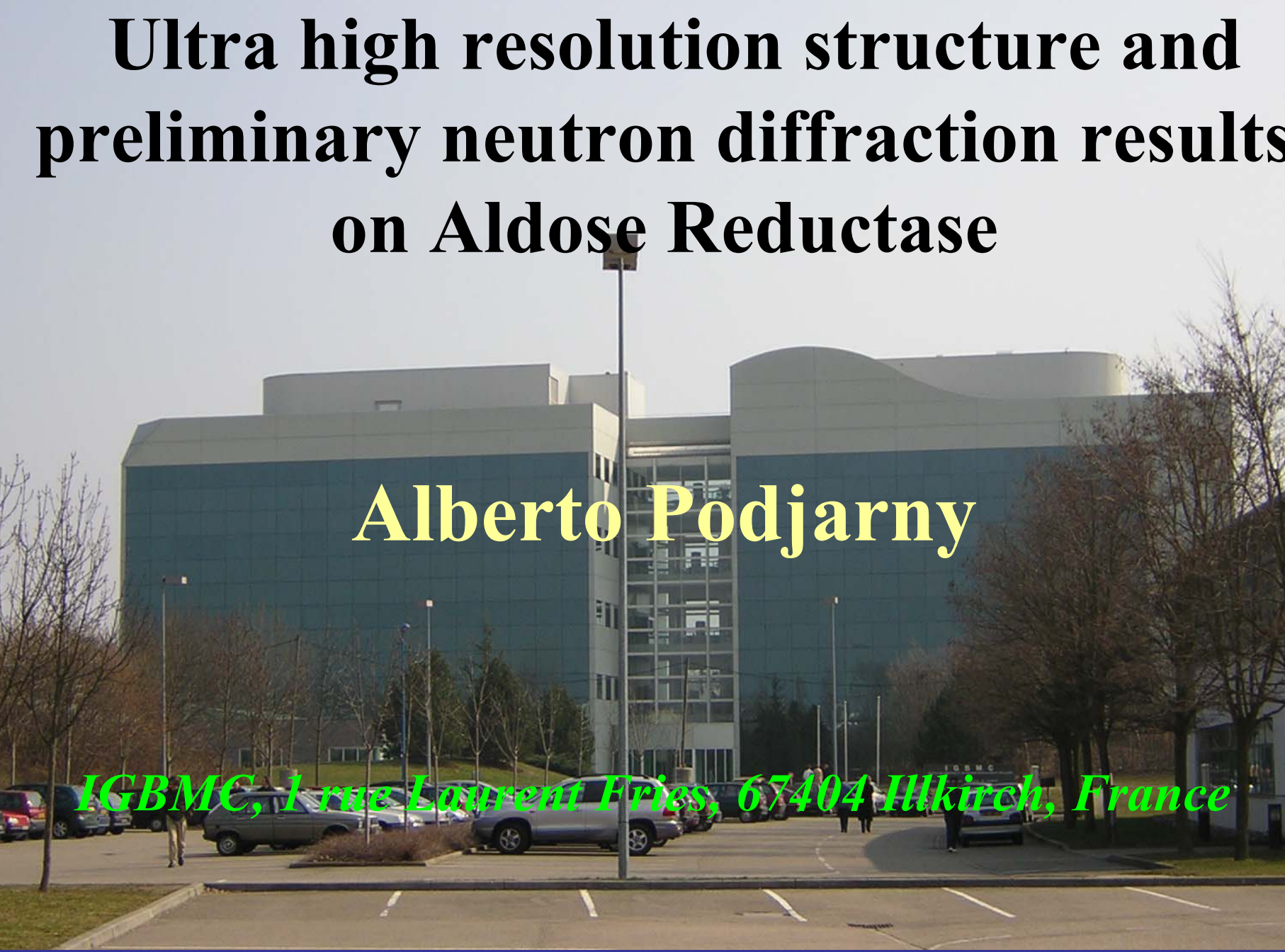
Ultra high resolution structure and preliminary neutron diffraction results on Aldose Reductase



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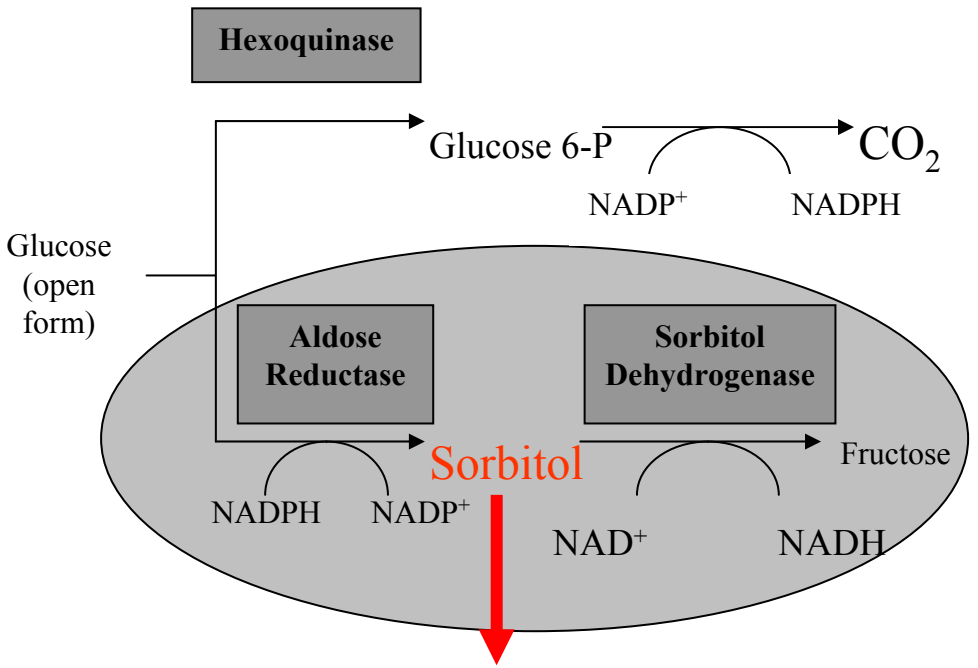
C. Schulze and T. Tomizaki

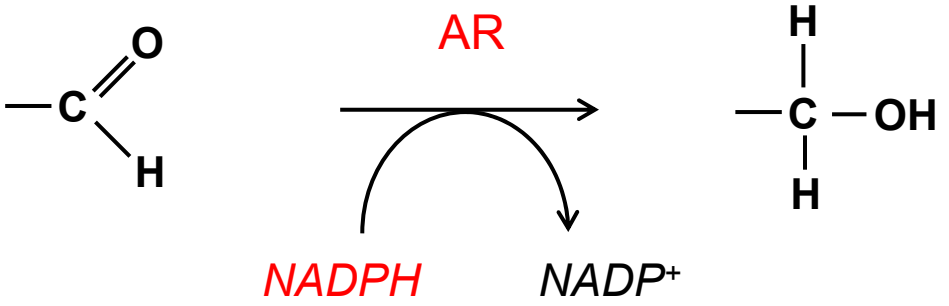
The Polyol pathway

Cellular glucose is phosphorilated into glucose 6-phosphate by Hexoquinase under normoglycemia conditions. Only a minor part of glucose goes into an alternative route named polyol-pathway (grey ellipsoid in the figure). Aldose is reduced to sorbitol by Aldose Reductase and sorbitol converted to fructose by Sorbitol dehydrogenase



