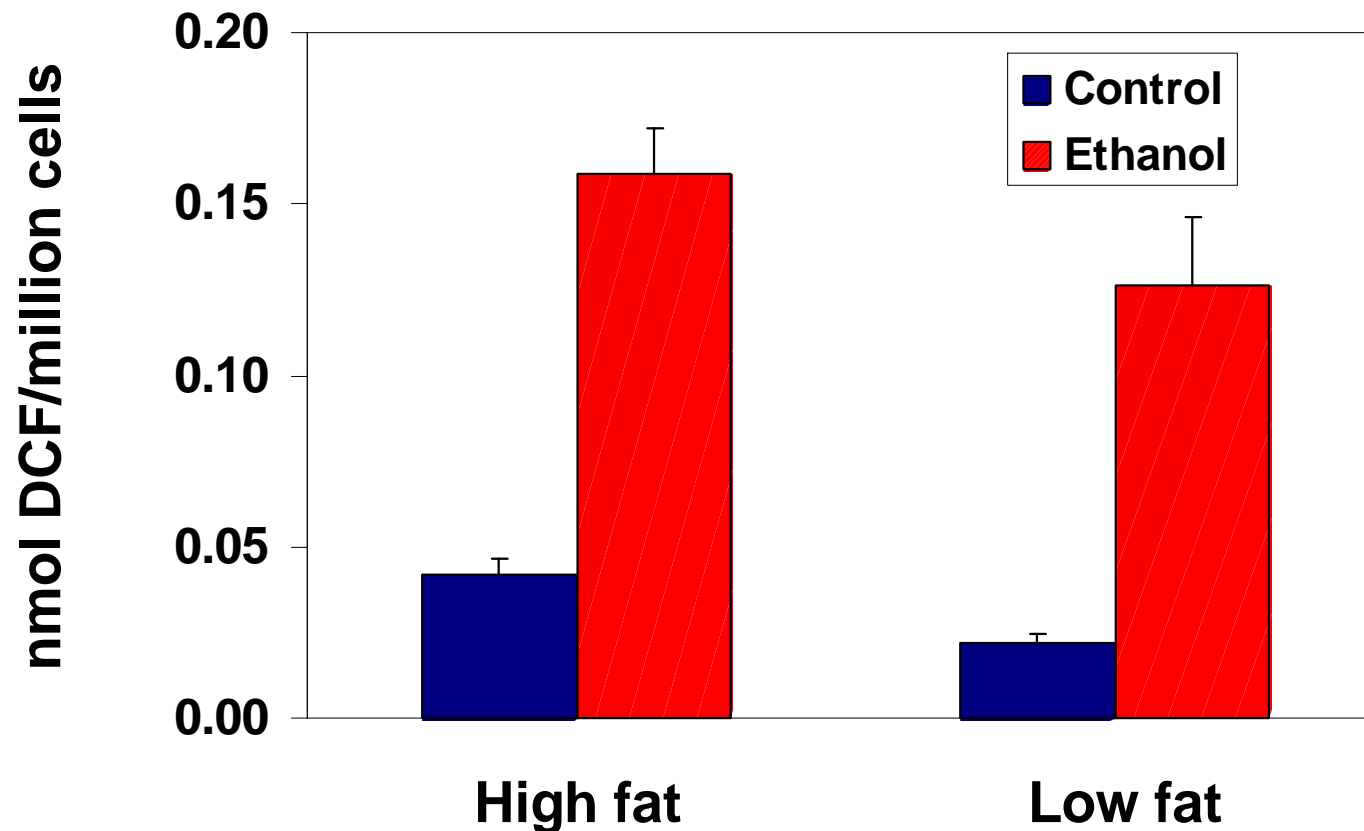
TCA cycle &
amino acid metabolism
 α -Ketoglutarate dease (lipoamide)
Glutamate dease
3-Hydroxyisobutyrate dease
Methylmalonate-semialdehyde dease

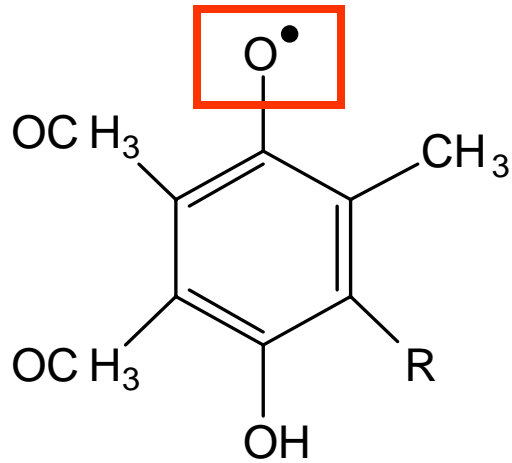
Oxidative phosphorylation

<u>NADH dease</u>	<u>Ubiquinol-cyto c reductase</u>	<u>Cytochrome c oxidase</u>	<u>ATP synthase</u>
ND1	Core protein 1	Subunit I	α & β
ND2	Core protein 2	Subunit II	γ
ND3	Heme protein	Subunit III	B-chain
ND4	Fe-S subunit	Subunit IV	OSCP
ND4L	14 kDa protein	Subunit V	D-chain
ND5	Cyto b	Subunit VI	F-subunit
ND6		Subunit VII	ATPase 6 & 8

Role of oxidative stress in Alcoholic Liver Disease

Chronic ethanol feeding increases hepatocyte ROS



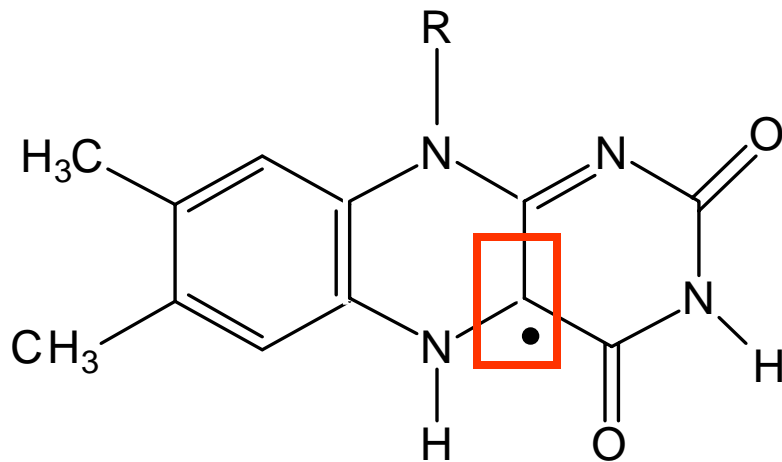


Semiquinone form of ubiquinone

Complex III

Mitochondrial ROS production

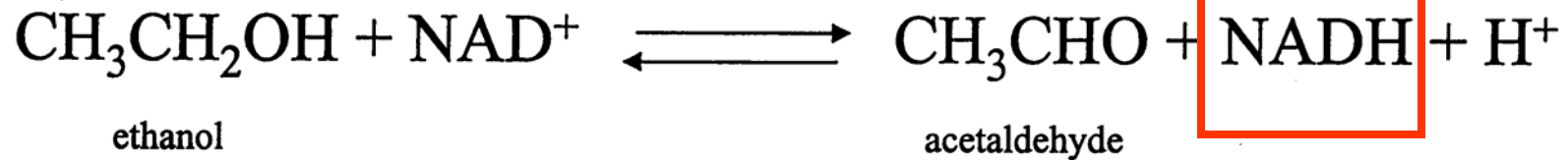
Unpaired electron transferred to molecular oxygen to form the superoxide anion free radical



