Mitglied der Helmholtz-Gemeinschaft

Jülich Centre for Neutron Science

JÜLICH FORSCHUNGSZENTRUM

What neutrons can do for you: From diffraction on protein crystals to small angle neutron scattering with contrast matching

Besucherführerschulung

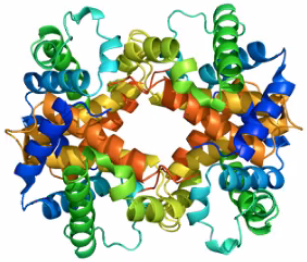
Sept. 18th 2023 | Tobias E. Schrader

Outline

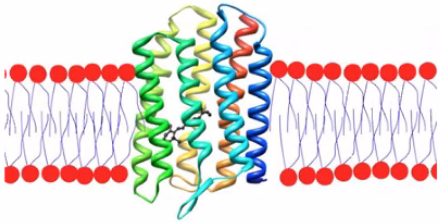
- Motivation: Why do we need protein structures at atomic resolution?
- neutron protein crystallography
- Application examples
- Summary

Proteine

- Etwa 20 000 verschiedene Proteine arbeiten im menschlichen Körper und erfüllen die unterschiedlichsten Aufgaben:

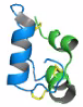


Hämoglobin,
Sauerstofftransport

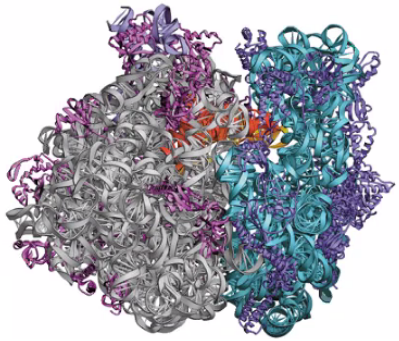


Rhodopsin,
Sehvermögen

Alkoholdehydrogenase,
Alkoholverdauung



Insulin,
Regulierung des
Zuckerhaushalts

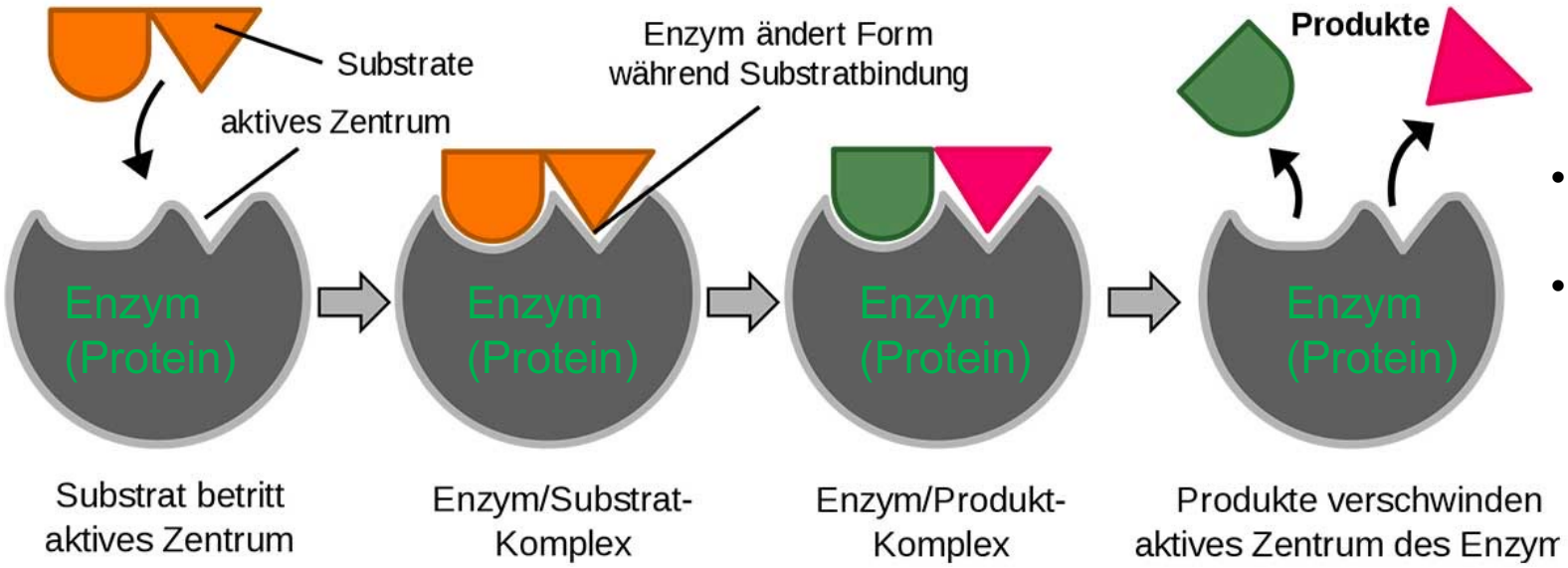


Ribosom,
Synthese von Proteinen

Einige von ihnen bieten Angriffspunkte für Medikamente...

Enzyme sind biologische Katalysatoren

- Sie beschleunigen chemische Reaktionen schon nahe Raumtemperatur



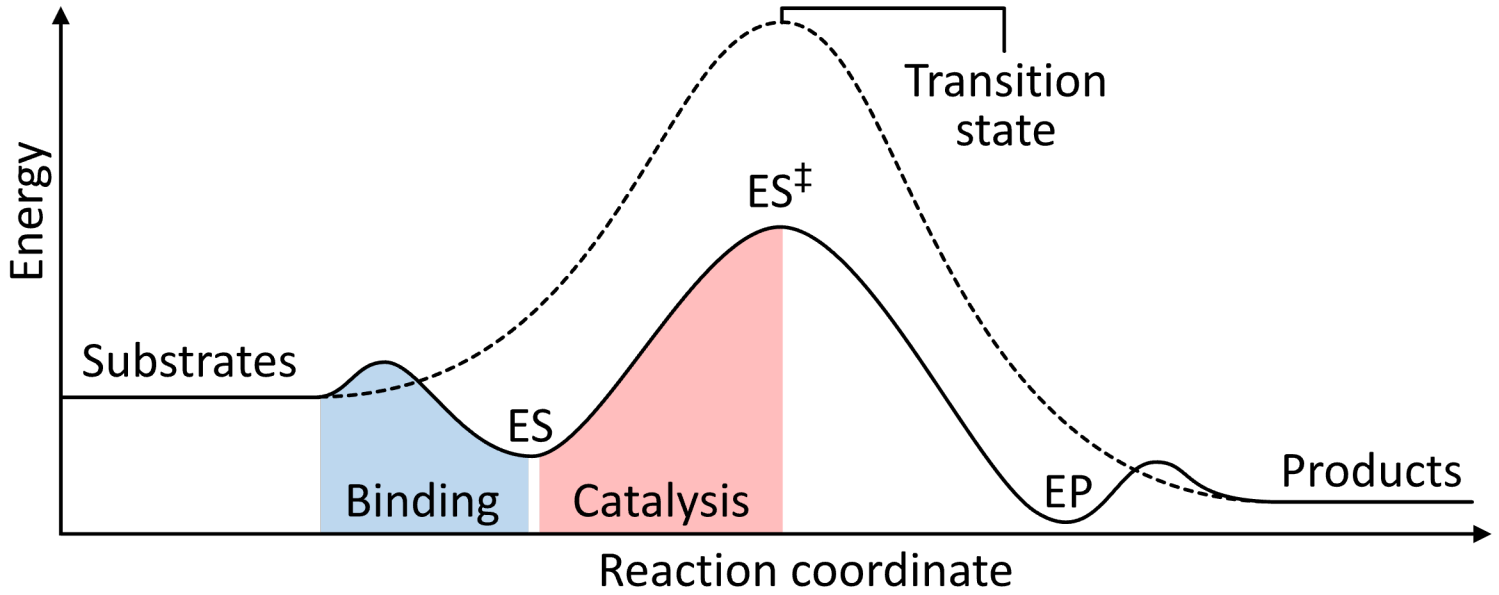
- Temperatur ca. 37°C
- Hoch substrat-spezifisch

- Im Vergleich dazu:

  Chemische Industrie

- ❖ Oft hohe Temperaturen notwendig (=nicht sehr energieeffizient)
- ❖ Mitunter viele unerwünschte Nebenprodukte, die mühsam abgetrennt werden müssen

Enzymes, a sub-class of proteins, accelerate chemical reactions



- ➡ very high substrate specificity
- ➡ rate enhancements up to 10^{18}
- ➡ enzymes can be “controlled”

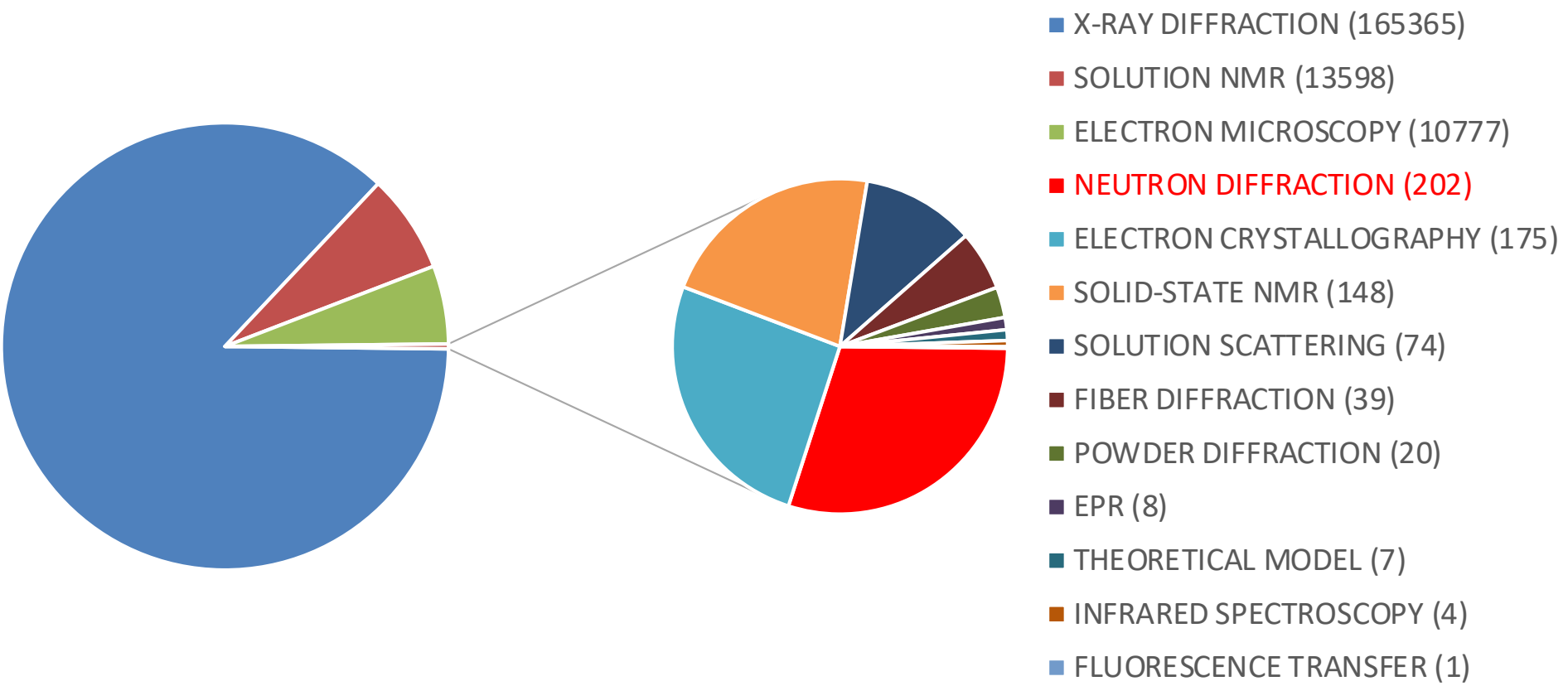
Why do we need to measure protein structures?
Are there no good simulations?
How do we measure protein structures?