LINKED TO DIABETIC COMPLICATIONS

The enzymatic reaction

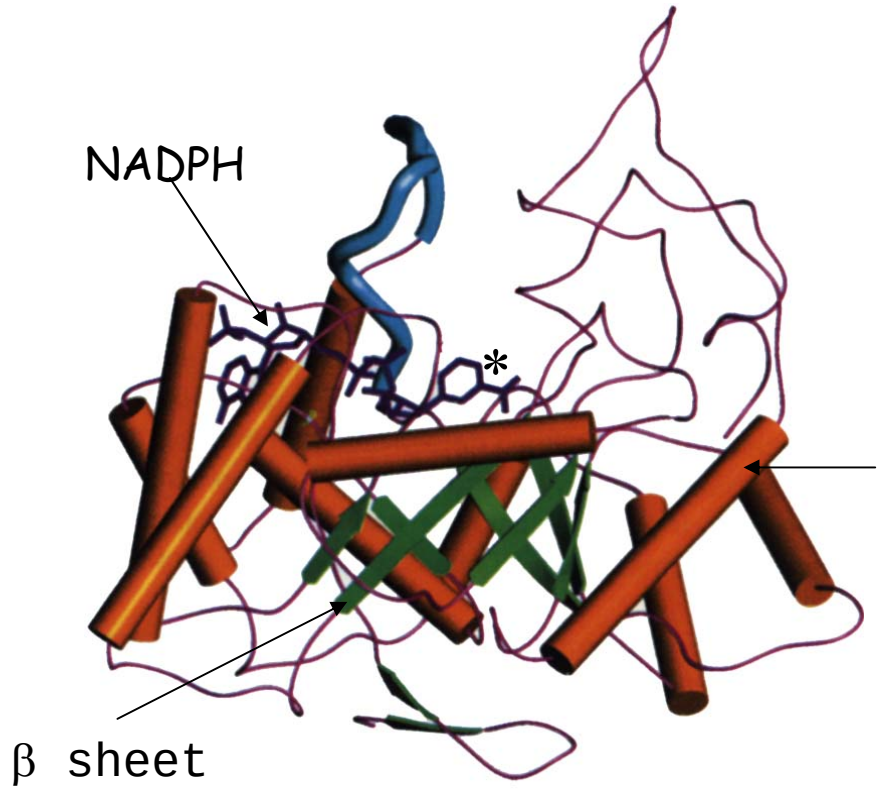


H^- (NADPH)

H^+ (enzyme)

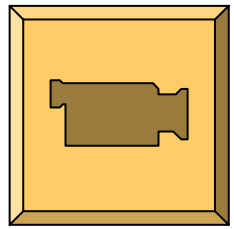
The enzymatic reaction involves a hydride donation from the NADPH coenzyme and a proton donation from the enzyme

3D structure



* Hydride donor

α helix



The structure originally solved from the pig enzyme (1989) showed a TIM barrel structure, with the coenzyme sitting on top of the barrel

' Novel NADPH binding domain revealed by the crystal structure of Aldose Reductase'. J.M. Rondeau,

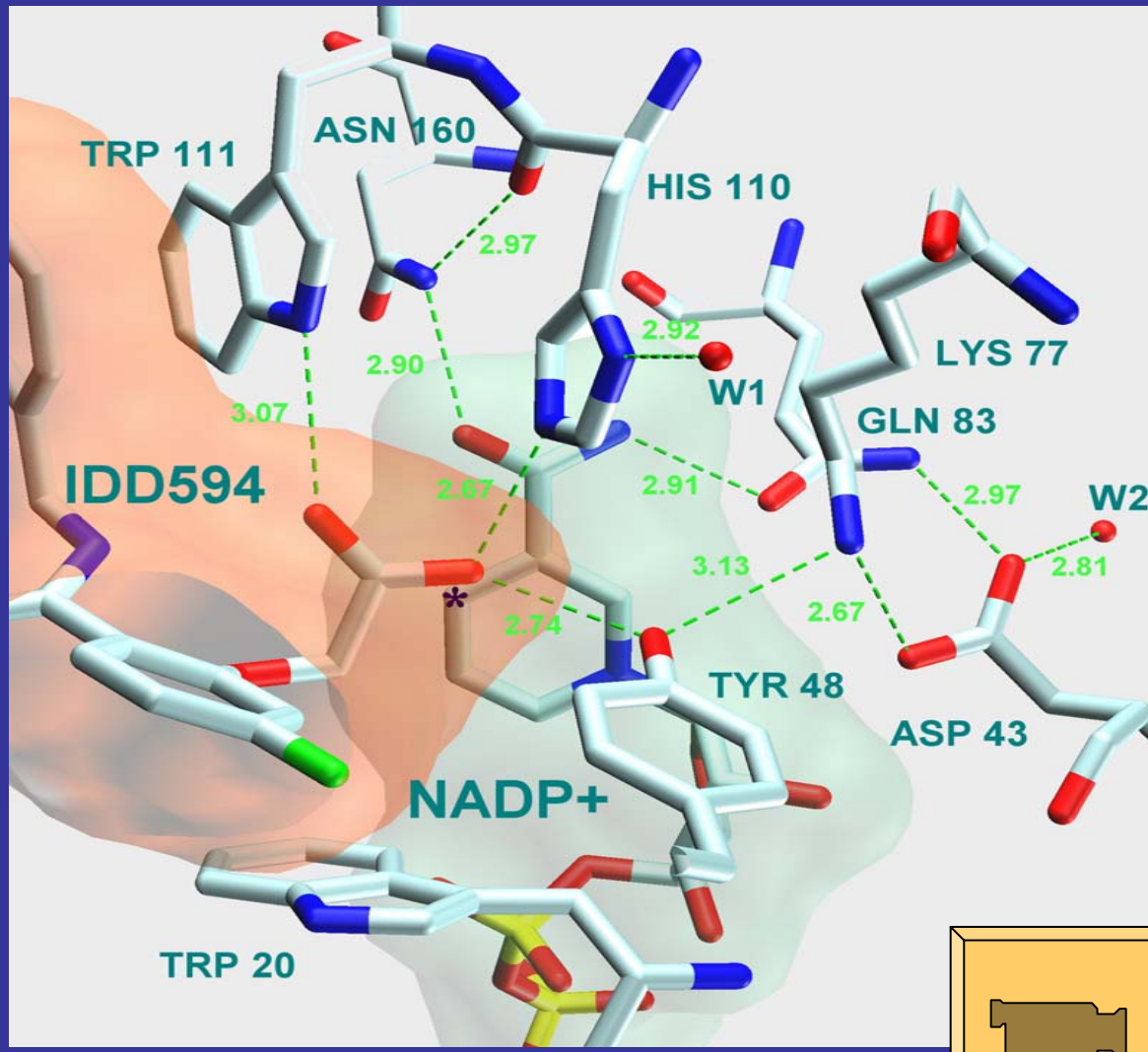
The catalytic site

The COO- head interacts in the catalytic site, which has a dense network of H-bonds around the NADP+