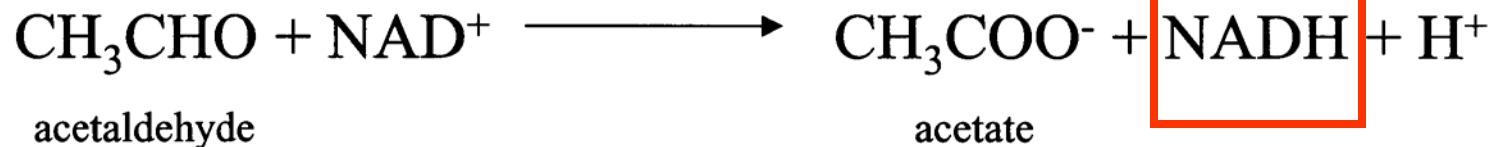
Complex I

Semiquinone form of Flavin mononucleotide

Alcohol dehydrogenase



Aldehyde dehydrogenase

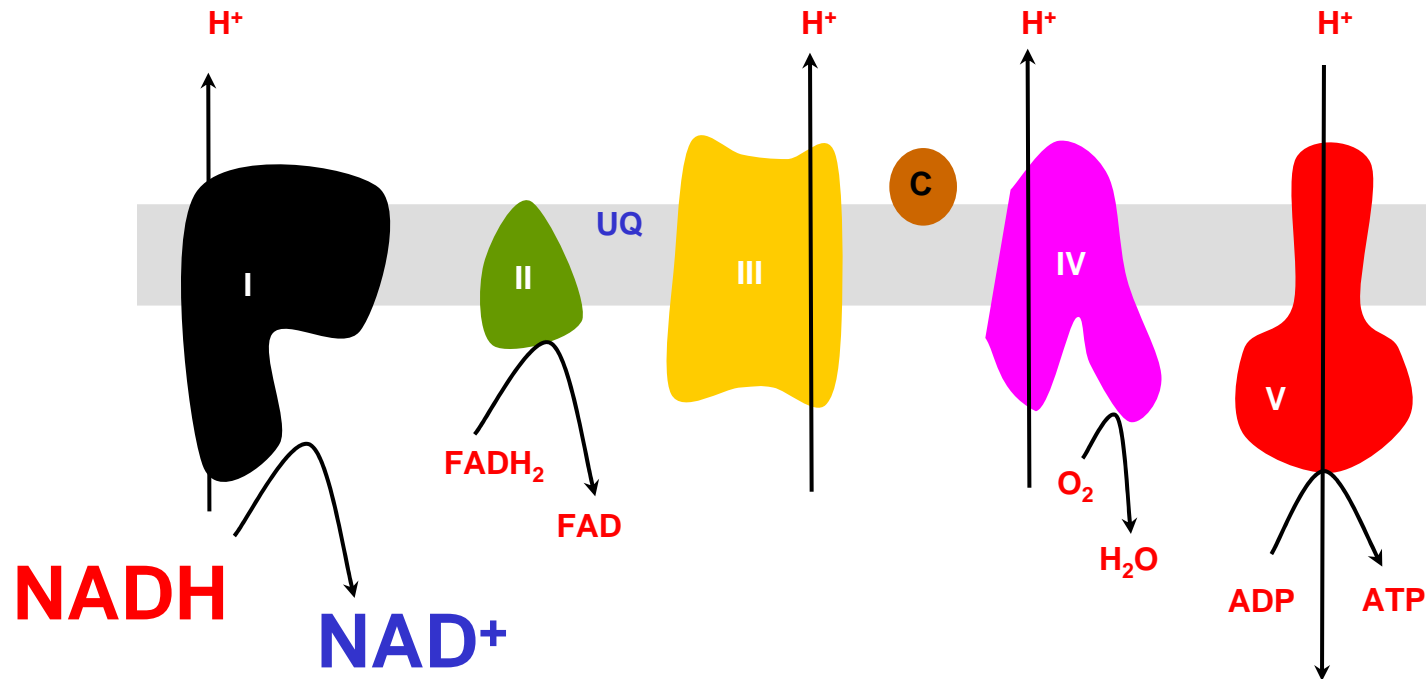


Hepatic Redox State:

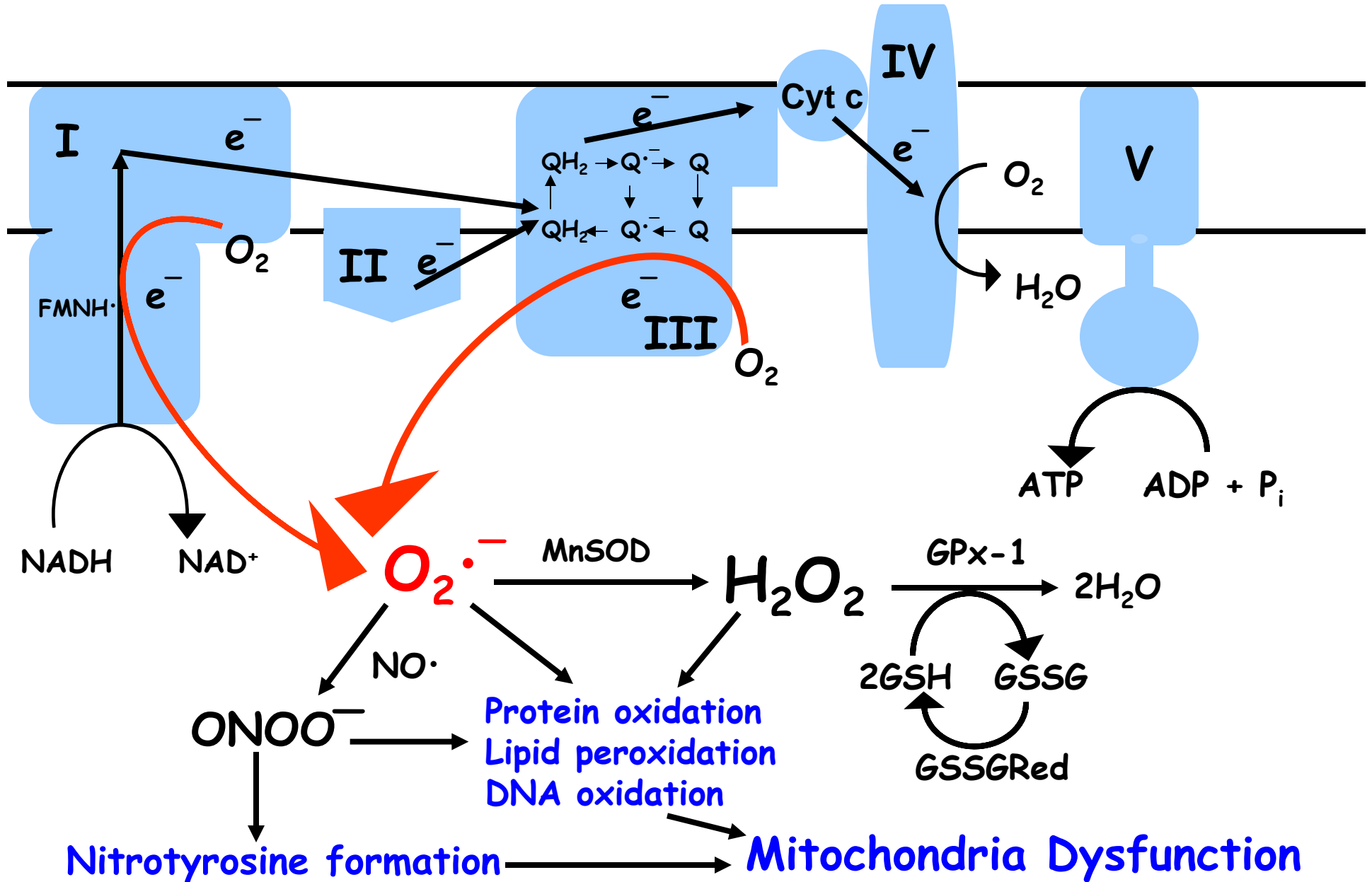
ADH and ALDH reactions use NAD^+ and produce **NADH**
Increase in the NADH/NAD^+ ratio in the cytosol and mitochondria
Effect – disruption of liver metabolism, lead to toxicity