Why do we need neutron studies on proteins?

- **MD-Simulations** suffer from non-perfect force fields: Especially the long range electrostatics is not reproduced very well. But proteins use defined and structure related electrostatics to move the acidity constants of side chains in order to make them fullfill their tasks. MD-simulations cannot model bond breaking and forming very well since the quantum chemistry nature of this process is not included in the theoretical foundation of MD.
- Alphafold can only predict based on existing structures which often lack exact hydrogen atom positions.
- **Ab initio quantum ch** still too demanding to model the complete a ncluding its substrate)

Most structures are obtained by x-ray crystallography, available neutron structures in protein data bank: ca. 200

Entries in the protein data base as of 9th of May 2022

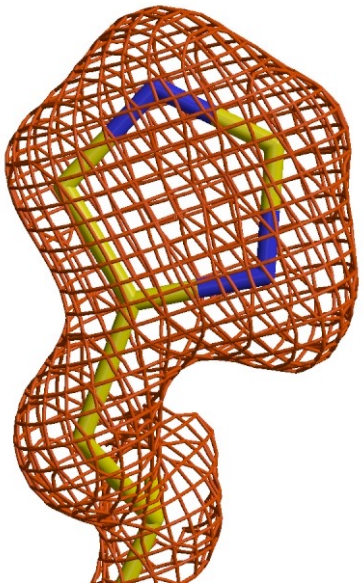


http://www.rcsb.org/

Total number of structures: 190418

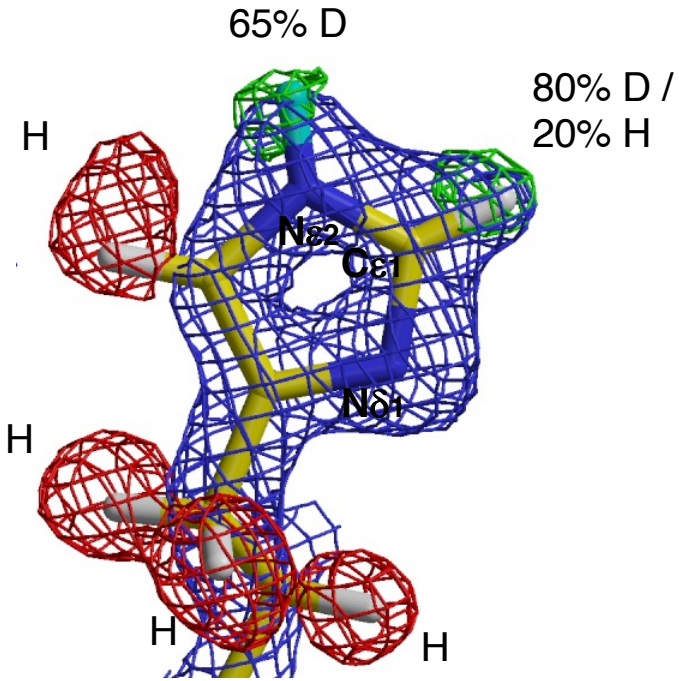
X-ray data versus neutron data on the same protein:

X-ray d_{min}= 1.5Å:



2Fo-Fc map; +1.5σ

neutrons d_{min}= 1.5Å:



2Fo-Fc map; +1.5σ

Fo-Fc omit-map; -3.0σ

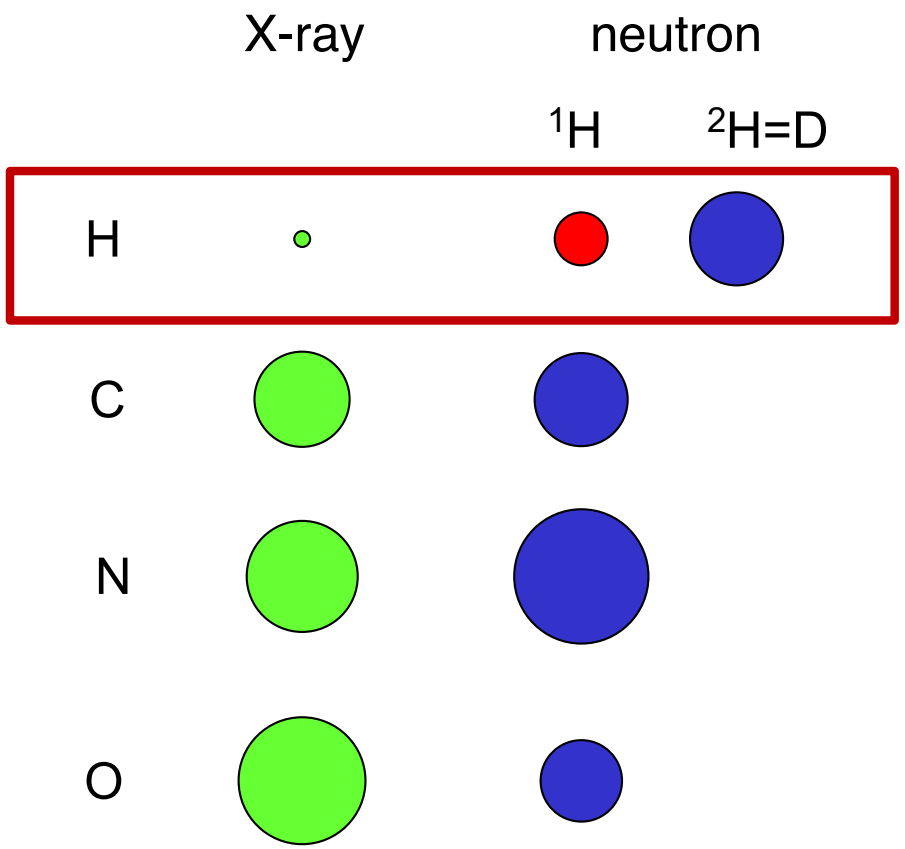
Fo-Fc omit-map; +3.0σ

Niimura N, Chatake T, Ostermann A, Kurihara K, Tanaka T. (2003) Z. Kristallogr. 218:96

Advantages of structure determination with neutrons:

Comparison of form factors (X-ray) and scattering lengths (neutrons):

Nucleus	atomic number	scattering length [10^{-12} cm]
^{1}H	1	**-0.378**
^{2}H	1	**0.667**
^{12}C	6	**0.665**
^{15}N	7	**0.921**
^{16}O	8	**0.581**



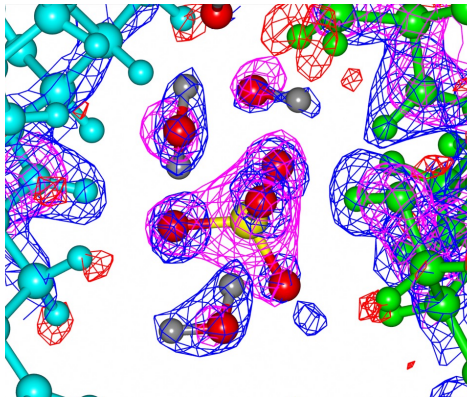
diameters correspond to:
form factor / scattering length
(scaled for C-atom)

σ_{coh} of ^{1}H is 1.8×10^{-28} m^2 but
σ_{incoh} of ^{1}H is 80.2×10^{-28} m^2
Large background from hydrogen atoms!

Advantages of Structure Determination with Neutrons

Hydrogen/deuterium atoms can be resolved even at a resolution of $d_{min} \approx 2.5$ Å (for ^2H). Therefore one can determine:

- protonation states of amino acid side chains and ligands
- deuterium exchange as a measure of flexibility and accessibility (discrimination between **H** / **D**)
- solvent structure including hydrogen atoms



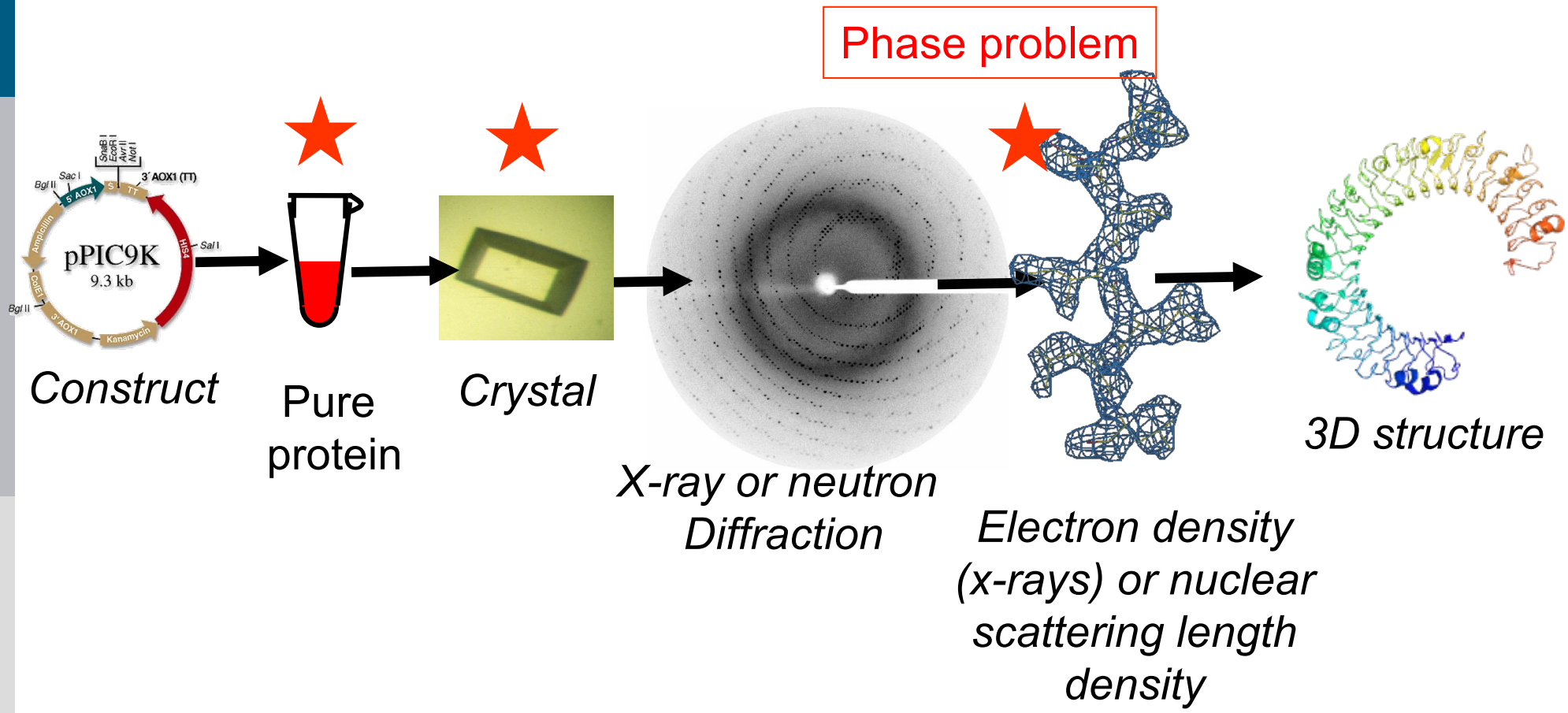
Water network in the contact region between two myoglobin molecules in the crystal.

x-ray map (magenta): contour level of +2.7σ
nuclear map (red): contour level of -1.75σ
nuclear map (blue): contour level of +2.3σ