The proton donor can

be either His 110 or Tyr 48

Note that the proximity of NADP^{+} and Lys 77



ID 19
APS frame

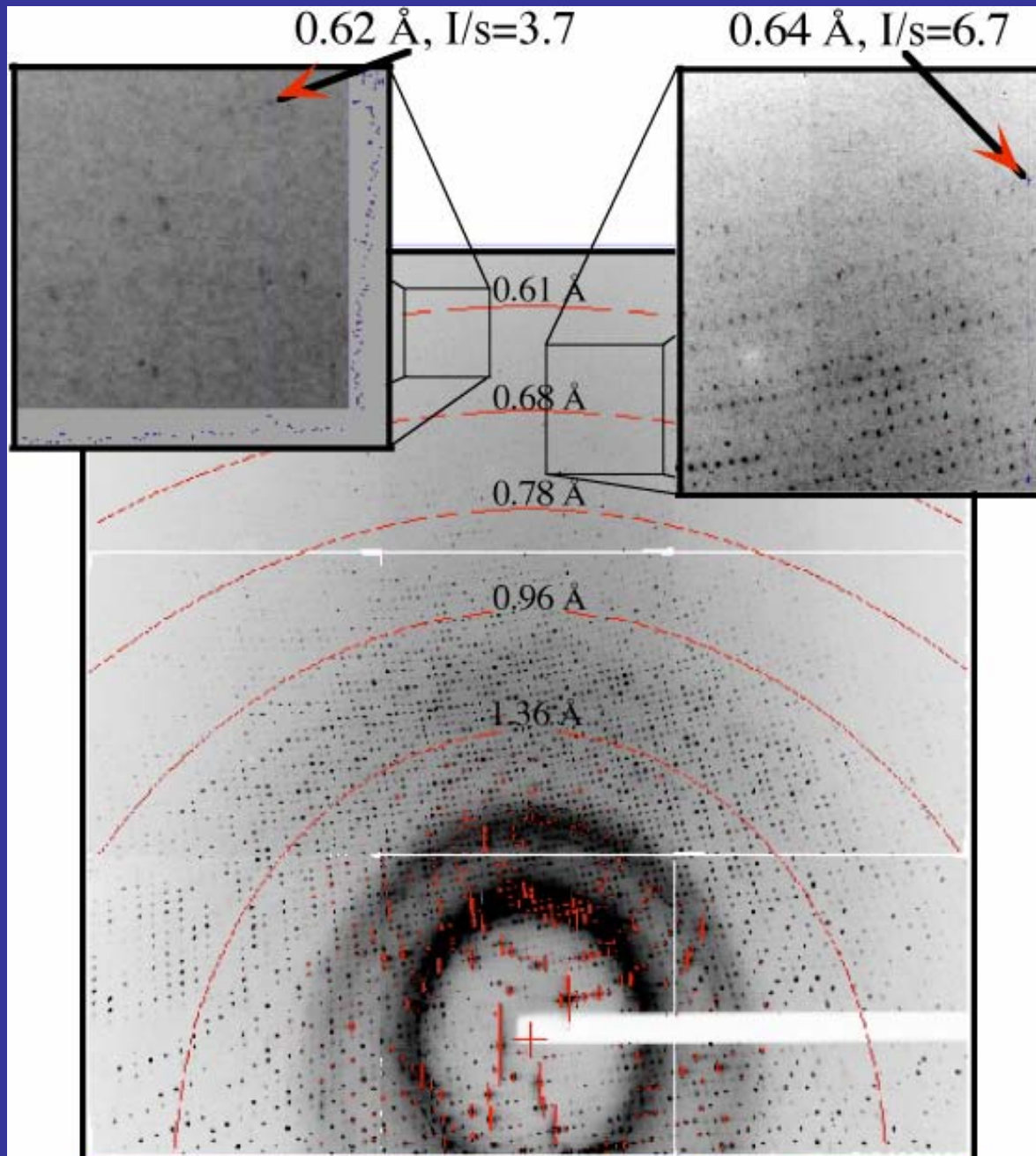
λ=0.65785 A

0.62 A
diffraction
is so far the highest for a
36 kDa enzyme

R-Factor: 8.4%

A. Joachimiak
N. Sanishvili
SBC

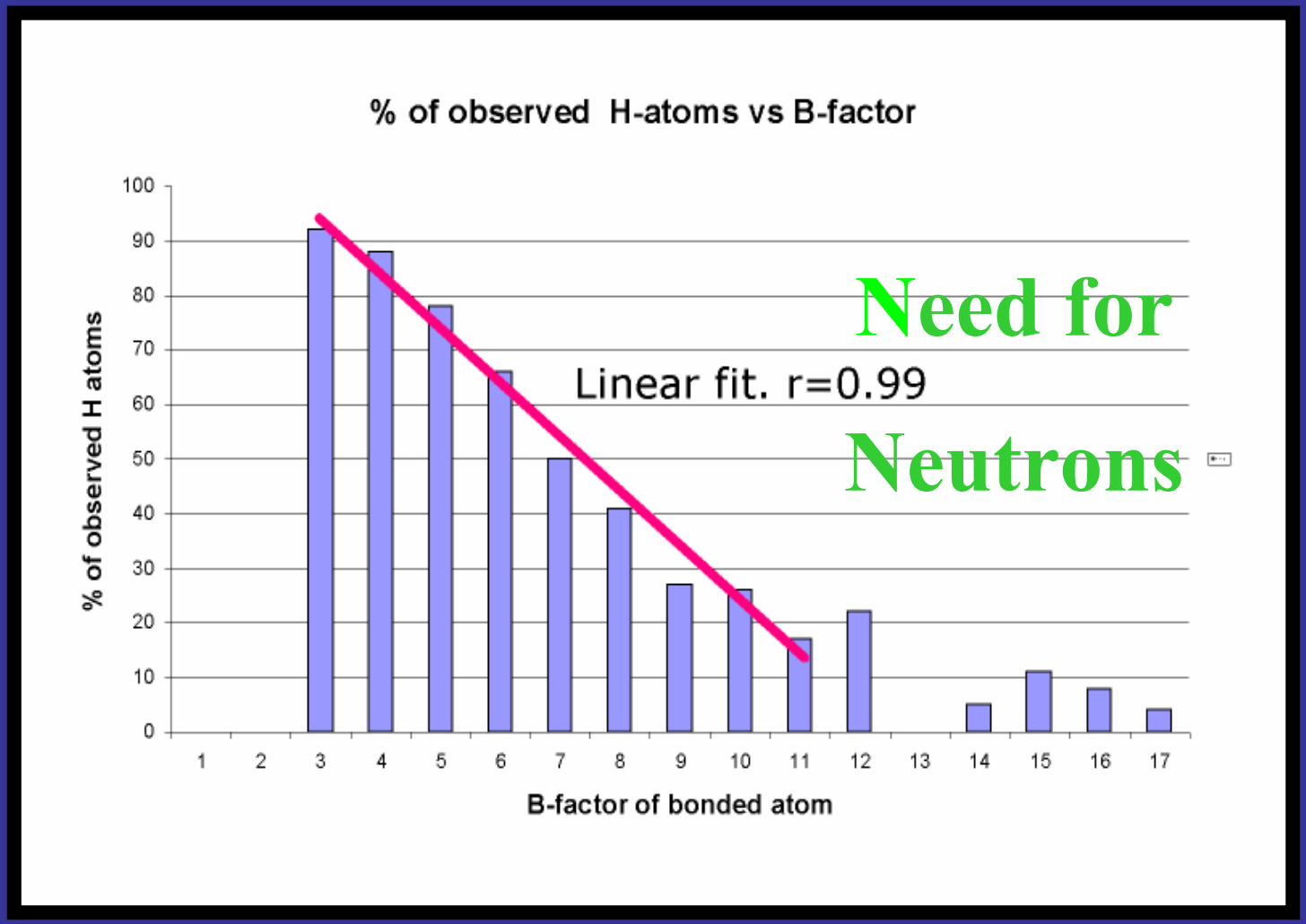
Helium-cooled 15K
R-factor: 7.2%
(T.Petrova)