Reoxidation of NADH necessary for maintenance of normal liver function

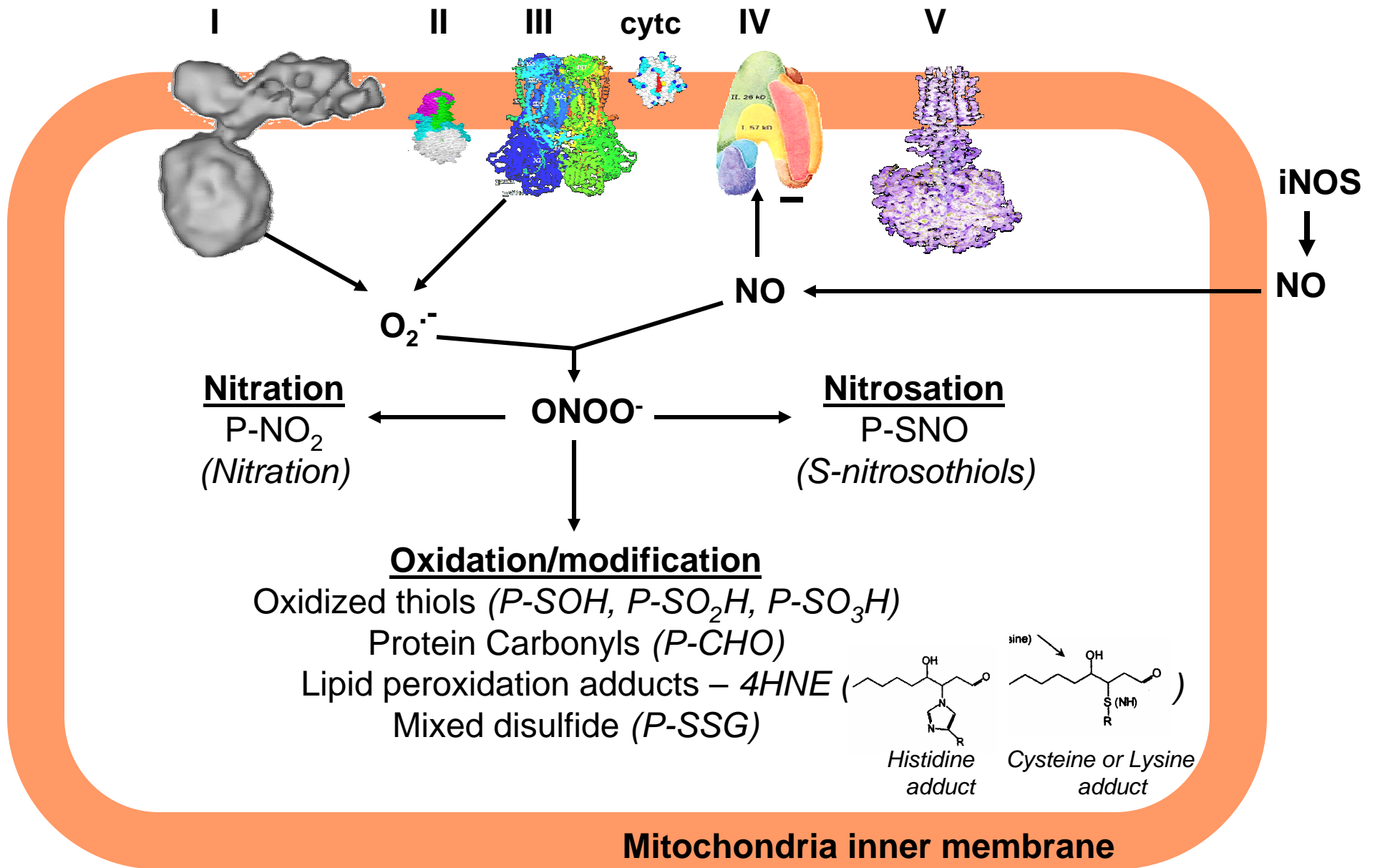
- There is a need to reoxidize NADH back to NAD⁺
- NADH is reoxidized by the **mitochondrial electron transport chain**