Much less radiation damage as compared to x-rays: Metallo-proteins can be measured without reducing the metal centres

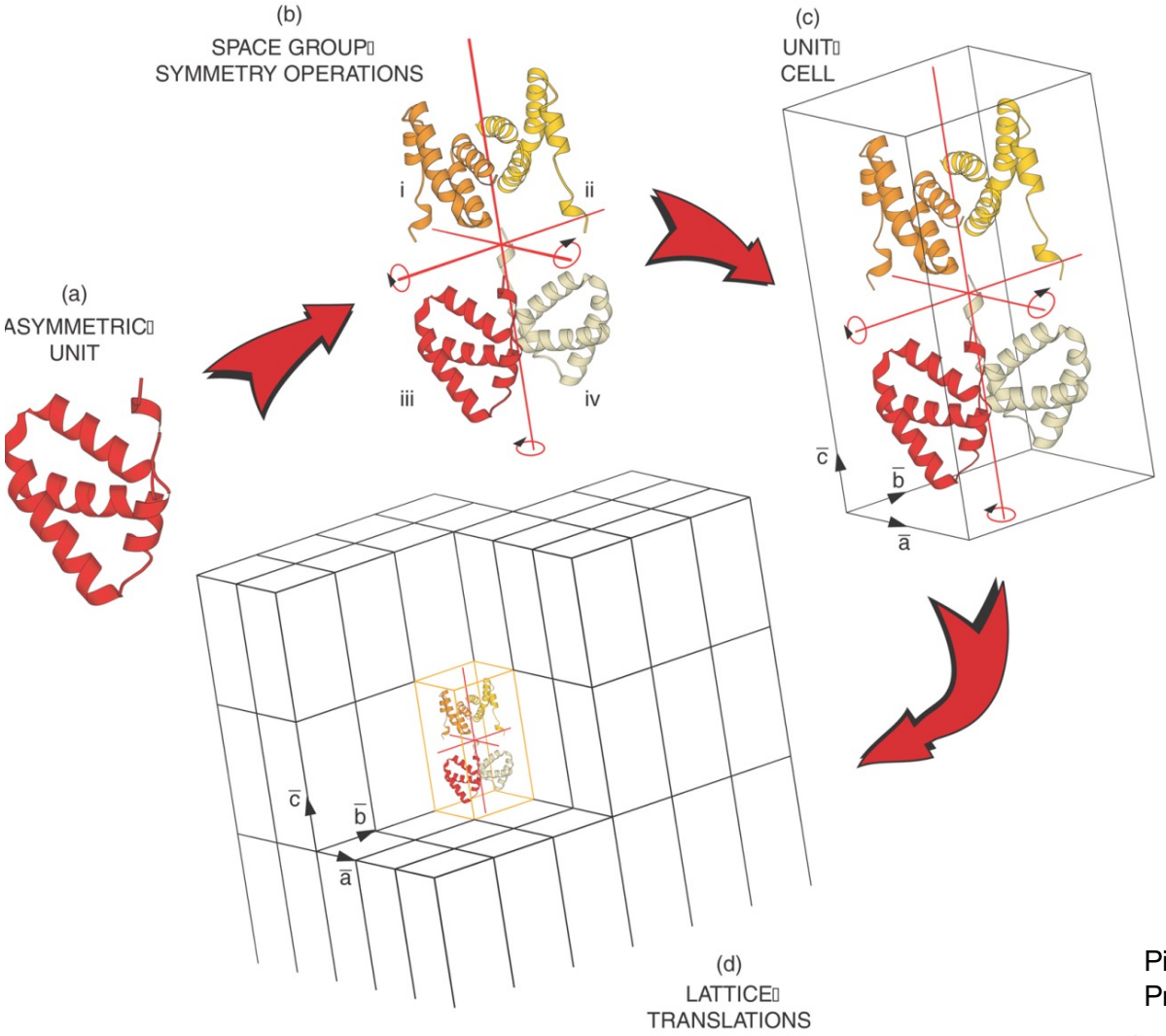
Protein crystallography in general, valid for both x-rays and neutrons as probes

Crystallography: Overiew over the process



Harma Brondijk, Crystal and Structural chemistry, Utrecht University

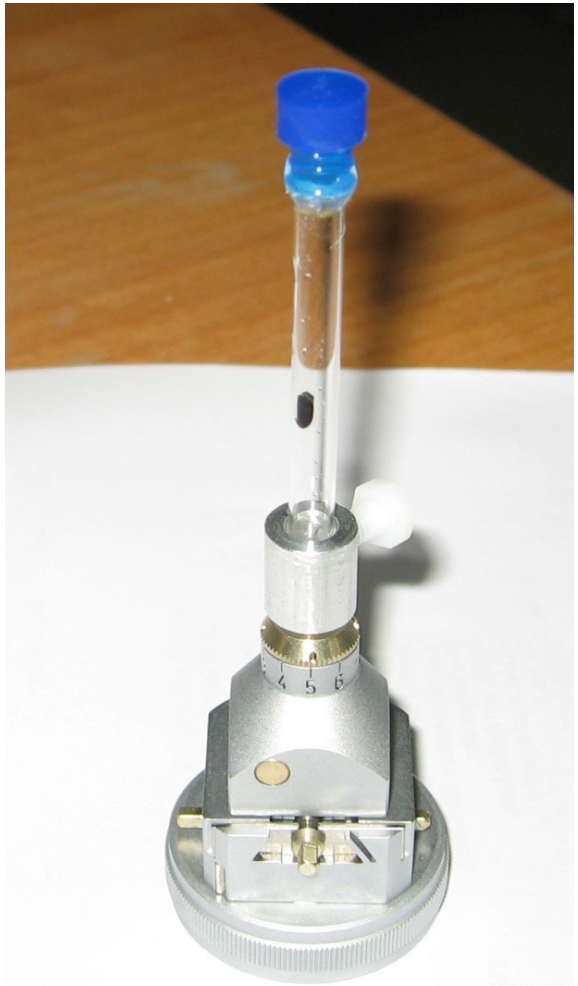
How a typical protein crystal looks like…



Picture taken from Lecture of Prof. Locher at ETH Zürich

fig 2.2

Size considerations of protein crystals

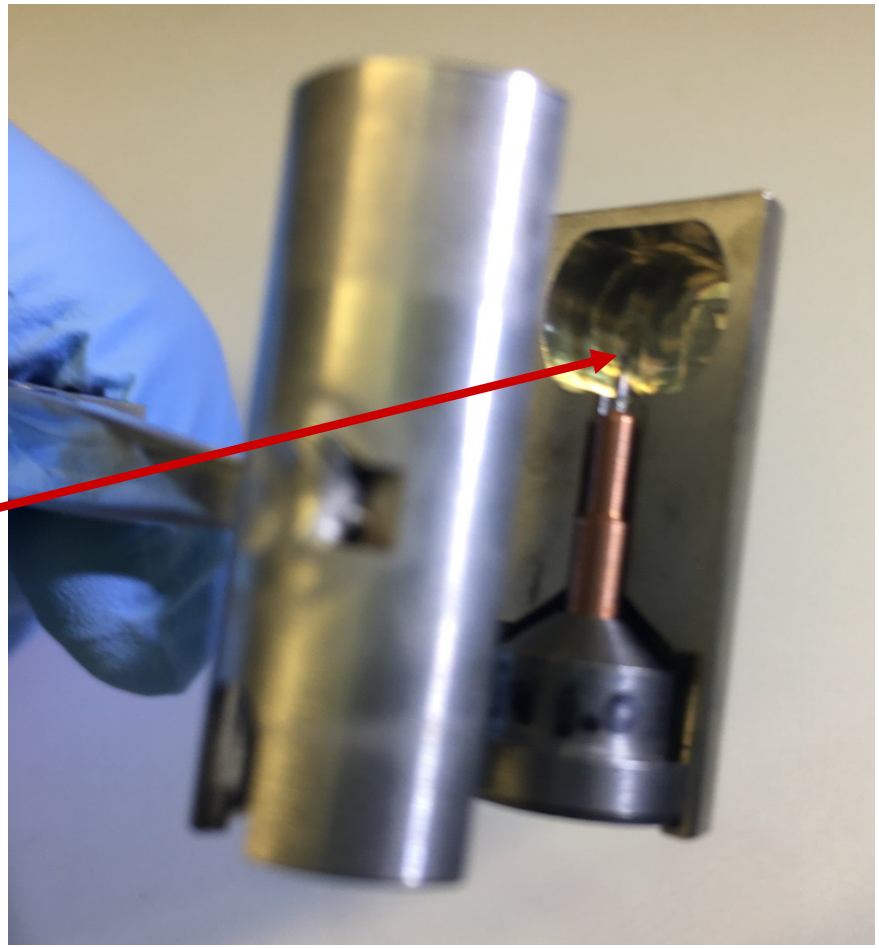


Outer diamter of the glas tube: 5 mm

size:
x-ray-crystallography:
ca. 10 µm x 10 µm x 10 µm
typically cryoprotectants needed to facilitate measurements at low (80 K) temperatures

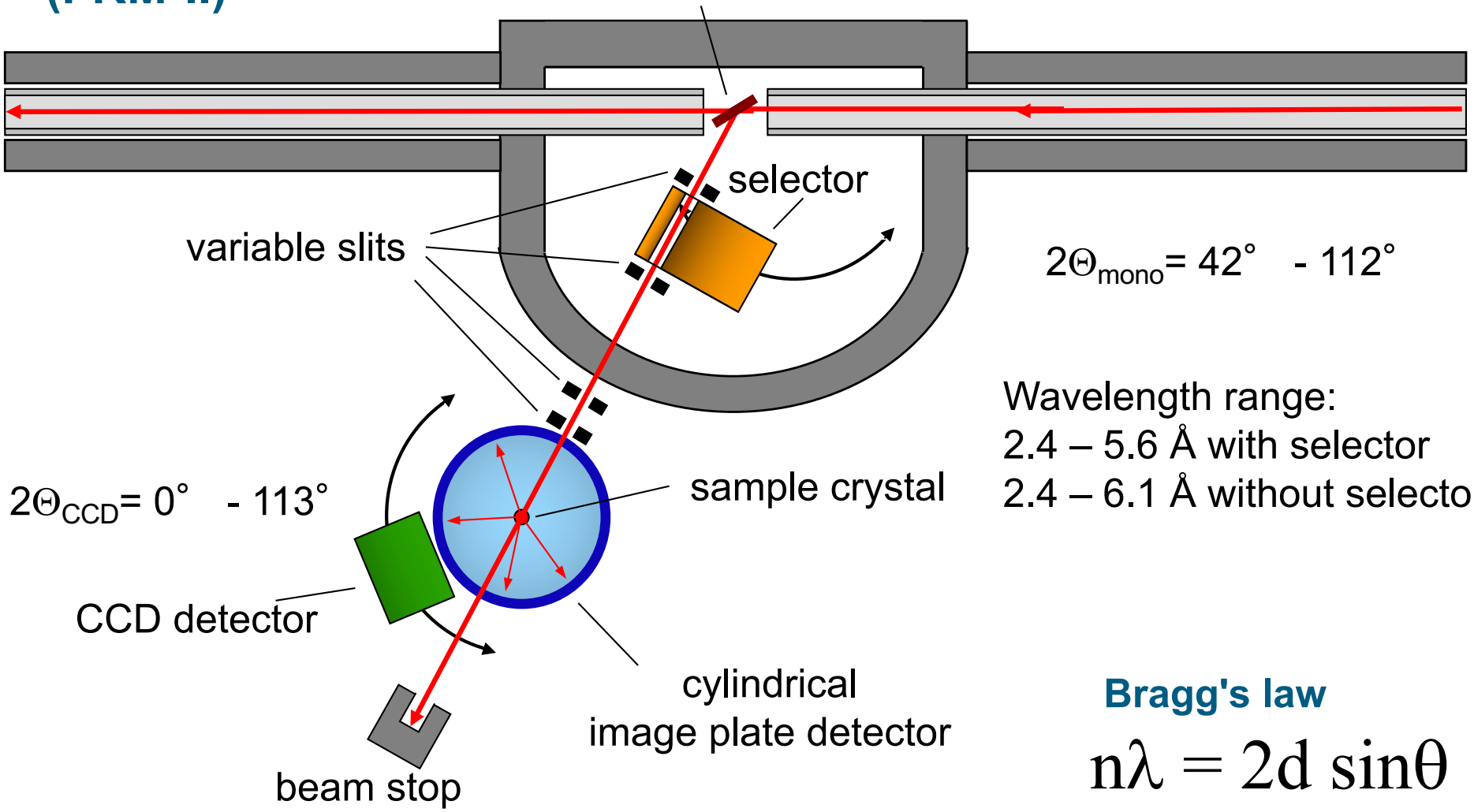
neutron protein crystalography:
The desirable size should be around
1 mm x 0.5 mm x 0.5 mm (depending on the protein/space group)

Cryo-mounting of large crystals



- Avoid hydrogenated polymers in the loop, use capton (Mitigen) or carbon meshes instead (especially when you have a fully deuterated protein)
- Make sure that your crystal fits into the cryoTong: We prefer the 18 mm one.