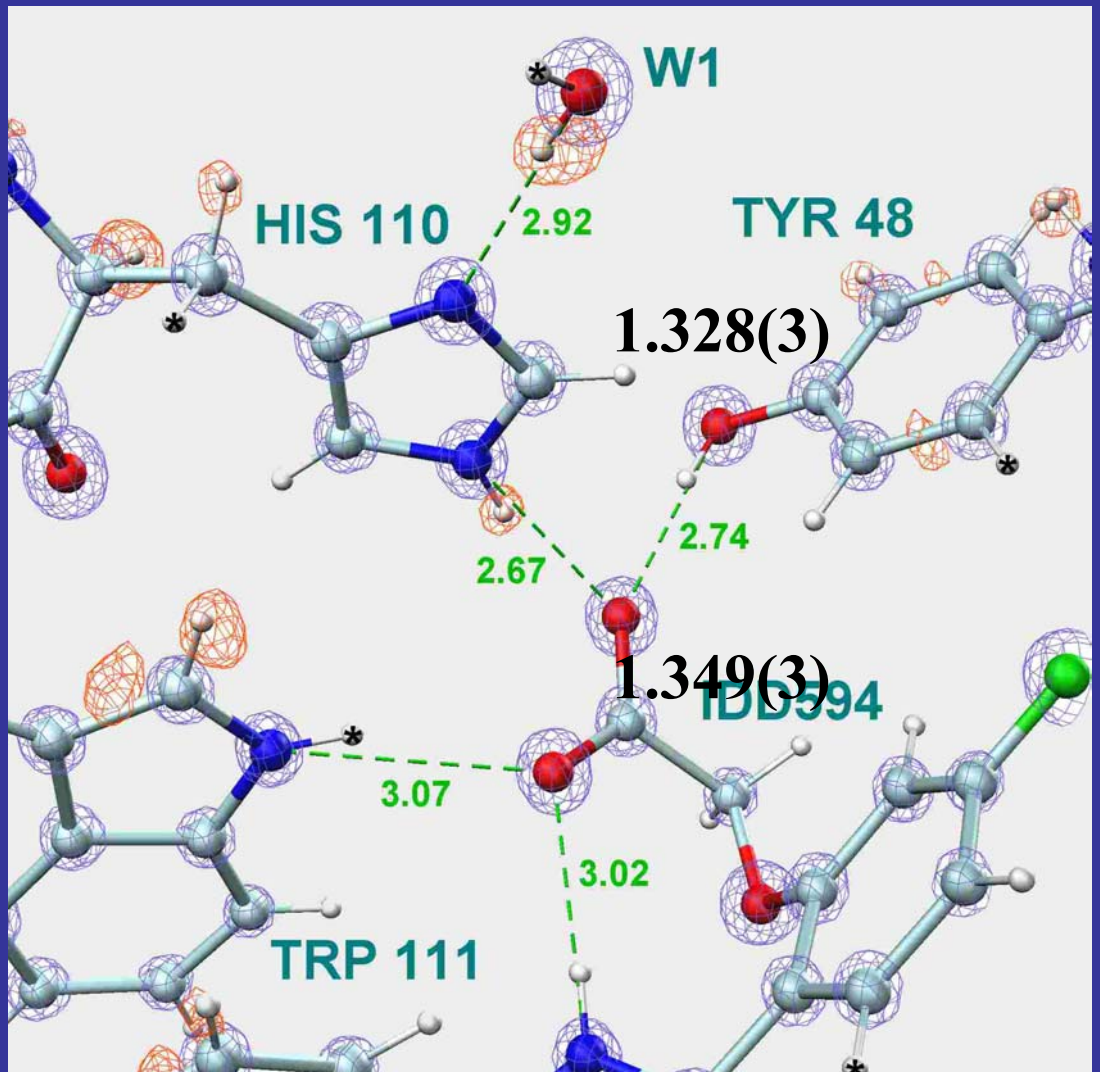
X-Ray diffraction of h-AR-IDD594 resolution 0.66 Å (100K) (M.W. = 36kD).

in the protein only 54 % of all hydrogen atoms are seen,

in the catalytic site 77 % , *but not always where you expect !.*



H-bonds of COO- head seen at 0.66 A resolution



Why Neutron diffraction of fully deuterated proteins ?

- H atoms are of great interest but they remain difficult to « see » by X Ray diffraction because of their weak diffracting power

- **Ordered D** atoms are localized unambiguously in the neutron diffraction maps **at positive peak heights** similar to those of C, N or O atoms.

