Progress Towards Crystallization of Transhydrogenase (TH) holoenzyme from *E. Coli*.

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Introduction

Nicotinamide nucleotide Transhydrogenase (TH) (EC 1.6.1.1) is an essential component of respiratory apparatus in the mitochondria and bacteria [1-4]. TH is a homodimer of α and β subunits, $\alpha_2\beta_2$ (200kDa) forming a tetramer on the membrane[5]. α subunit (52kDa) consists of over 500 residues whereas, β (48kDa) subunit comprises of over 450 residues. The soluble domain of α and β subunits that is involved in nucleotide binding and hydride transfer is referred as domain I and domain III respectively. The membrane integrated region involved in proton translocation is called domain II [6]. TH is essentially involved in maintenance of physiological levels of NADP(H) for biosynthesis and detoxification in the cytoplasm. It is also actively involved in the regulation/production of reduced glutathione in the mitochondria to prevent/overcome oxidative stress through free radicals/peroxides by supporting glutathione peroxidation reaction [7-10]. A microcycle connecting TH with isocitrate dehydrogenase has also predicted its role in regulation of Krebs cycle in many cell types [11]. In *E. coli* defects in TH gene or gene product may lead to altered pathways of amino acid metabolism. Recent studies on mice with impaired control of insulin secretion and glucose homeostasis leading to diabetes type 2 has revealed the role of TH in this phenotype [12].

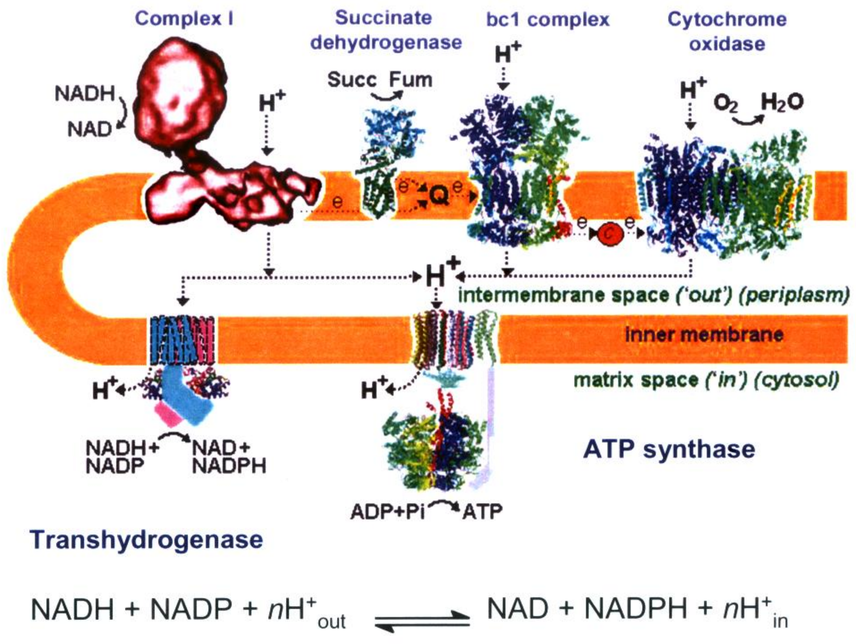

Fig.1 Respiratory complexes in mitochondria

TH couples hydride equivalents transfer between NAD(H) and NADP(H) through its two soluble domains (domain I and III) respectively with proton translocation associated conformational changes with the integral membrane component (domain II). One proton is translocated across the membrane through transhydrogenase per hydride ion transferred between nucleotides [4]. TH, like ATP synthase, uses the proton motive force in the inward direction to maintain a high NADPH concentration inside mitochondria. Earlier works with 3D-structures of soluble domain of dI_2-$dIII_1$ has revealed the mechanism of hydride transfer in great detail [13,14] . Now the aim of current work is to crystallize TH holoenzyme to understand the biochemistry of proton translocation in conjunction with hydride transfer.