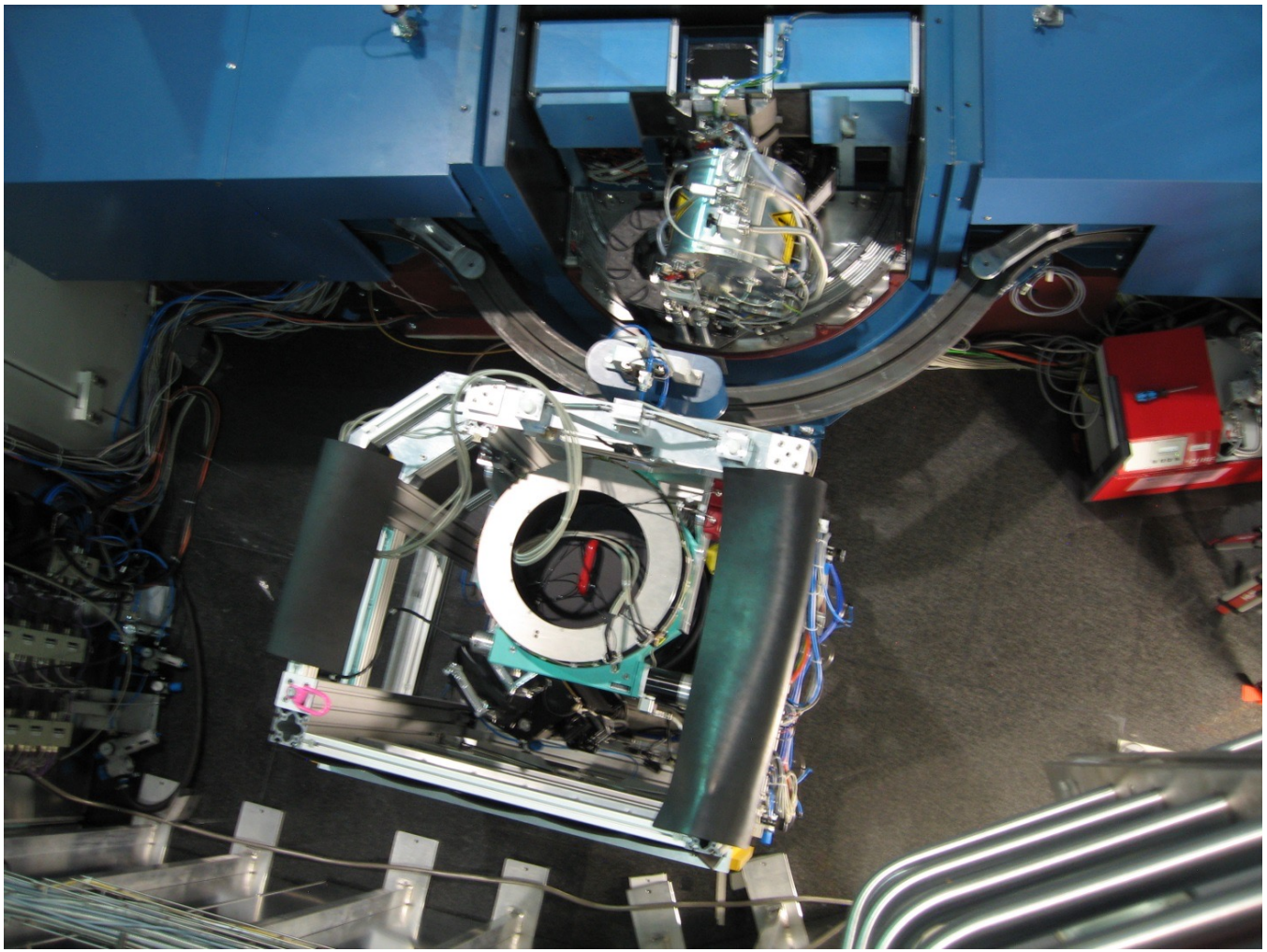
Schematic overview over BioDiff: A neutron protein diffractometer: collaboration between JCNS and TUM (FRM-II)



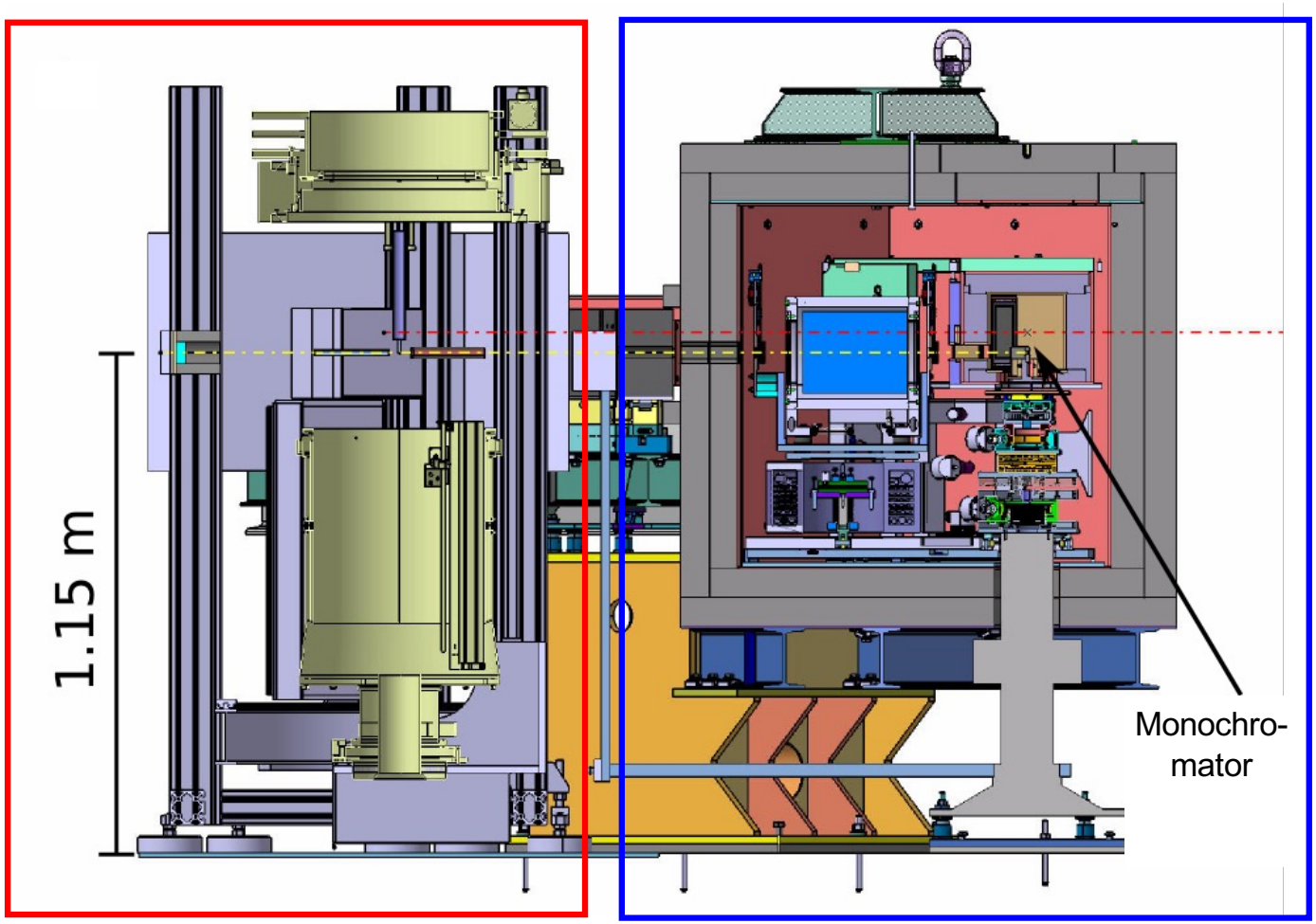
Bragg's law

$$n\lambda = 2d \sin\theta$$

BioDiff, the corresponding view in reality:



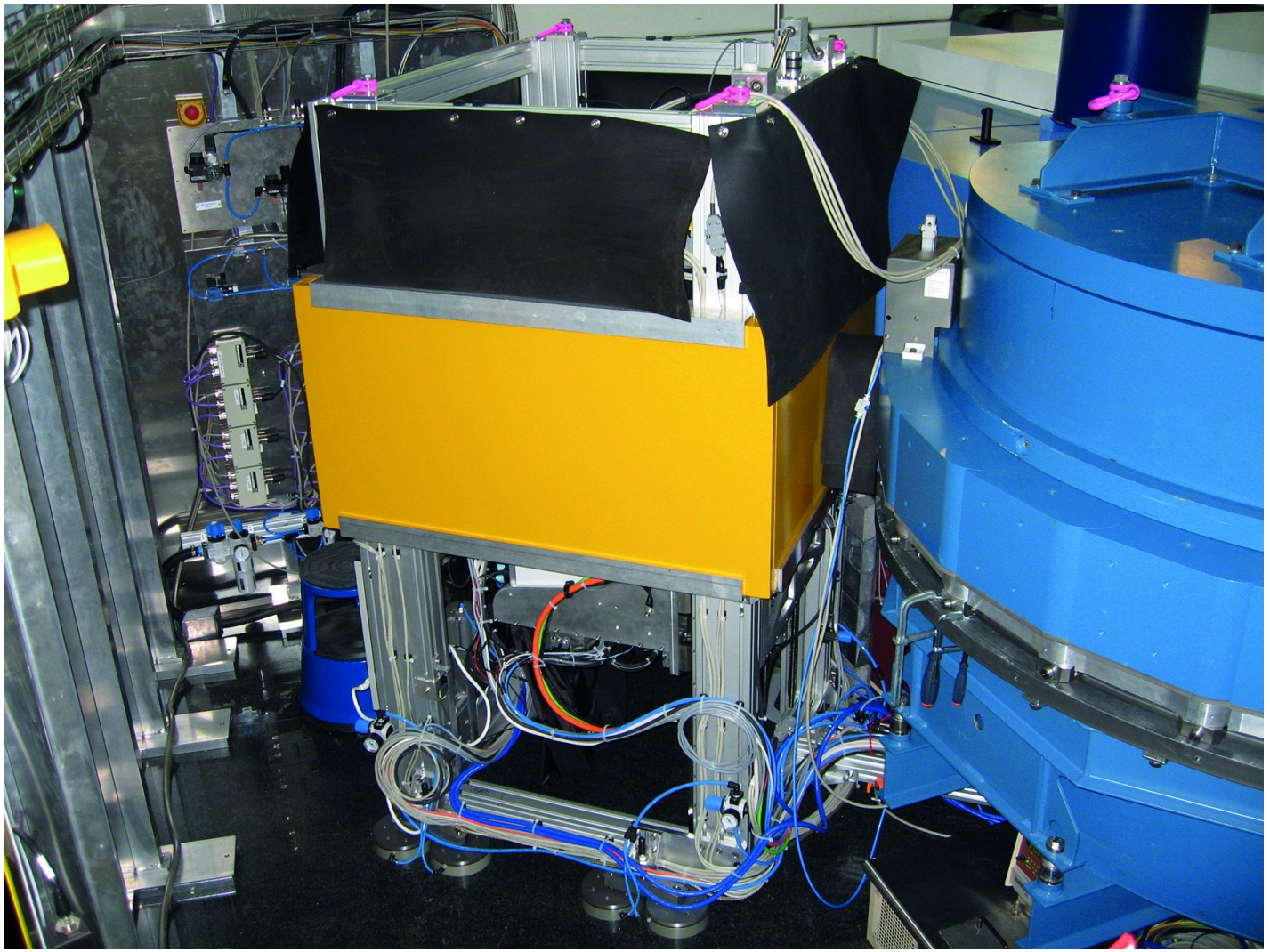
The Simultaneous Construction-phase in Garching and Jülich