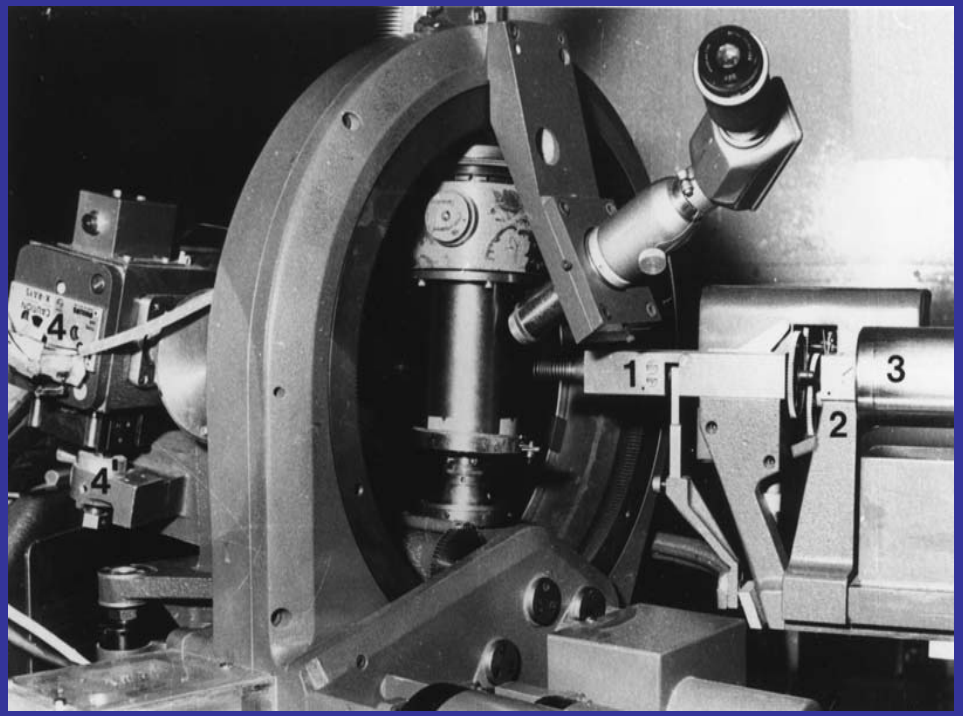
- D-atoms have a much lower incoherent scattering (background) than H-atoms

- Main problem: Low Flux, needing large crystal size!!!

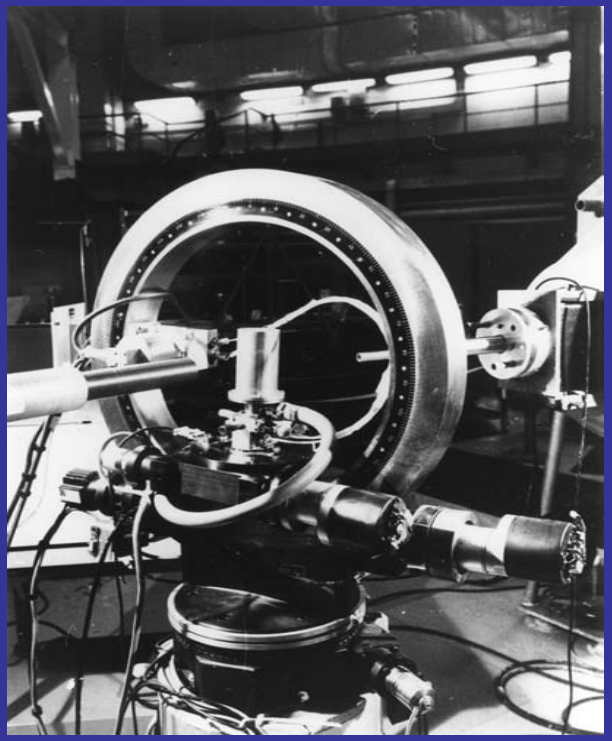
A short point of history (1984)

In **CHEMISTRY**, for organometallic molecules,

Andre Mitschler already combined X-Ray and Neutron data



X-Rays, in-house, Strasbourg



Neutrons, ILL, Grenoble

Rees, Mitschler . J.A.C.S., 98, 7918 (1976)
Mitschler, Rees and Lehman. J.A.C.S., 100, 3390 (1978)
Mitschler, Rees, Wiest and Benard. J.A.C.S., 104, 7101 (1982)

Neutron diffraction

•Scattering centres are the nuclei, **not electrons**

•Each nucleus has a characteristic strong force interaction with a neutron.

•Less variation between the elements # X-Ray

•Interaction can be different for isotopes of the same element.

Atom type	Neutron scattering length, *barns* (10^{-12} cm)	
H	**- 0.374**	**80.27**
D	**0.667**	**2.05**
C	**0.665**	**0**
N	**0.936**	**0.49**
O	**0.580**	**0**
S	**0.285**	
Fe	**0.945**	
Ca	**0.470**	
Mn	**-0.373**	

Direct observation of H or D

• Enzymatic reactions and structure-function relationships

Myoglobin (Shu, 2000),

Endothiapepsin (Coates, 2001)

• Solvent structure and H bonding

Concanavalin A (Habash, 2000 & Blakeley, 2004),

Lysozyme (Bon, 1999)

• Structural kinetics through H / D exchange

Trypsin (Kossiakoff, 1981),

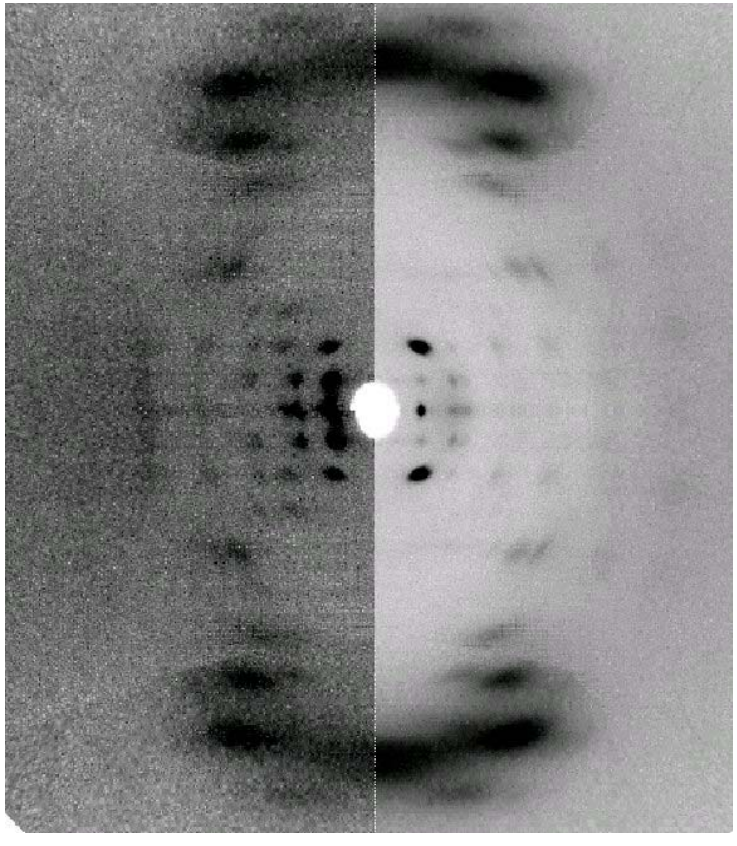
Crambin (Teeter,1984)

Hydrogenated proteins

A-DNA **(H) / H_2O**

A-DNA **(p-D) / D_2**

- Approx. 50% of a protein atoms are H
- About ~25% can be exchanged only
- H = large incoherent scattering cross section
  = large background on detector
  = large reduction of S / N ratio in data.
- H = weak **negative** coherent scattering length,
  = weak negative peak height in maps



Need to produce fully deuterated proteins!