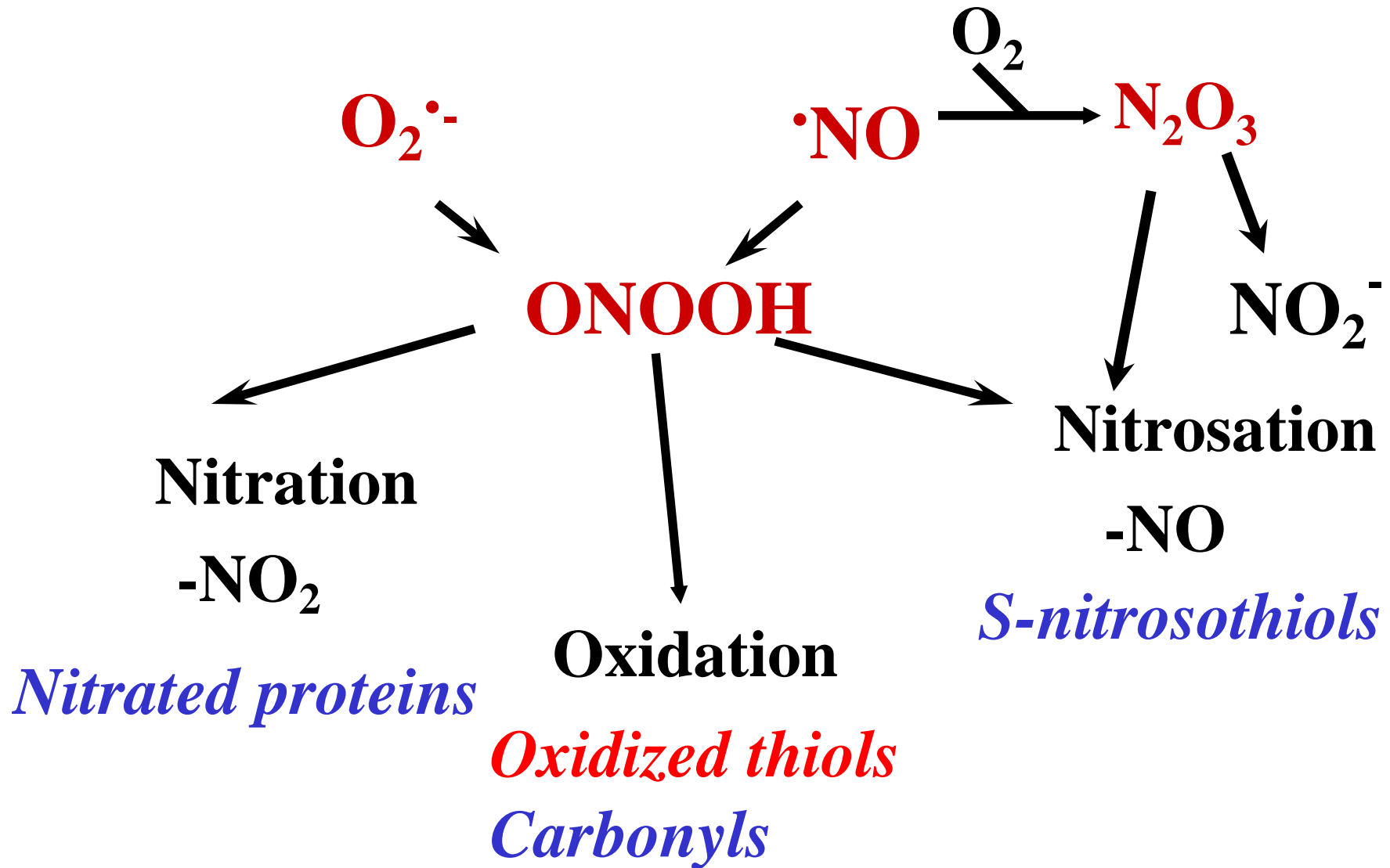
Ethanol stimulates ROS production at Complex I and III



Role of post-translational modifications in alcoholic liver disease



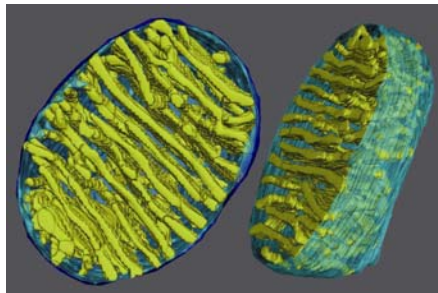
Post-Translational Modifications



Mitochondria & Protein Thiol Status

Changes in mitochondrial protein thiol status

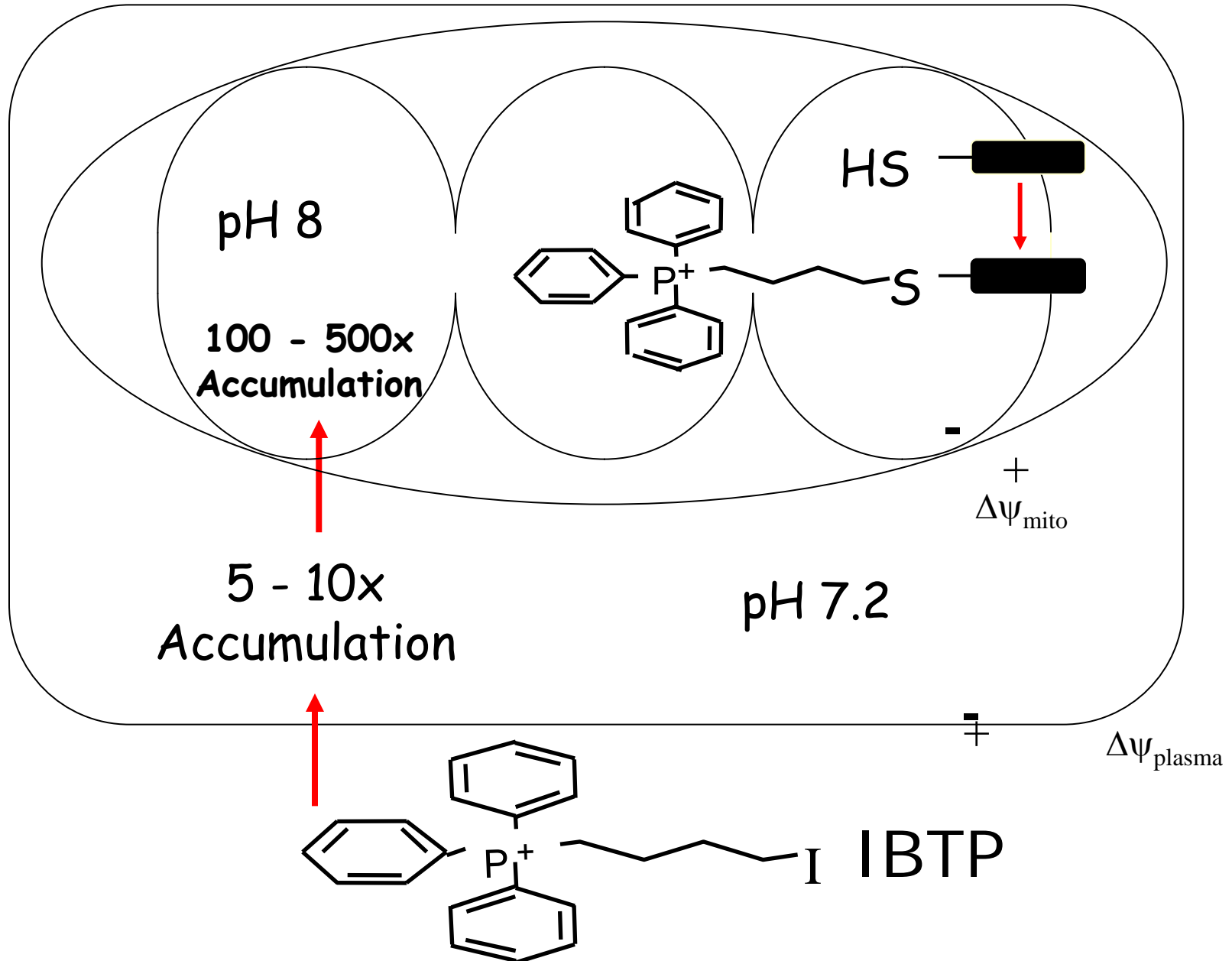
- ❖ MPT
- ❖ Cell death
- ❖ Oxidative stress
- ❖ NO responsiveness
- ❖ TNF α signaling
- ❖ Regulation of respiratory chain function