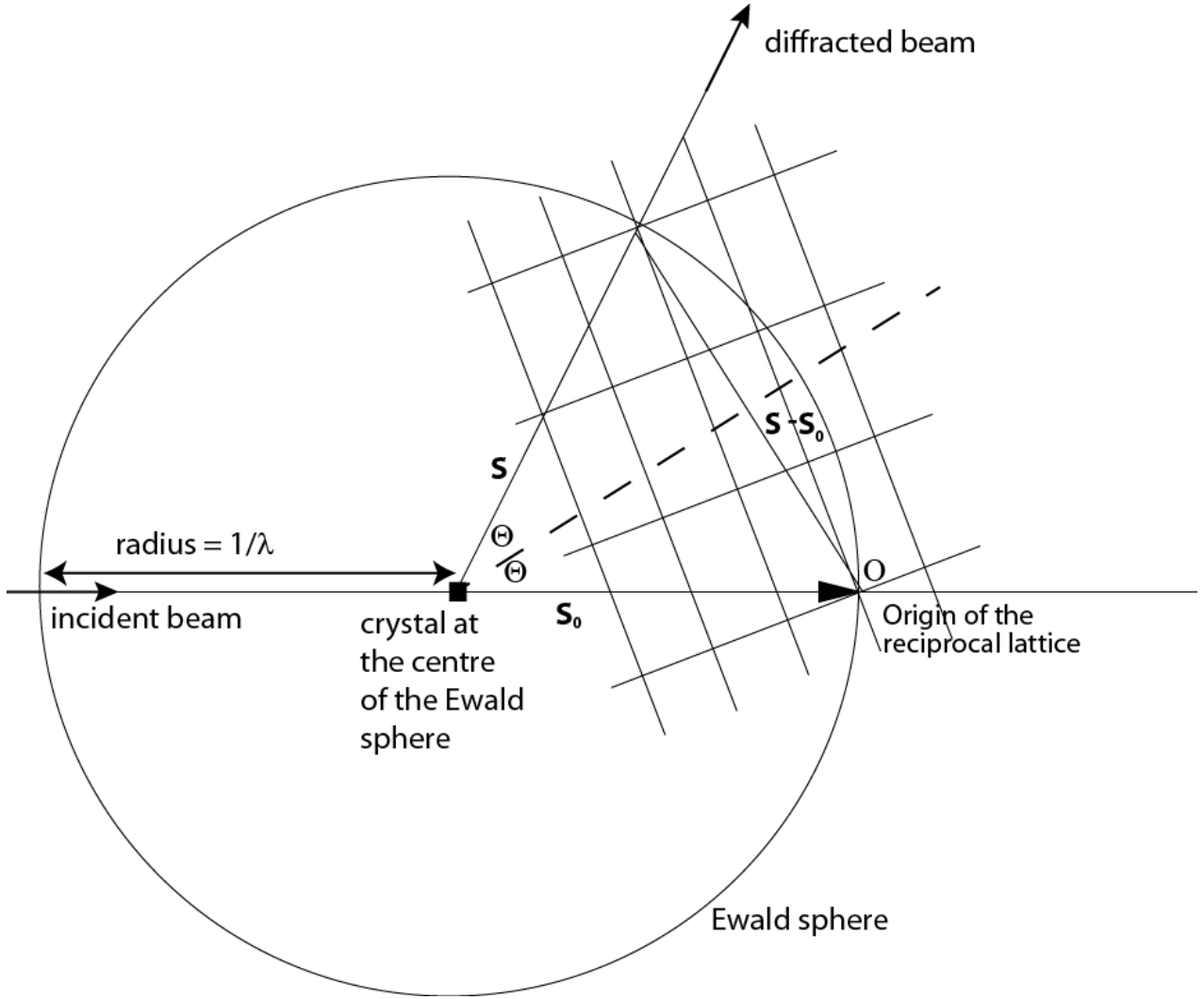
Detector unit, constructed and built in Garching (Ph. Jüttner, MLZ)

Monochromator-shielding, constructed and built in Juelich (B. Laatsch, ZEA-1 Engineering)

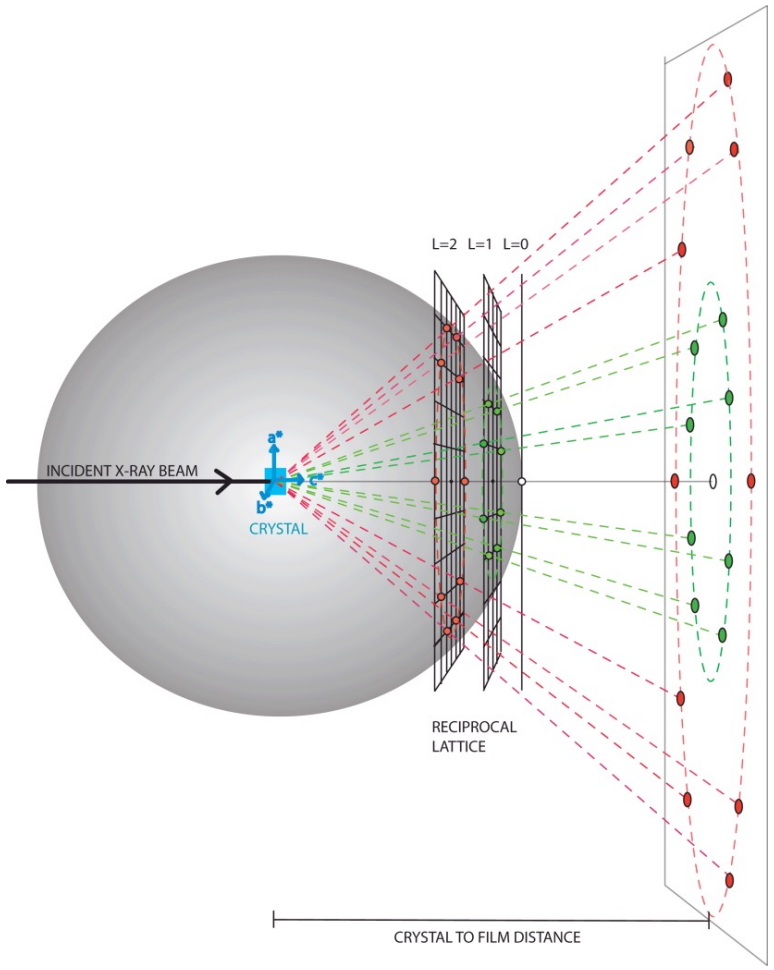
A Most Recent View of the Instrument BioDiff

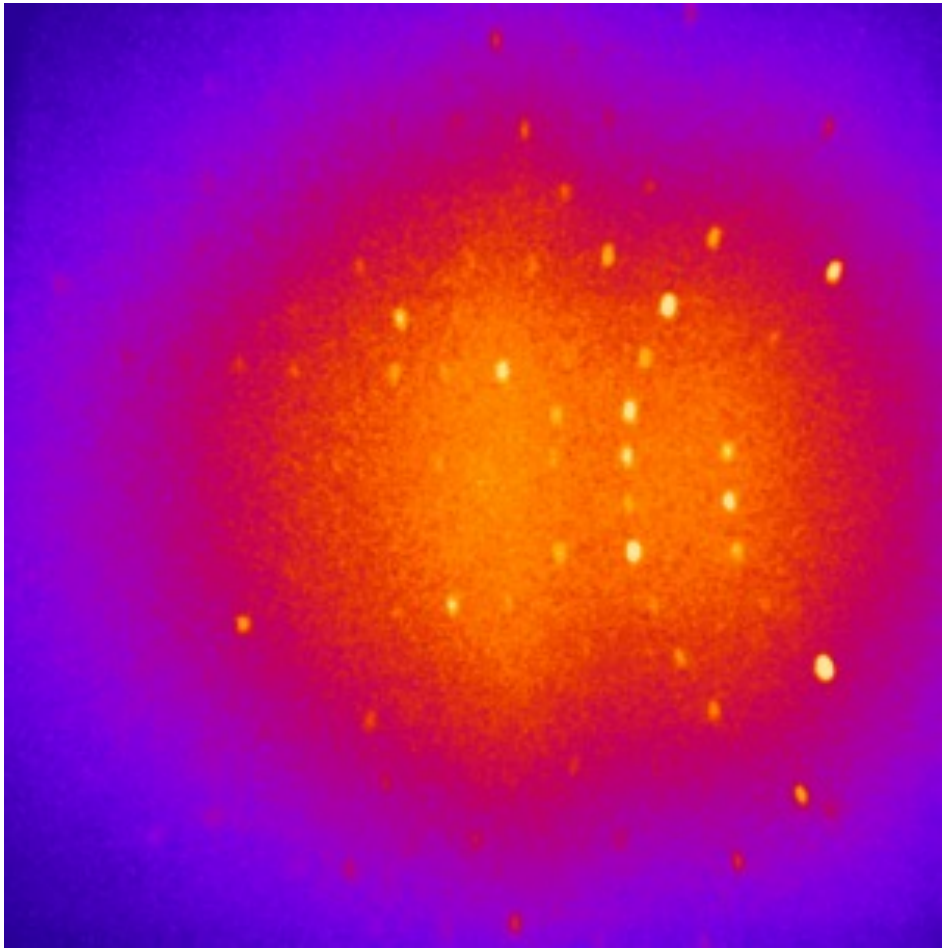


Ewald construction and Bragg‘s Law



Myoglobin protein crystal (deuterated mother liquor) full data set recorded with CCD-camera