- All H atoms are exchanged ***in vivo*** for **DEUTERIUM**
- Huge reduction of background = diffraction data can be measured !
- No cancellation of density but cooperative enhancement of positive nuclear density

Full deuteration : Culture comparison

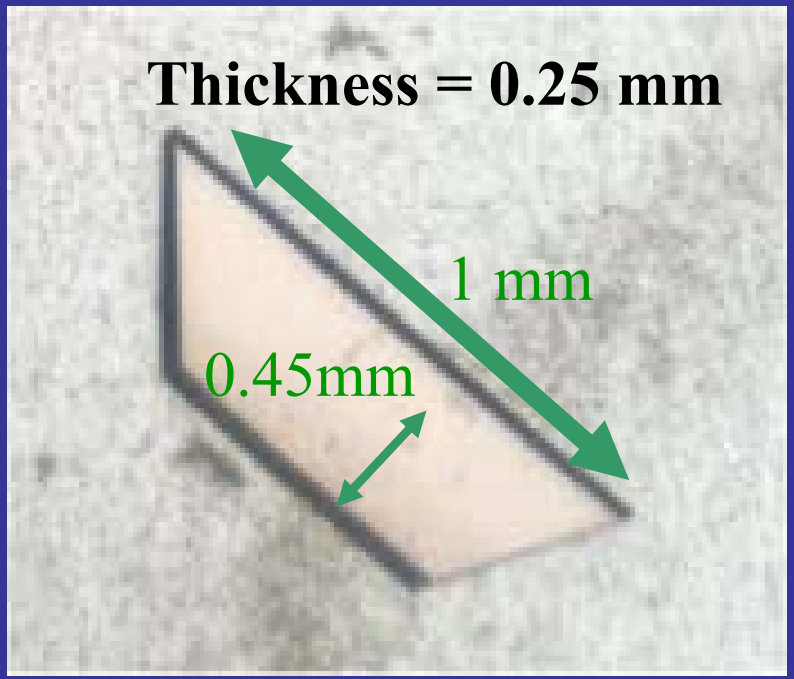
	Deuterated culture (**ILL protocol** *)	Hydrogenated culture
Plasmide	pET28b	pET15b
Strain	E.coli BL21	E.coli BL21
Media	$(NH_4)_2SO_4$ KH_2PO_4 Na_2HPO_4 $(NH_4)_2$ citrate $MgSO_4$ Metal salts : $CaCl_2$ $FeCl_3$ $ZnSO_4$ $CuSO_4$ $MnSO_4$ $CoCl_2$ Na EDT + **deuterated succinic acid**	LB Broth (*Invitrogen*) (Peptone-Yeast extract-NaCl)
Mode	**HCDC** - FERMENTOR (1L)	Erlenmeyer (6x1L)
Induction	0.2 mM IPTG when DO_{600}~3	1 mM IPTG when DO_{600}~0.6
Quantity	60 mg	25 mg

**ILL: I. Hazemann, F. Meilleur, M. T. Dauvergne, M. Haertlein,D. Myles*

Neutron Laue diffraction ternary complex h -AR-NADP $^{+}$- IDD594 $^{-}$ crystals grown by I. Hazemann