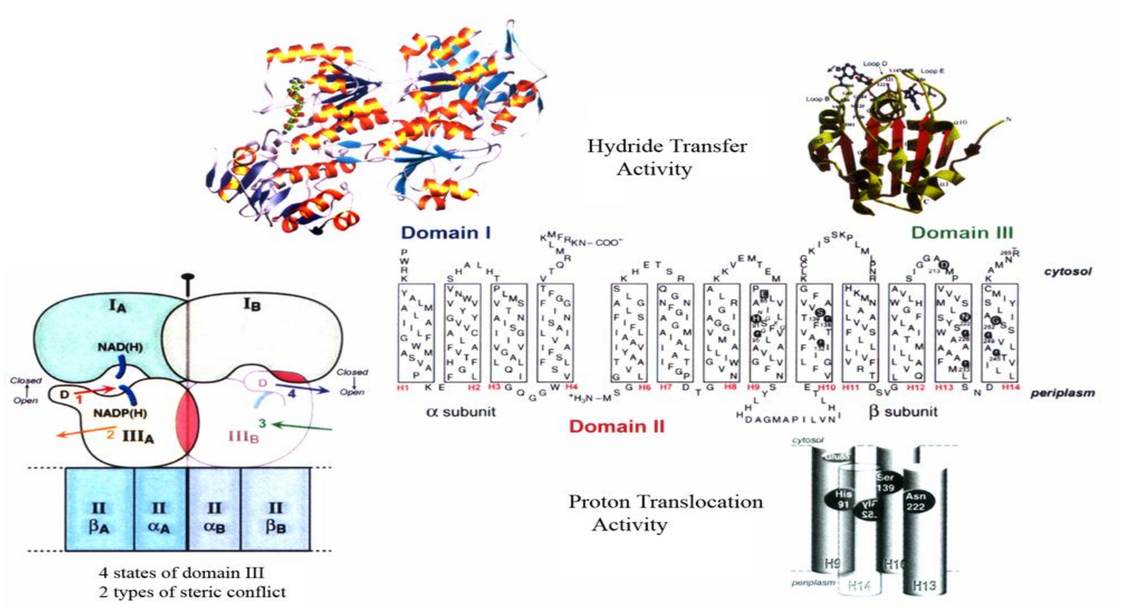

Fig.2 Structures of TH components

Methods

Like any other integral membrane protein crystallization, the challenge towards crystallization of TH was over expression and purification of highly pure, viable, active and homogeneous sample. We have developed a standard lab manufacturing protocol to purify stable and active sample for the crystallization experiments of TH complex.

O/N Culture grown in LB supplemented with ampicillin

⇩

1% O/N culture inoculation in fresh LB amp media, growth at 37^0C, 200rpm

⇩

Induction with 1mM IPTG at OD_{600nm} 1.4-1.5 and continue growth for 4-5 hrs at same temperature

⇩

Sonicate the pellet of E.Coli in Buffer (20mM Tris-Cl, 300mM NaCl, lysozyme 100ug/ml, protease inhibitor cocktail)

⇩

Remove unbroken cells and cell debris at 9,800g

⇩

Collect supernatant and ultracentrifuge at 129,000g for 30 min at 4^0C

⇩

Collect pellet and solubilize in buffer (20mM Tris-Cl, 300mM NaCl, 2%DHPC, protease inhibitor cocktail for 2h at 4^0C

⇩

ultracentrifuge at 129,000g for 30 min at 4^0C

⇩

Collect solubilized fraction and incubate with the pre equilibrated His Bind Resin (Novagen) for12-15h at 4^0C

⇩

Wash resin with the suitable detergent in buffer 20mM Tris-Cl, 300mM NaCl, 10% glycerol (50/100/150mM imidazole)

⇩

Elute in 250mM imidazole in buffer (20mM Tris-Cl, 300mM NaCl, 10% glycerol) in the presence of detergent.