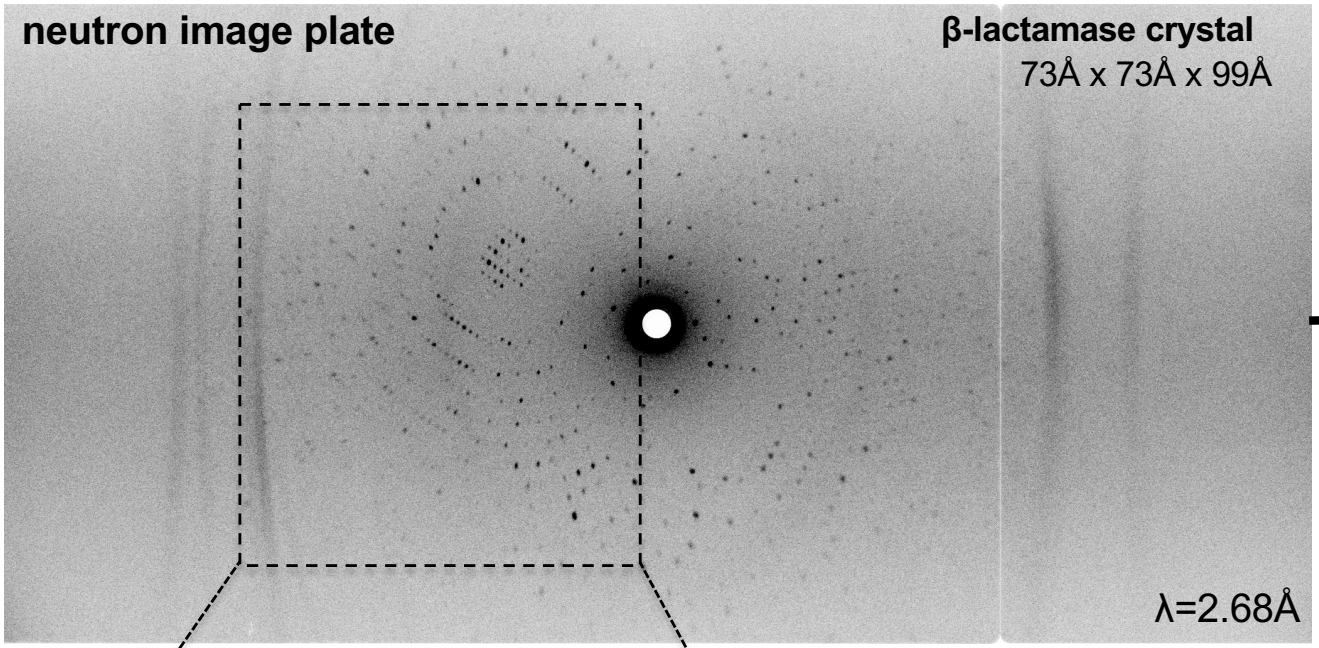


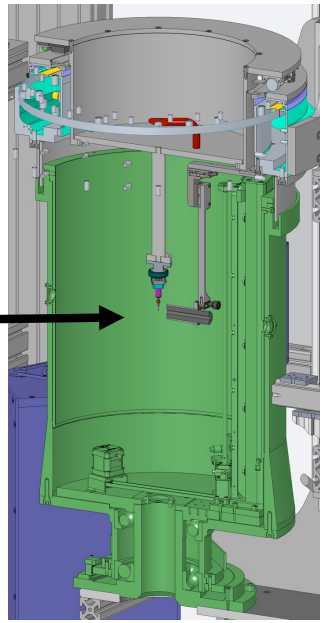


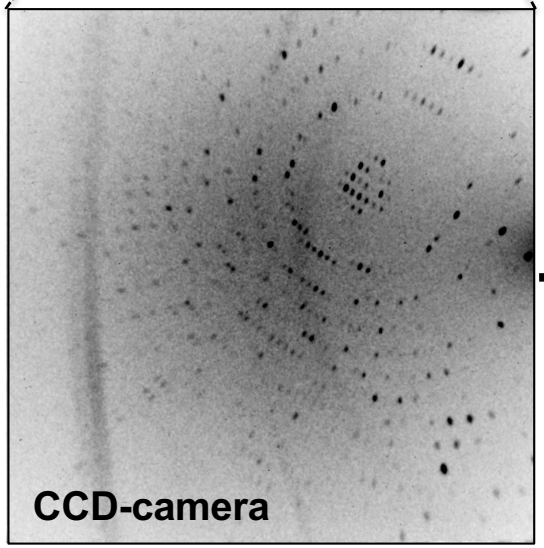
prim. beam

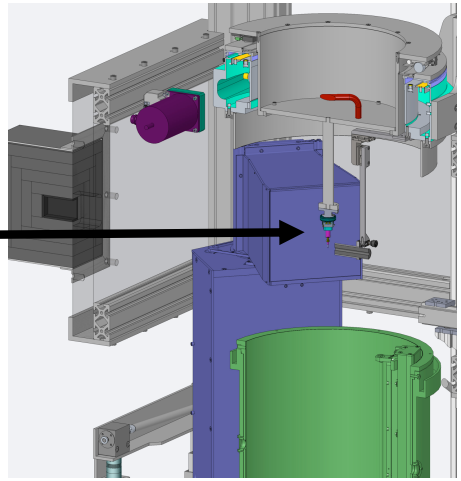
BioDiff: exposure time per frame: 20 minutes, sample: Myoglobin in deuterated mother liquor

The Two Detectors of BIODIFF









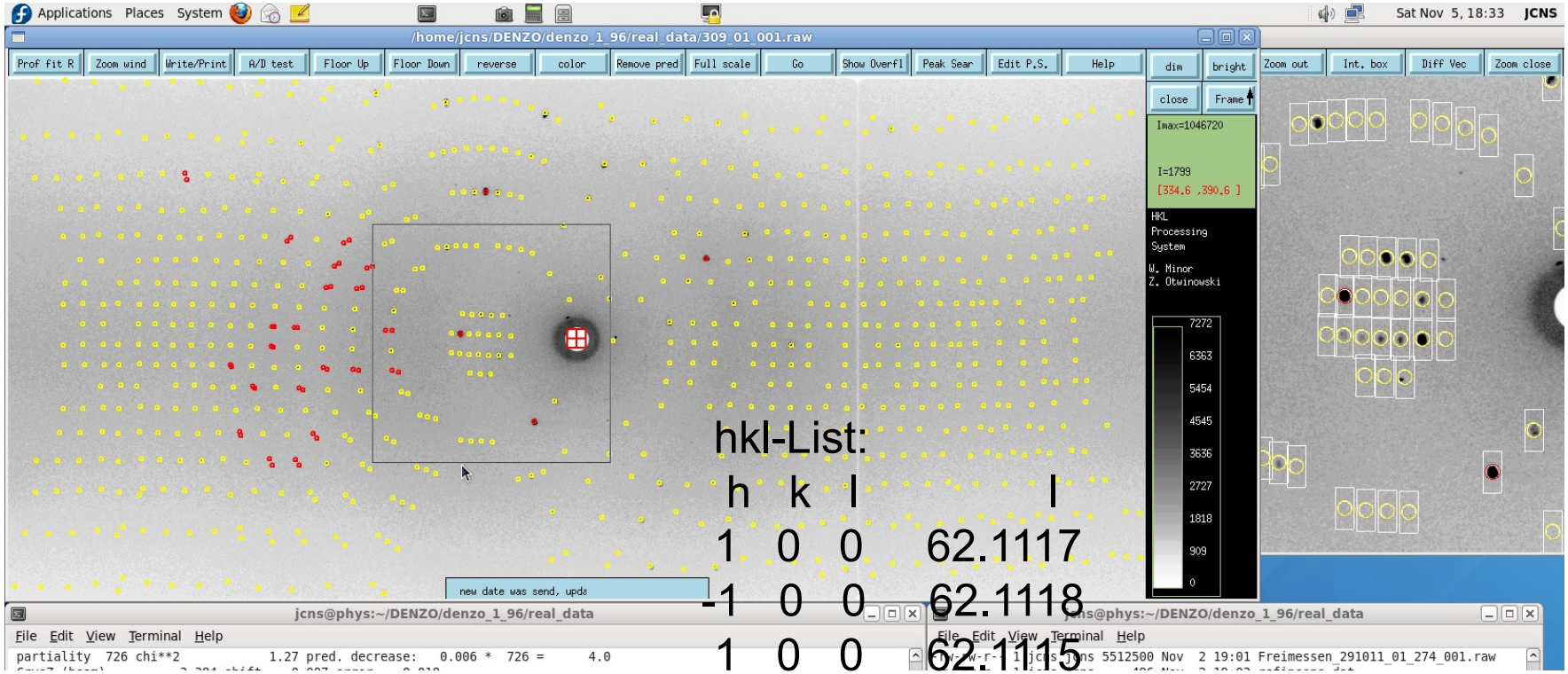
NIP-scanner

- larger solid angle
- readout time ≥ 4 min

CCD-camera

- smaller solid angle
- readout time ≥ 1 sec

Integration of partial Bragg peaks with the commercial software hkl-denzo up to d_{min}=1.5 Å



hkl-List:

h	k	l	I
1	0	0	62.1117
-1	0	0	62.1118
1	0	0	62.1115
-1	0	0	62.1120
0	0	-1	33.5555
1	0	-1	33.5589
0	0	1	33.5533
-1	0	1	33.5511

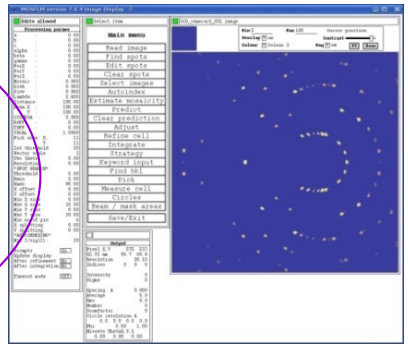
ca. 300 images

Flow chart of data treatment and model building

Scans at varying crystal orientation
Scan := Series of detector images

Data reduction

- determination of crystal orientation, unit cell dimensions etc.
- Calculating integral of reflection intensities



-MOSFLM
-HKL-denzo
- HKL2000
(comercial)

hkl-list for each scan:
h k l Intensity Intensity error

Scaling of each hkl list to match each other

-SCALA (CCP4-program package)

Unified hkl-list of measurement := complete data set

Calculation of a first map ⟷ Additional information from the solution of the phase problem

Structure refinement

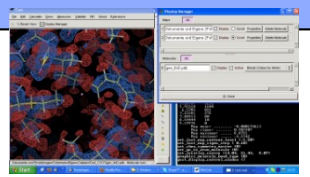
-Refinement of atom coordinates displacements
- Calculation of scattering density maps (netrons) or electron density maps (x-rays)

⟷

Map-plotting

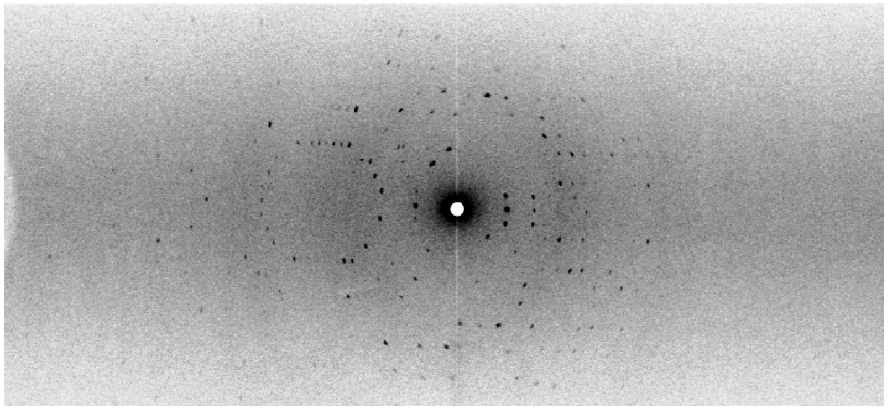
- inspection of model to fit the map)
- real space changes and refinement to the model