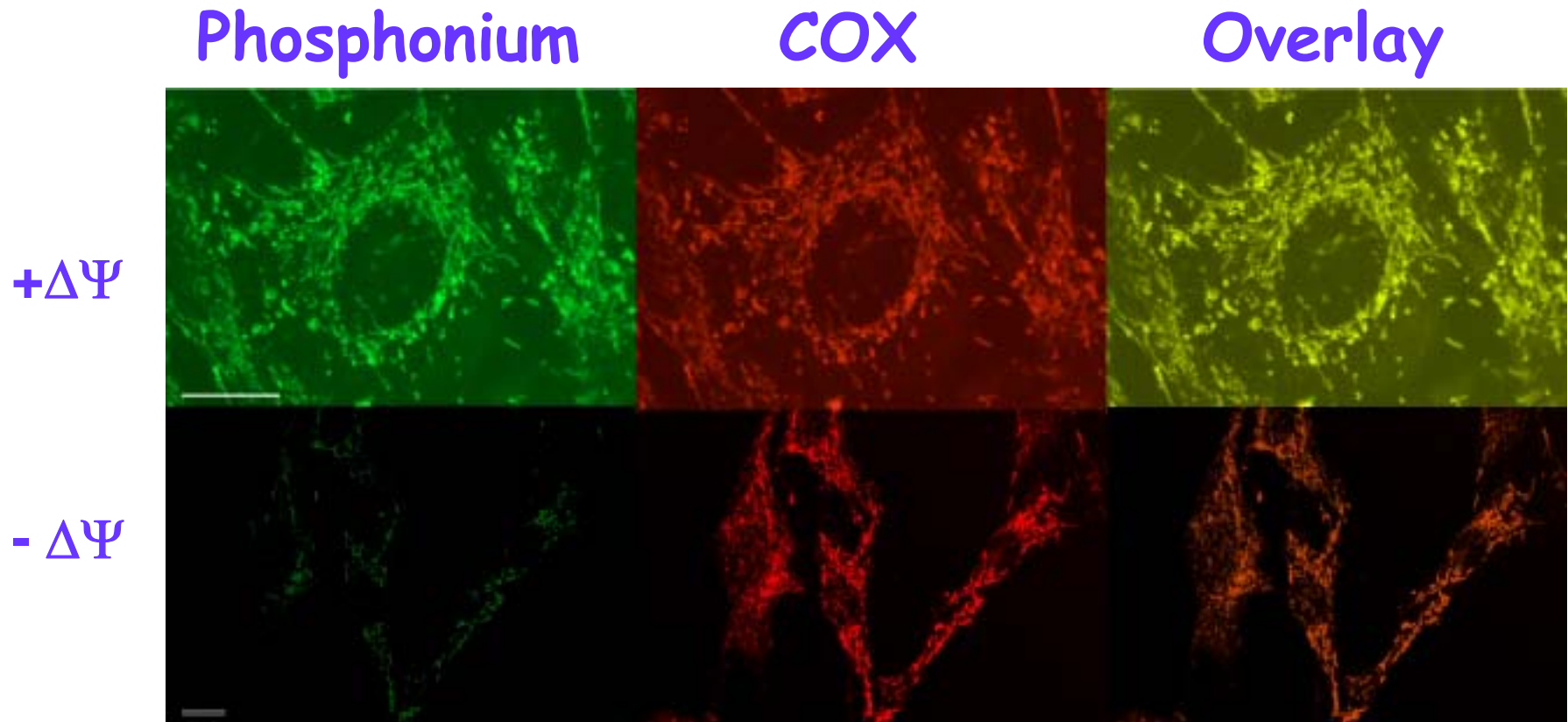
Hypothesis

Post-translational modification to mitochondrial protein thiols disrupts mitochondrial function and contributes to cell injury in response to chronic alcohol.

Labeling Mitochondrial Protein Thiols with IBTP



IBTP Labeling to Mitochondrial Protein Thiols in Cells



Lin et al. *J. Biol. Chem.* 277: 17048-17056, 2002

Experimental Design – IBTP labeling of mitochondrial protein thiols

Mito isolated from liver of control and ethanol-fed rats



Mito (1 mg/mL) in respiration buffer (succinate/ADP)