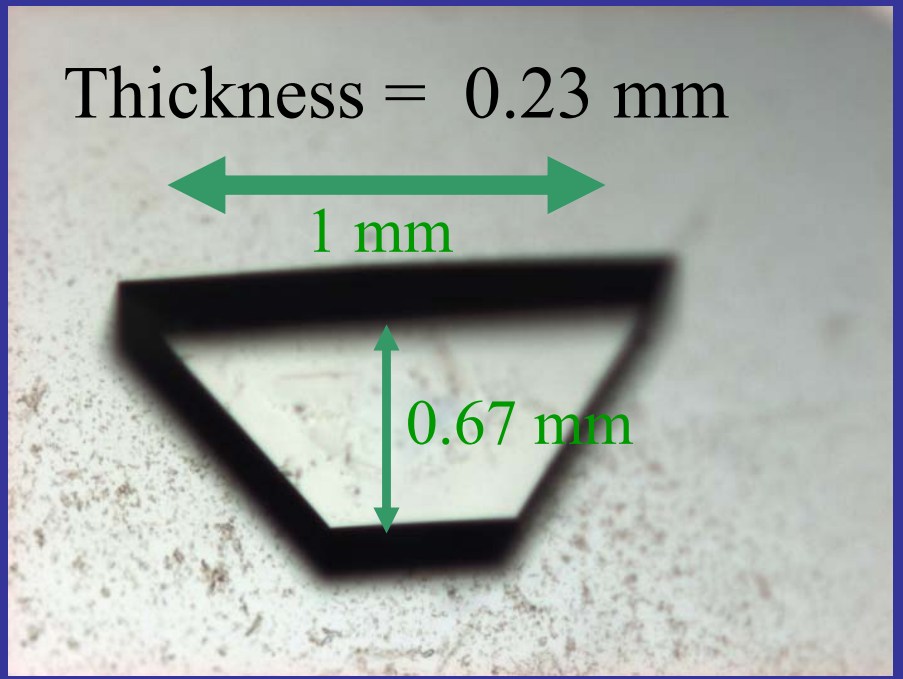
Partially deuterated protein

NO SUCCESS



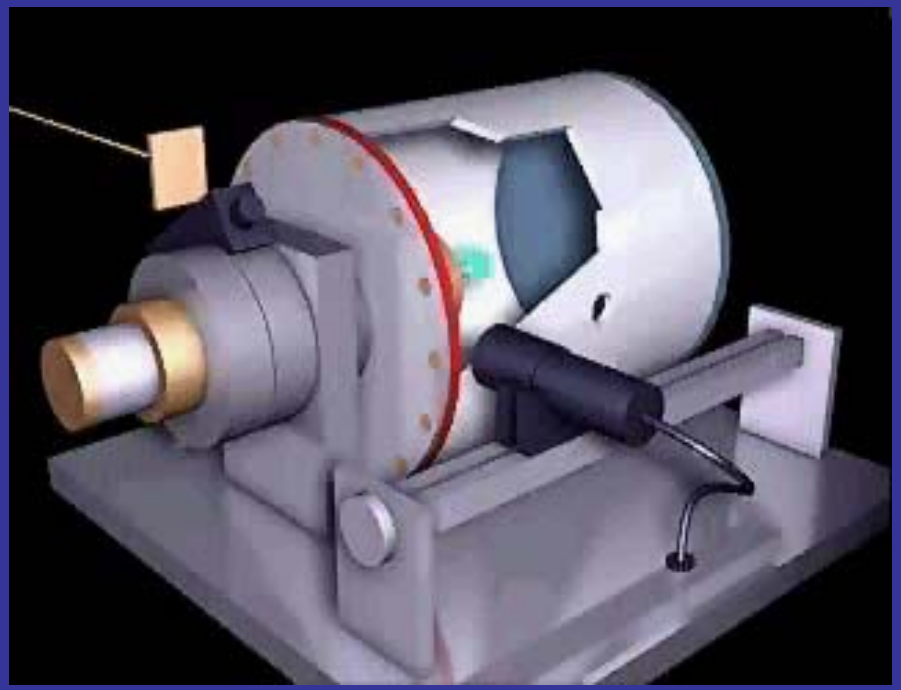
Low resolution : 4.5Å, V = 0.11 mm^3

Fully deuterated protein



Medium resolution : **2.2Å**, V = **0.15** mm^3

The Laue Diffractometer, LADI at ILL –Grenoble - France



• Radius	159.19 mm
• Length	400 mm
• Active area	800 x 400 mm^2
• Angle subtended	144° in T, 52° in v
• Pixel size	200 x 200 μm^2

Monochromators

Laue White beam

Quasi-Laue ($\delta\lambda\backslash\lambda < 30\%$)

Ti/Ni multilayer bandpass filters

Collimation

Pinholes 0.5 to 4mm

Detector

Cylinder covered with image plates

NIP

Gd_2O_3 doped $BaF(Br.I):Eu^{2+}$

Sample

Flux at specimen =3 x 10^7 n cm^{-2} s^{-1}

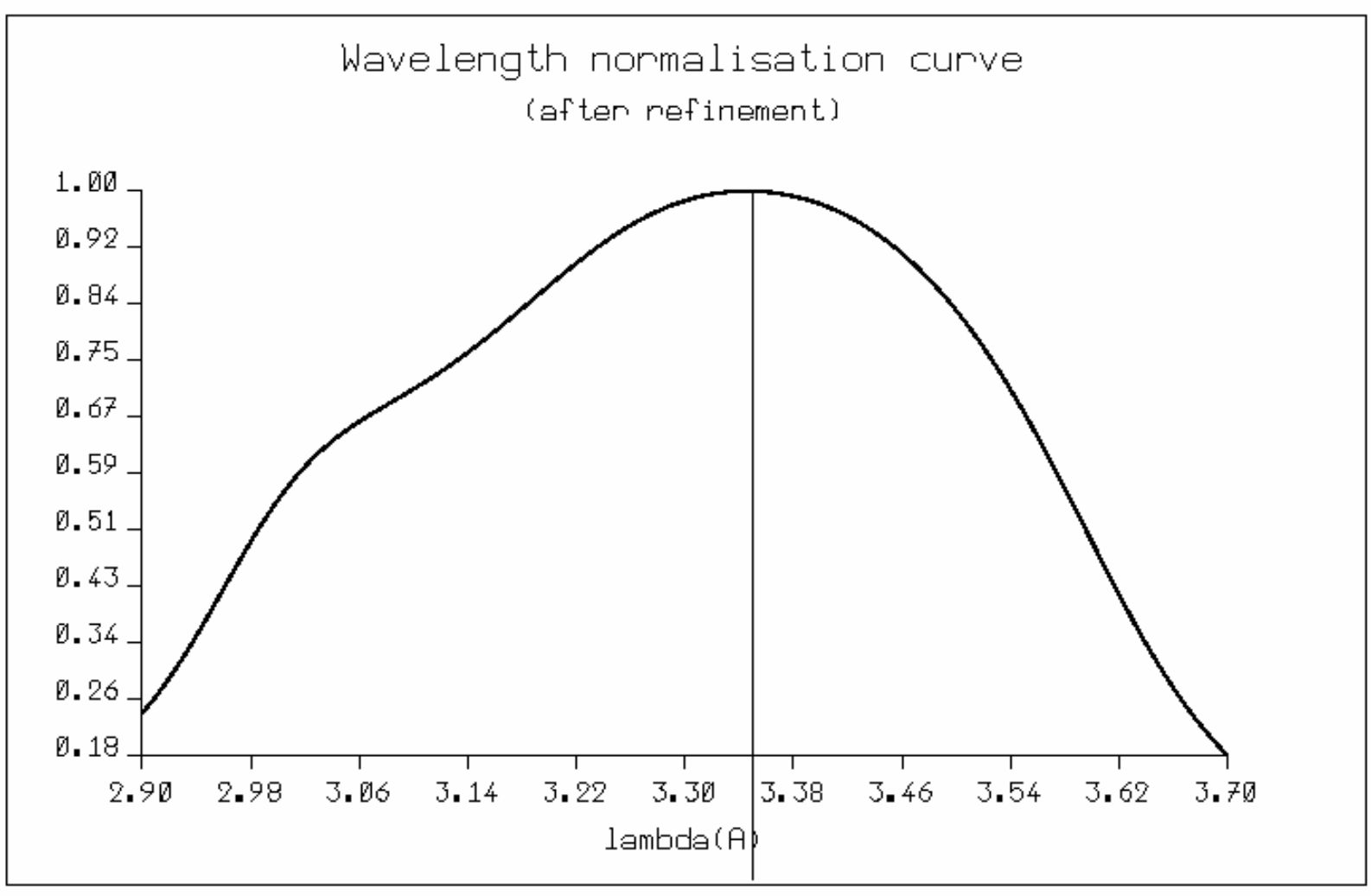
(λ=3.5Å, $\delta\lambda\backslash\lambda$ = 20%)

Sample environment

Displex cryostat under vaccum

going down to approx. 12K

LADI Diffractometer at ILL - Grenoble - France
Neutron LAUE wavelength normalization curve