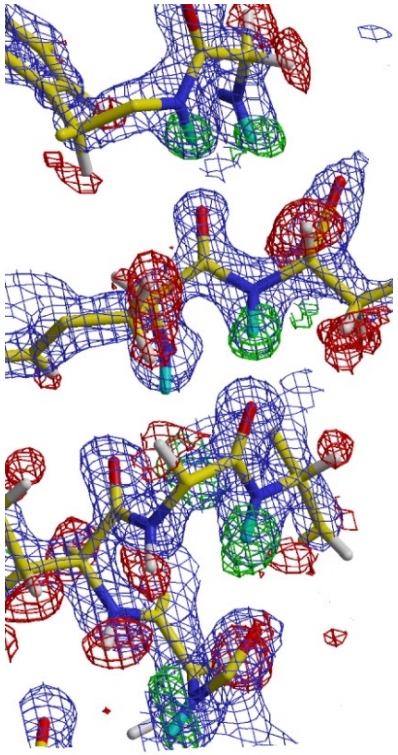
-nCNS
-PHENIX



-XtalView
-Coot

The phase problem:





$$\rho(x,y,z) = \frac{1}{V_E} \sum_{h,k,l} F_{hkl} \cdot e^{-2\pi i(h\cdot x + k\cdot y + l\cdot z)}$$

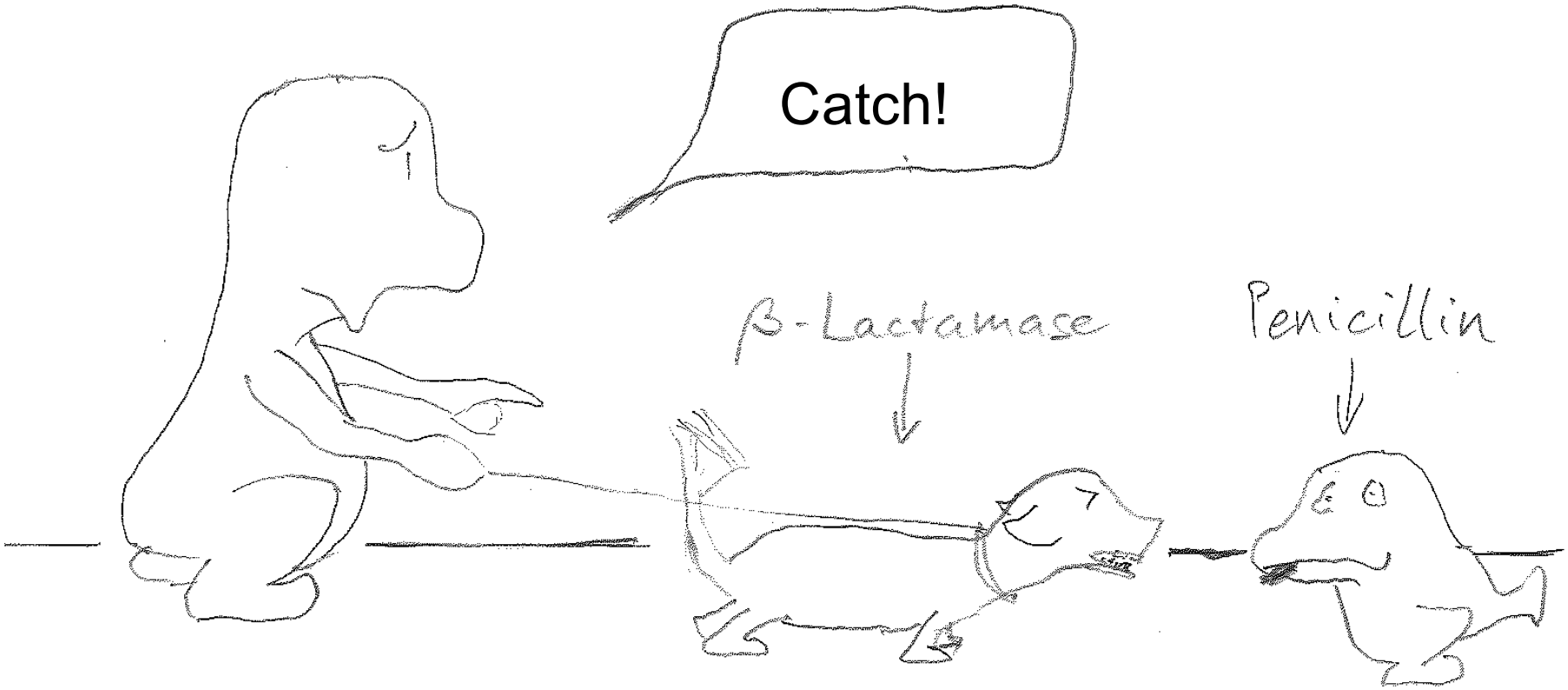
Structure factors are complex numbers: $F_{hkl} = \|F_{hkl}\| e^{-2\pi i \alpha_{hkl}}$

with amplitudes $\|F_{hkl}\|$ and phases α_{hkl}

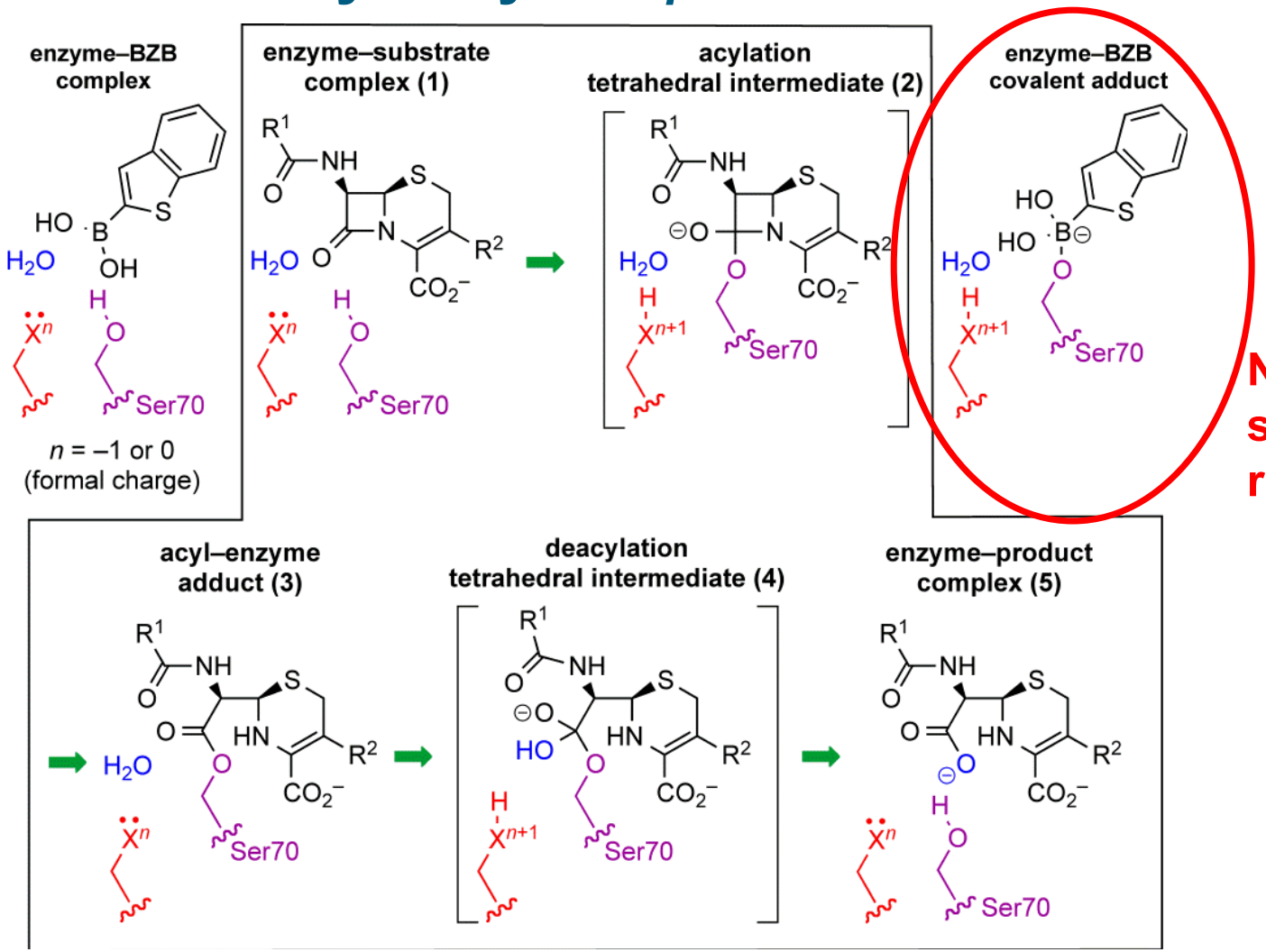
→ Phase Problem, because we only record intensities: $I = \|F_{hkl}\|^2$

Application Example: Protonation state of amino acid residues

The protein β-lactamase



β-lactamase: hydrolyses β-lactam antibiotics

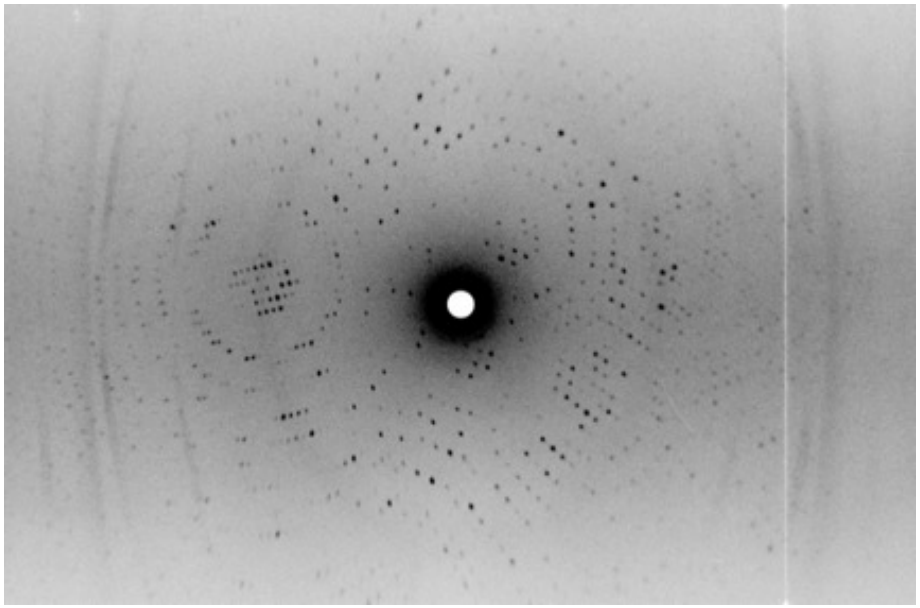


Neutron structure recorded

The catalytic cycle of a class A β-lactamase illustrated for a cephalosporin substrate (inside box) and the mode of inhibition by BZB (outside box). The general base employed is not necessarily the same for acylation and deacylation. The overall reaction pathway for β-lactam hydrolysis of a cephalosporin-like substrate by the class A β-lactamase enzymes.