Incubation with IBTP (5 μ M) for 10 min



Reaction stopped with iodoacetate

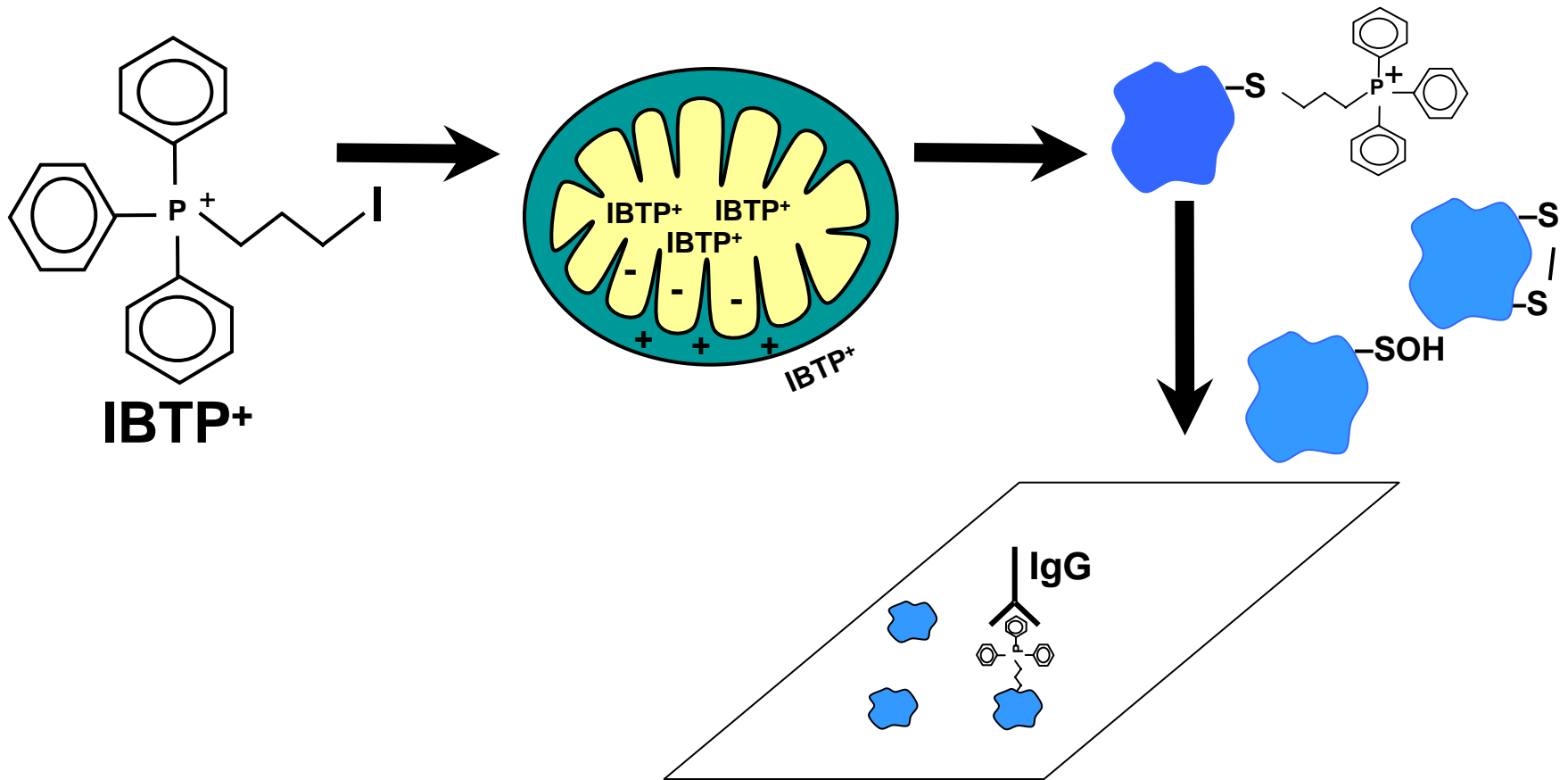


Mito centrifuged, pellets stored -80°C



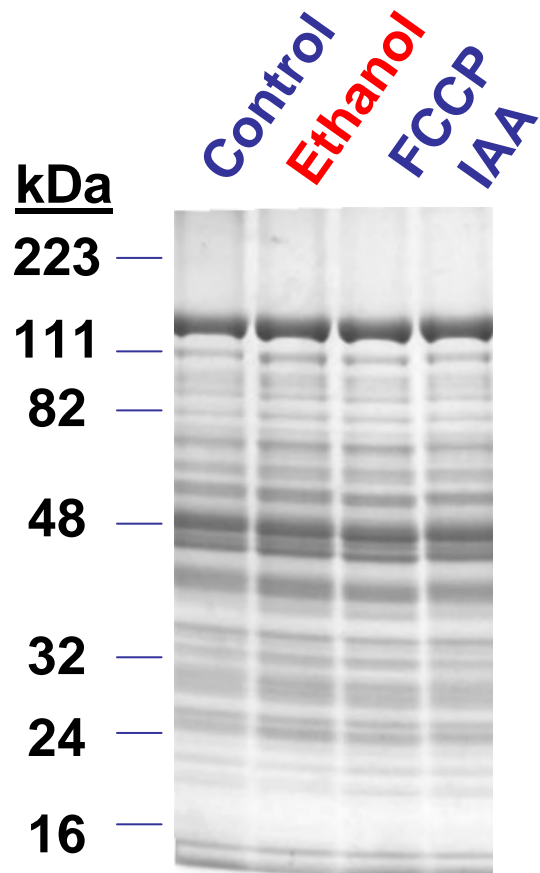
Gel electrophoresis and immunoblotting with anti-TPP

Immunoblot detection of IBTP-labeled proteins

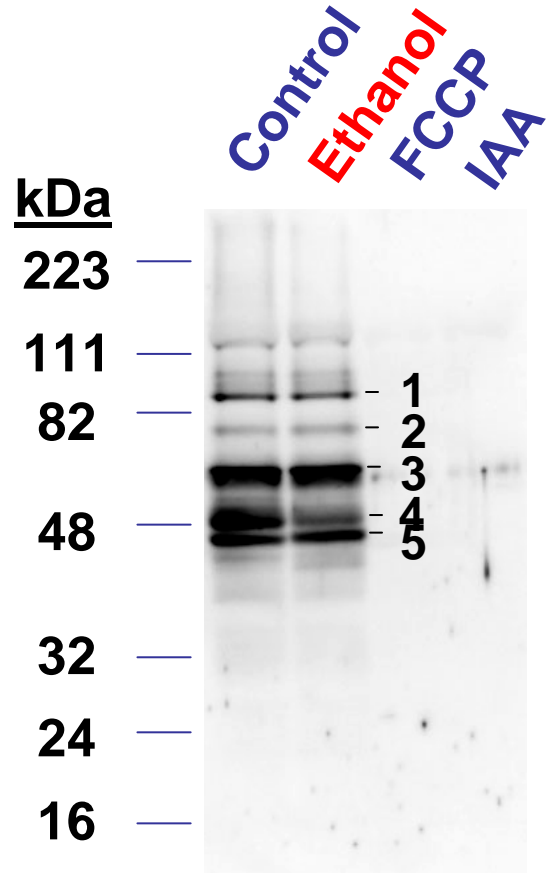


Decrease in labeling indicates oxidized/modified thiols

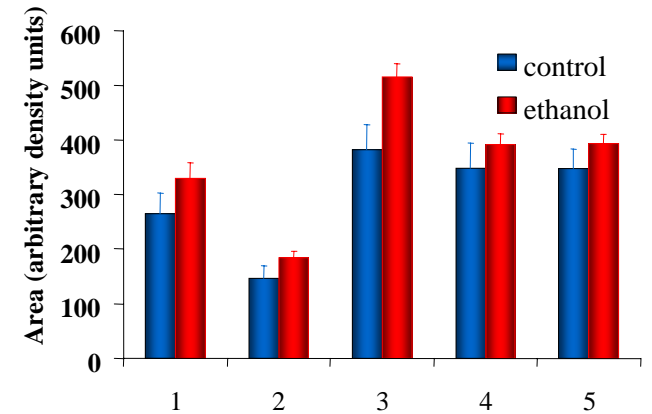
Labeling of Mitochondria Protein Thiols with IBTP