⇩

ultracentrifuge at129,000g for 30 min at 4^0C to remove high molecular mass particles and aggregates

⇩

Concentrate protein using 50kDa mwco membrane

Buffer exchange to the crystallization buffer

⇩

Airfuge at 40psi to remove aggregates before setting up drops

Buffer exchange to 20mM Tris-Cl (pH 8.0), 50mM NaCl, 10% glycerol+detergent

⇩

Bind to DEAE-Sepharose resin, wash with suitable buffer and elute in desired buffer with detergent

⇩

Concentrate using 50kDa mwco membrane and buffer exchange to crystallization buffer

⇩

Airfuge at 40psi to remove aggregate particles and set up drops

Results

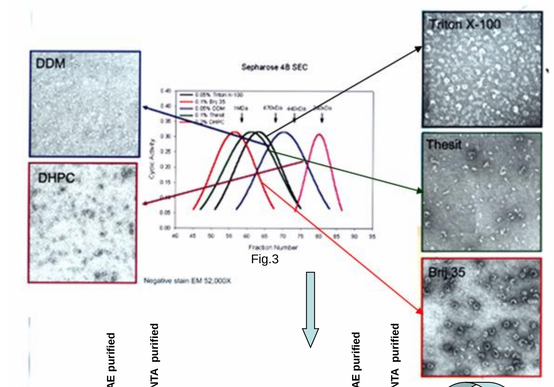

Fig.3

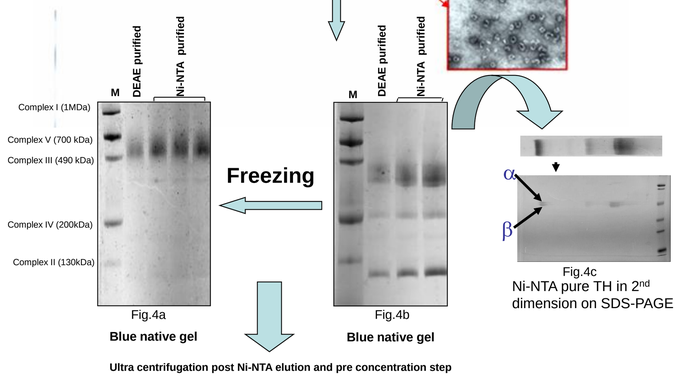

Fig.4a Fig.4b

Fig.4c
Ni-NTA pure TH in 2nd dimension on SDS-PAGE

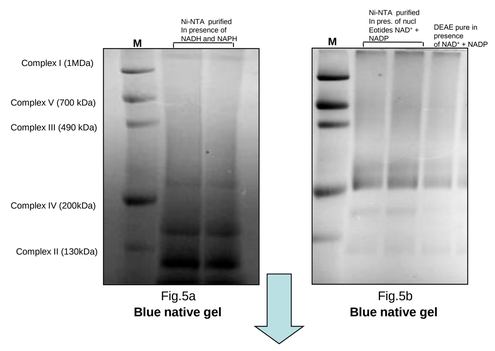

Fig.5a Fig.5b

Detergents	TH in Low Salt 25mM	TH in High salt 500mM
Beta-OG	Unstable (precipitate)	Unstable (precipitate)
Beta-NG	Unstable (precipitate)	Unstable (precipitate)
Cyglu-3	Unstable	Unstable
Cymal-5	Unstable	Unstable
Cymal-6	Unstable	Unstable
Lysofoscholine-14	Unstable (precipitate)	Unstable (precipitate)
LDAO	Polypeptides dissociate	Polypeptides dissociate
Zwittergent 3-12	Polypeptides dissociate	Polypeptides dissociate
DM	Less Stable	Less Stable
DHPC	Stable	Stable
DDM	Stable	Stable
Foscholine -12	Stable	Stable
Foscholine -14	Stable	Stable
Novel detergents (from Qinghai Zhang)		
179 Fos	Stable	Stable
138 Fos	Stable	Stable
234-Chol	Stable	Stable