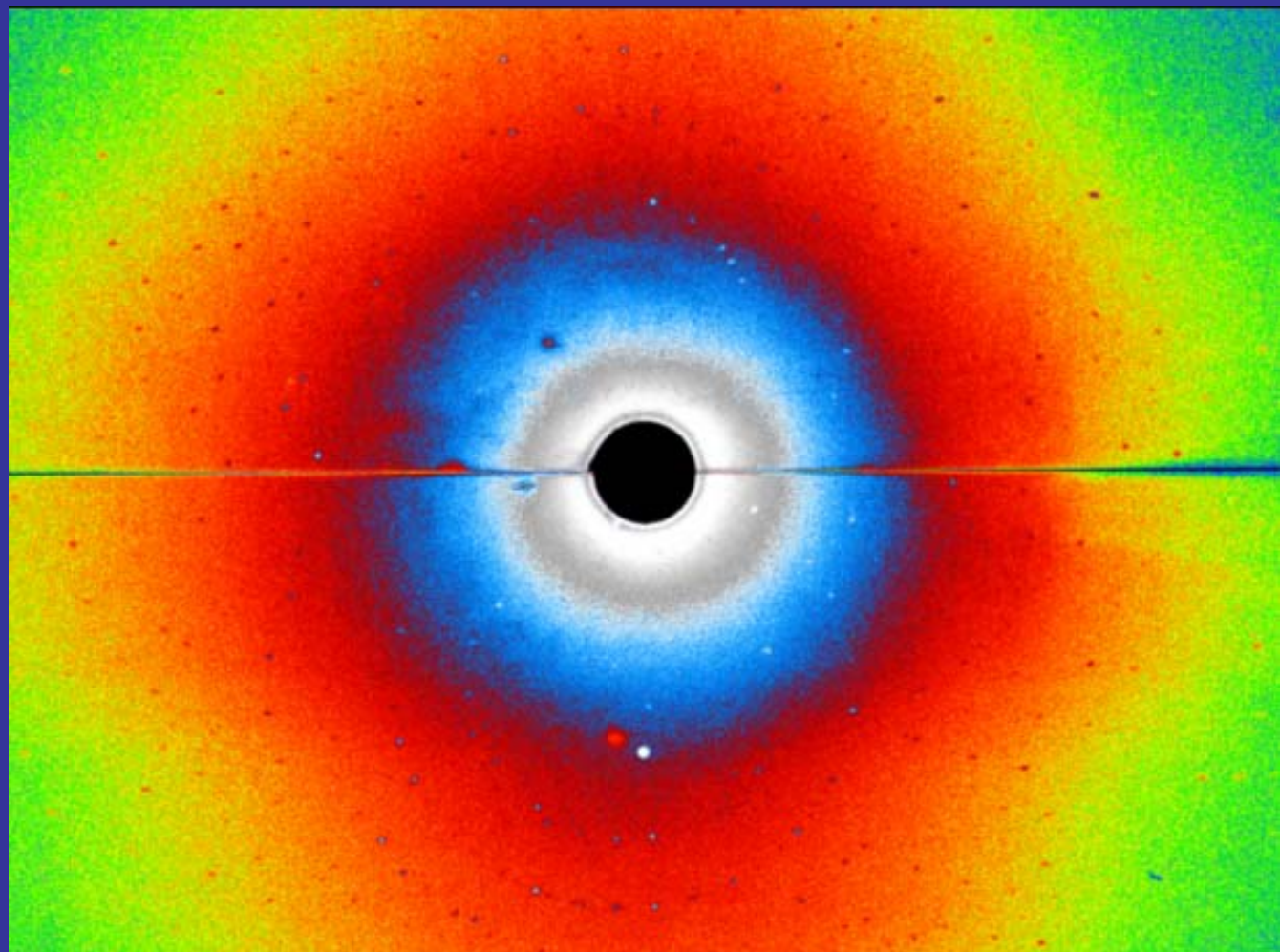


Neutron diffraction spots of fully deuterated crystal

With respect to D_2O/H_2O exchange:

Gain in peak height: 2.5

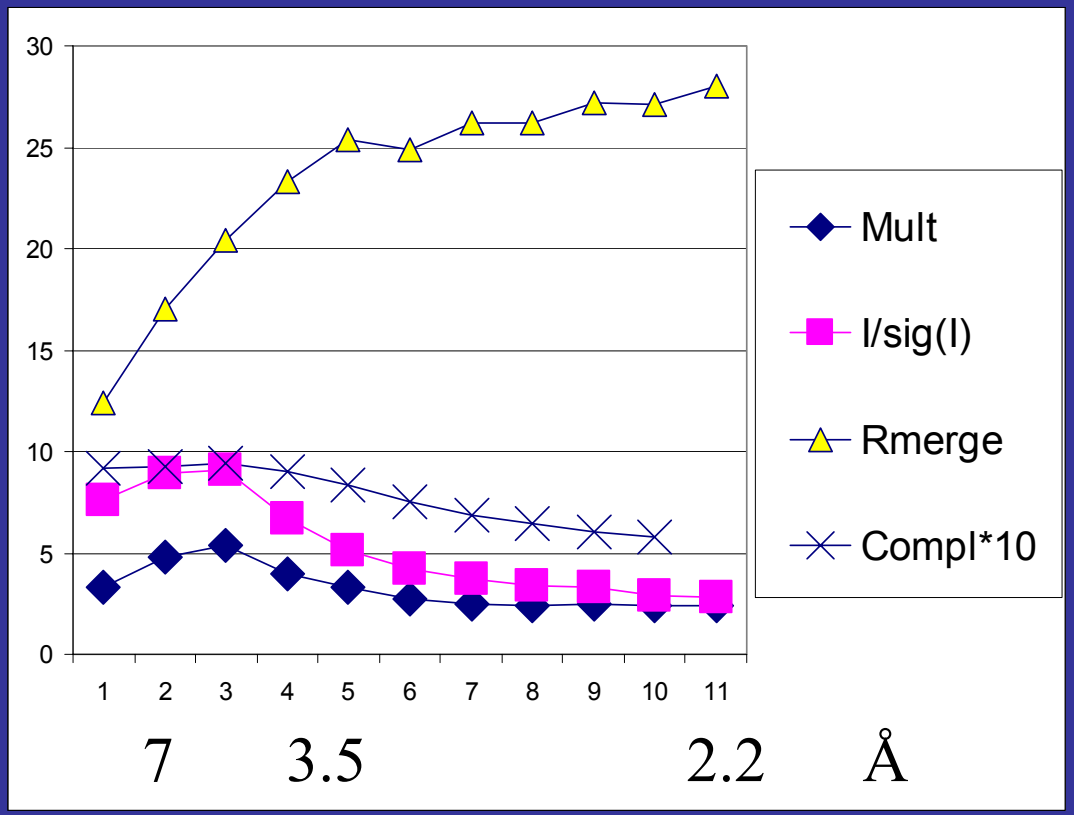
Gain in Background: 2.0



Neutron Laue data processing from 62 LADI frames at 293K(2.2 A

M. Blakeley EMBL-Grenoble, *F. Meilleur* ILL

with a **fully deuterated** crystal of **tiny** volume = 0.15mm³ = 1 x 0.67 x 0.23 mm³



Total reflections measured **(I/σ(I)>3)** : 11885 (73.5 % completeness

Exposure time for each « still » frame was **36 hours** (covering angle # 7°)

Perspectives

- Growth of bigger crystals (volume >> 0.15 mm^3) to improve neutron diffraction resolution (better than 2.2 A), first at RT, then at 15K.

- Solve neutron structures of other inhibitor complexes to confirm mobile protons along « shuttle pathways » « expected » by subatomic resolution X-Ray structures.

- Looking for new specific inhibitors.