Gel

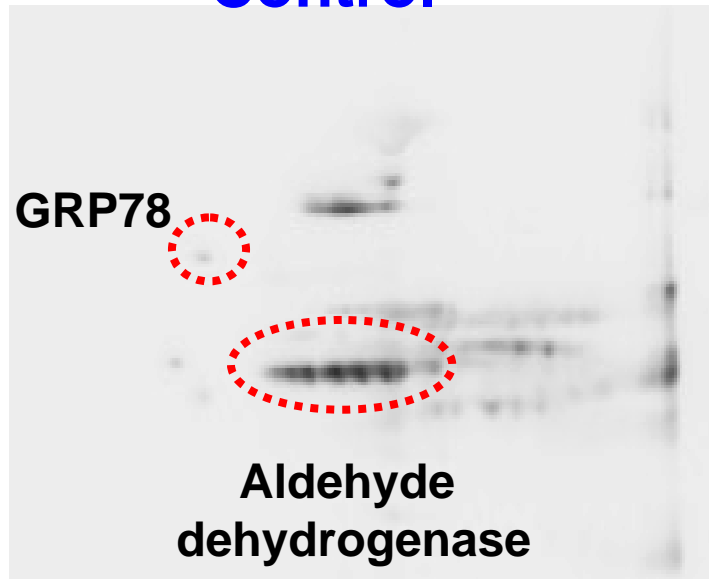


Blot

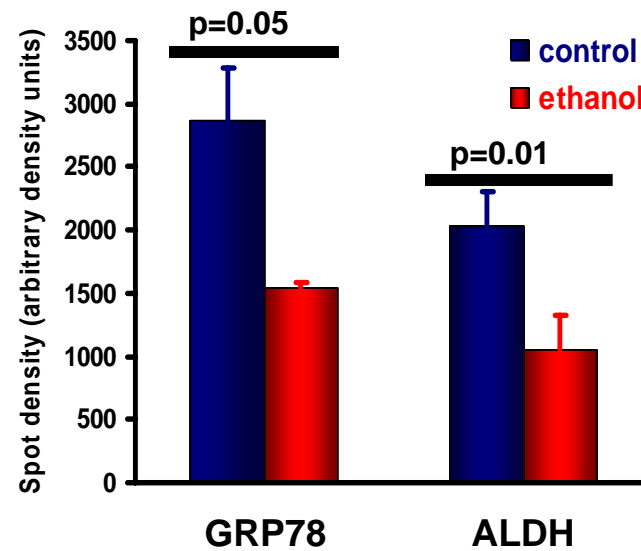


Decreased IBTP Immunoreactivity in GRP78 and ALDH

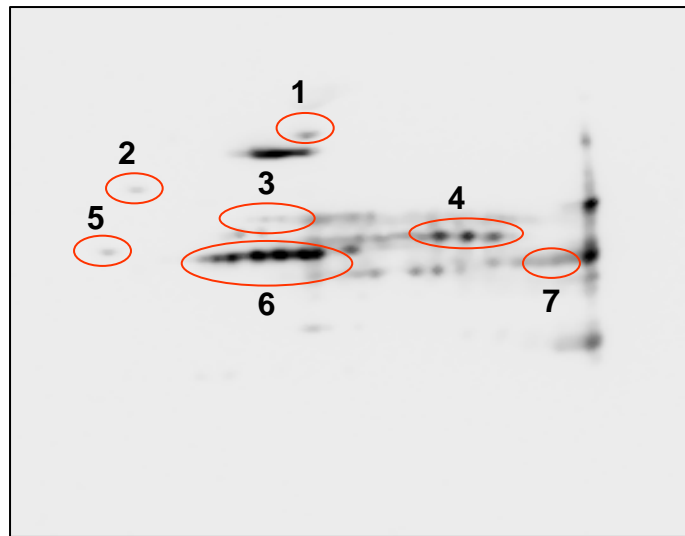
Control



Ethanol



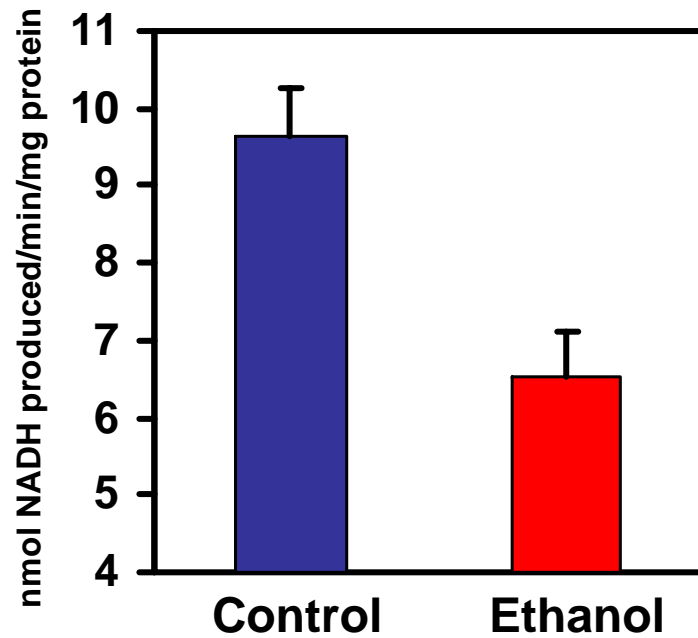
Mitochondrial proteins identified as containing IBTP-reactive thiols



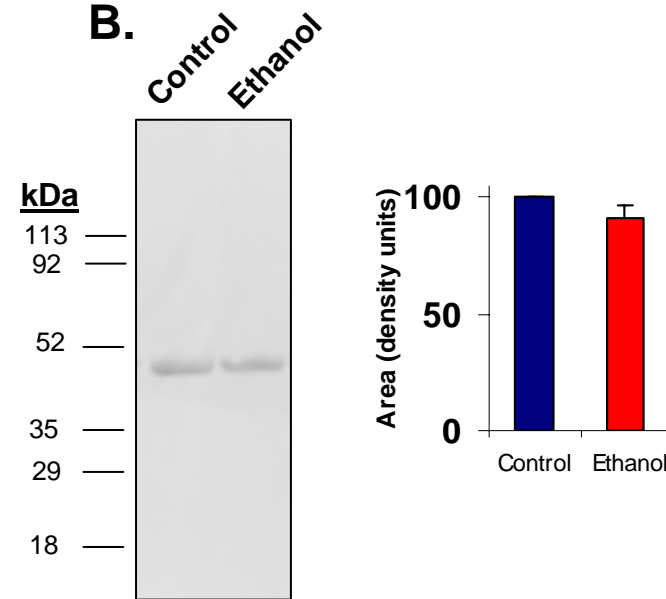
<u>Spot no.</u>		Mass (kDA)	MOWSE score	No. peptides matched/un- matched	Fold change ethanol
1	Pyruvate carboxylase	129.6	195	28/49	1.43(0.2)
2	GRP78	72.1	194	28/74	0.57(0.1)
3	Hsp60	60.9	90	8/13	0.91(0.1)
4	Glutamate dehydrogenase	56	98	8/15	0.53(0.2)
5	Protein disulfide isomerase	56.9	123	10/9	
6	ALDH	53	135	12/15	0.56(0.1)
7	Acetyl-coenzme A acyl transferase 2	41.8	79	7/13	1.67(0.7)

Chronic alcohol decrease low K_m ALDH activity

A.