Table1

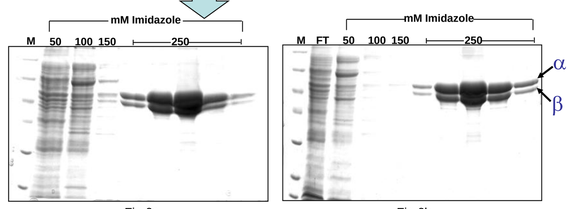

Fig.6a Fig.6b
SDS-PAGE: Ni^{2+}-NTA purification of TH in DDM SDS-PAGE: Ni^{2+}-NTA purification of TH in Focholine-14

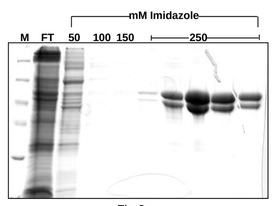

Fig.6c
SDS-PAGE: Ni^{2+}-NTA purification of TH in DHPC

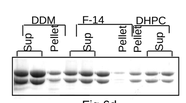

Fig.6d
SDS-PAGE analysis of TH post airfuge in detergents

- Solubilization of membrane in Triton X-100, Thesit and Brij35 form vesicular structures and aggregates of very high molecular mass ranging to 1MDa (fig.3).
- DDM and DHPC form more homogeneous Particles ranging between 200-500kDa (fig.3).
- DHPC forms particles that migrates as 240kDa complex (fig.3).
- Blue native PAGE analysis on TH sample revealed heterogeneous mixture of species in DDM ranging from 100-400kDa (fig.4b).
- Freezing the samples leads to more aggregation and formation of 400kDa and higher molecular mass particle (fig.4a).
- Blue Native PAGE sample in 2nd dimension on SDS-PAGE (fig.4c) revealed (αβ) as the building unit of multimers or high molecular mass species; α=β. It further revealed heterogeneous species as (αβ), $(\alpha\beta)_2$, $(\alpha\beta)_3$ and $(\alpha\beta)_4$.
- An ultra centrifugation step post Ni-NTA purification removes high molecular mass species. Further concentration of sample for crystallization remains homogeneous.
- Addition of nucleotides during solubilization and further purification maintains TH in $(\alpha\beta)_2$ form (fig.5b). The sample is suitable for crystalli -zation purpose. However, such phenomenon does not hold true for N-terminal his tag sample (fig.5a).
- Following the standardized protocol many detergents were tested for their ability to stabilize TH for crystallization purpose in high as well as low salt. Detergents marked in Pink in the table 1 didn't stabilize TH, detergent in green could stabilize TH marginally while detergents in cyan stabilized TH efficiently.
- Airfuge analysis among DDM, DHPC and foscholine-14 revealed that foscholine-14 is one of the most efficient detergent for TH to keep it in solution at higher concentration
- Detergent efficiency: Foscholine-14>DDM>DHPC (fig.6d)

Conclusion

- ✓ DHPC is the detergent of choice for solubilization of TH membrane extract
- ✓ αβ is the assembly unit for higher oligomers
- ✓ Freezing of TH leads to aggregation
- ✓ Nucleotides are necessary for maintaining homogeneity of TH sample
- ✓ Ultracentrifugation post Ni-NTA is essential to remove aggregates from the chelating column elutes
- ✓ Foscholine-14 and DDM stabilize TH and are favorable for crystallization experiments

Our approach toward crystallization of TH has addressed over-expression, yield optimization, membrane extraction, and purification together with control of cysteine oxidation and maintenance of homogeneity and monodispersity, which are evaluated at each stage of the preparation. Key aspects of the biochemical procedures include the use of several His-tagged constructs, introduction of stabilizing mutations, use of osmolytes, ultracentrifugation, and selection of detergents. Enzyme activity is evaluated in conjunction with negative stain electron microscopy, Blue-native PAGE analysis, and size exclusion and QHP ion exchange chromatography to monitor homogeneity. These experiments have established that monodisperse TH assembles as a 100 kDa αβ monomer, which dimerizes in the membrane or upon concentration in detergents.

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