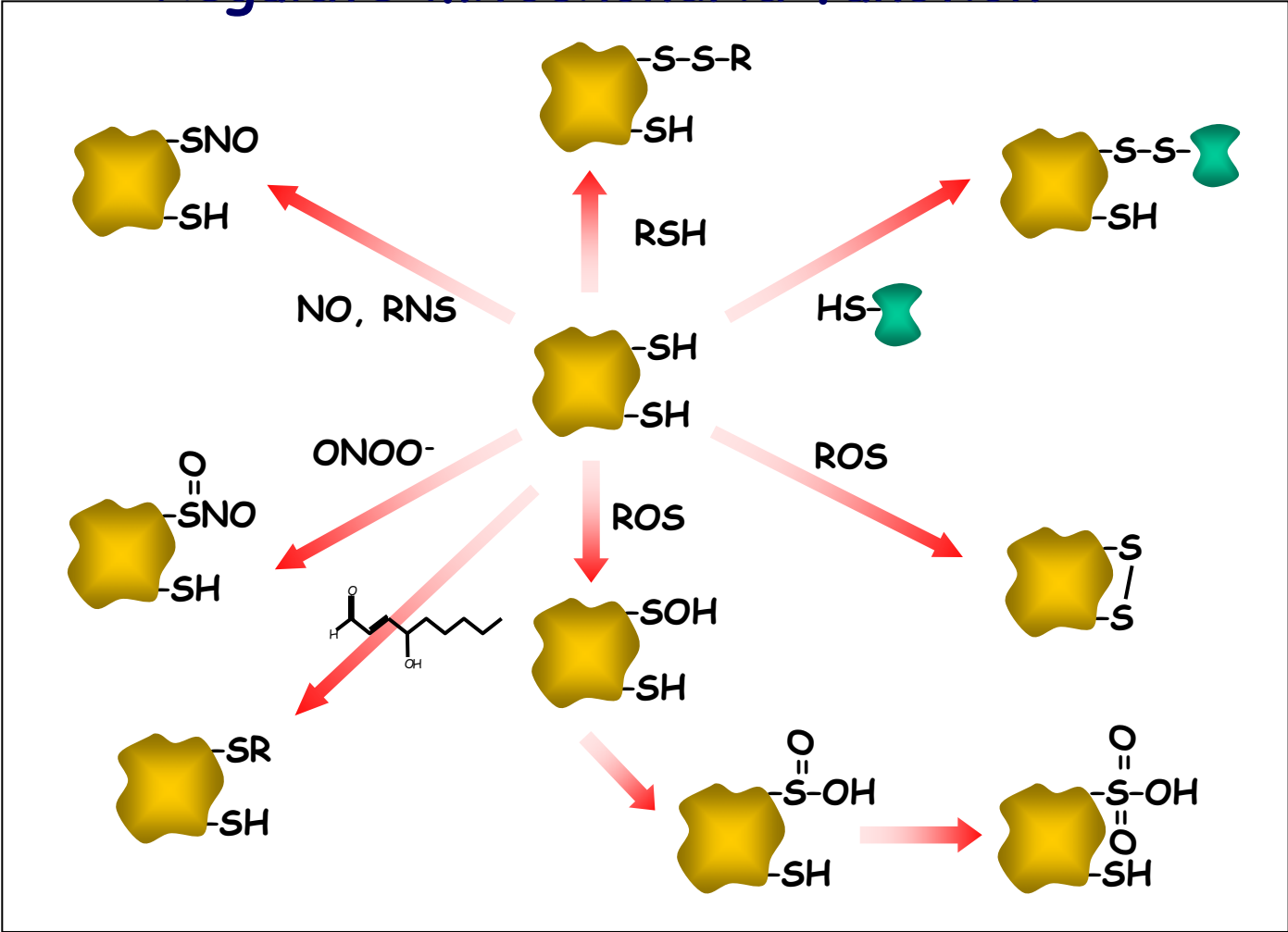
B.



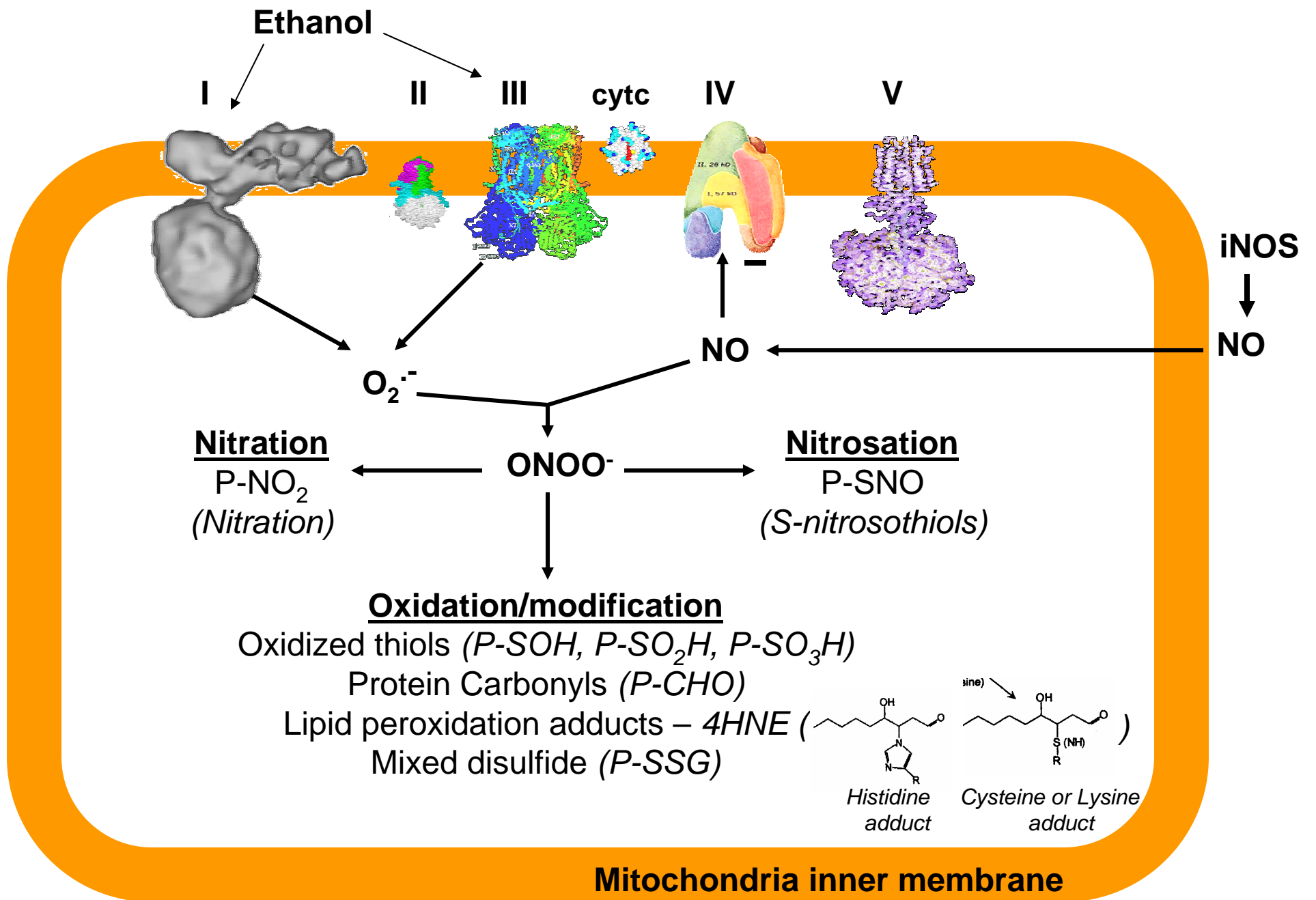
Irreversible oxidation to active site Cysteine

Protein Thiols As A Molecular Switch

Regulate mitochondria function



Cooper et al. Trends Biochem. Sci. 2002



Mitochondria Proteomic Approaches

1. Abundances
2. PTMs