Figures from: Tomanicek et al., doi: 10.1074/jbc.M112.436238

Data-set: β-lactamase with bound inhibitor



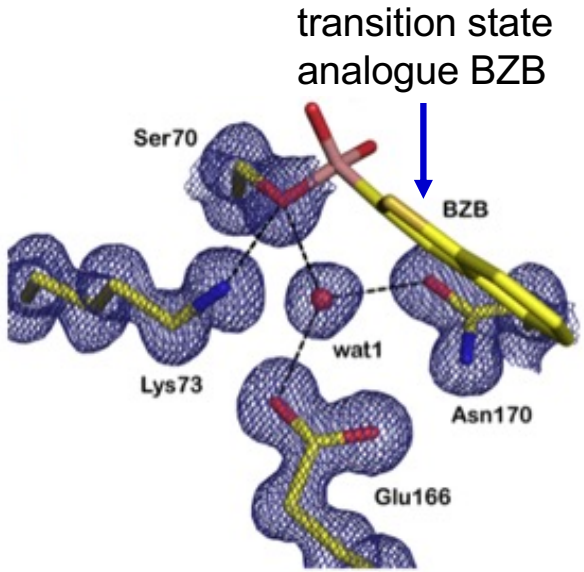
- unit cell: 73.4Å, 73.4Å, 99.1Å $P3_221$
- fully deuterated protein
- crystal size: 2.7mm^3
- Collection time: 9d

d_{min}	I/σ(I)	N_{meas}	mult.	compl. in shell %	R_{merge} %
4.31	27.8	12685	5.6	97.6	4.9
3.42	19.0	11941	5.5	98.0	8.0
2.99	10.3	10378	4.9	96.9	14.6
2.71	7.6	8757	4.3	95.5	18.7
2.52	5.9	7820	3.9	92.8	21.2
2.37	5.4	7099	3.8	89.2	21.6
2.25	5.0	6095	3.5	84.6	23.0
2.15	4.5	5906	3.4	82.9	24.7
2.07	4.1	5673	3.2	82.0	27.2
2.0	3.7	5059	2.9	81.2	27.9
overall	**7.4**	**81413**	**4.0**	**90.2**	**14.7**

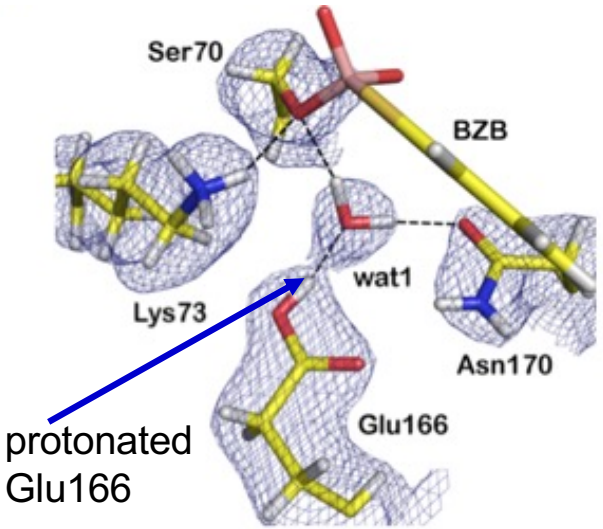
R_{pim} = 7.9% (17.9%)

Tomanicek et al., J. Biol. Chem., 288, 4715 (2013).

Catalytic Proton Network of the Toho-1 β-Lactamase



electron density map



nuclear density map from BioDiff

Glu166 acts as the general base during the catalytic action of the enzyme.

Stephen J. Tomanicek, Robert F. Standaert, Kevin L. Weiss,
Andreas Ostermann, Tobias E. Schrader, Joseph D. Ng, and Leighton Coates
J. Biol. Chem. 2013, 288:4715-4722

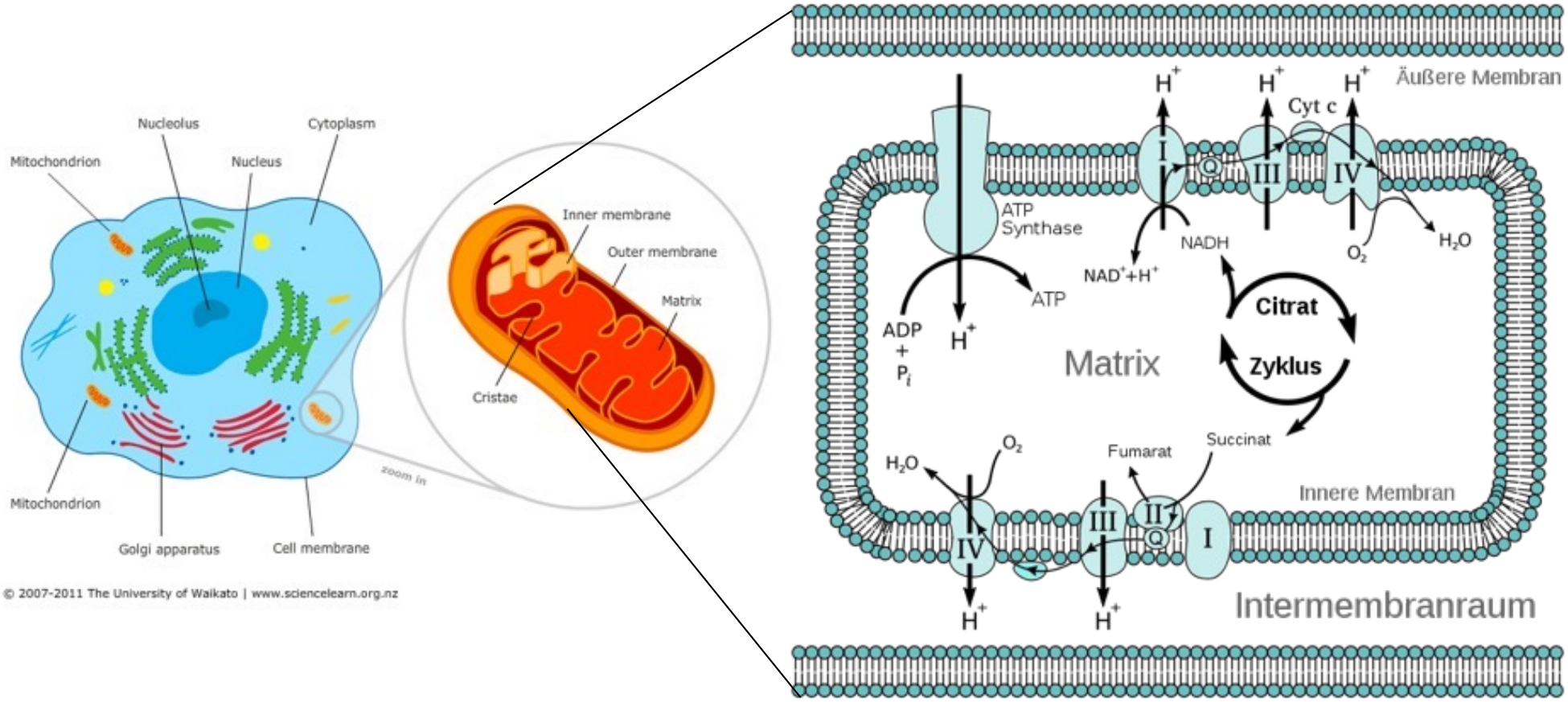
An example for a metallo-protein:

Cytochrome *c* peroxidase, or CcP is a water-soluble heme-containing enzyme of the peroxidase family that takes reducing equivalents from cytochrome *c* and reduces hydrogen peroxide to water:

$$CcP + H_2O_2 + 2\ \text{ferrocytochrome } c\ (Fe^{2+}) + 2H^+ \rightarrow CcP + 2H_2O + 2\ \text{ferricytochrome } c\ (Fe^{3+})$$

(taken from http://en.wikipedia.org/wiki/Cytochrome_c_peroxidase)

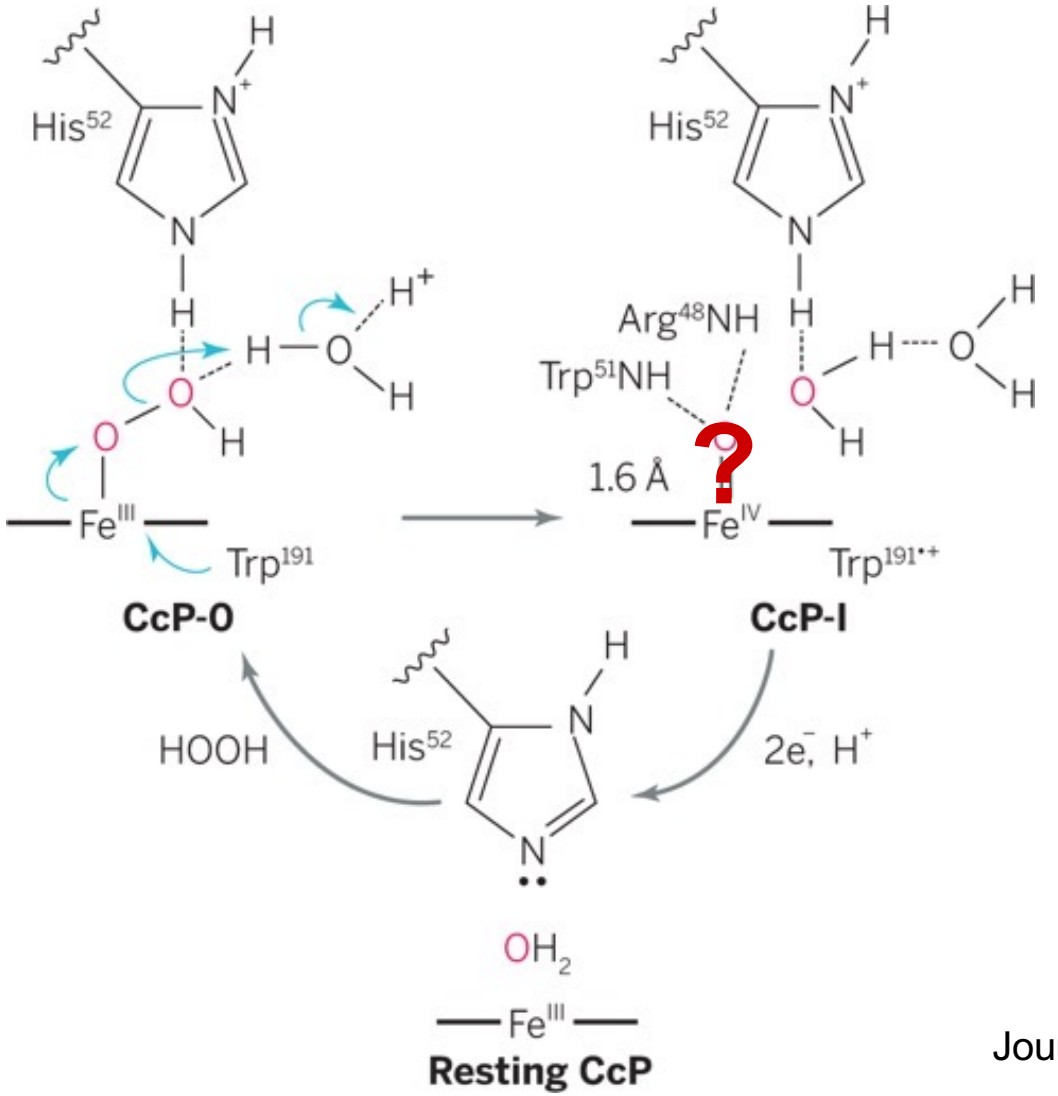
Mitochondria are the power plant of a cell (production of ATP):



http://de.wikipedia.org/wiki/Atmungskette

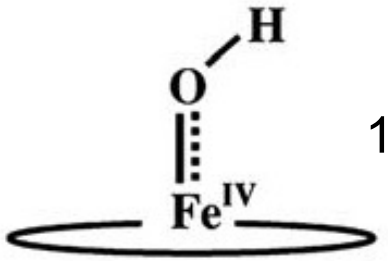
- Cytochrome C serves as an electron transporter in the respiratory chain.
- Cytochrome c Peroxidase uses two ferro-cytochrome C proteins to reduce H_2O_2 to water and two ferricytochrome C molecules

Proton-mediated mechanism.Reaction of ferric CcP with H_2O_2 first gives CcP-0, followed by O-O bond scission driven by external protonation to afford CcP-I.



J T Groves, and N C Boaz Science 2014;345:142-143

Alternative Hypothesis:

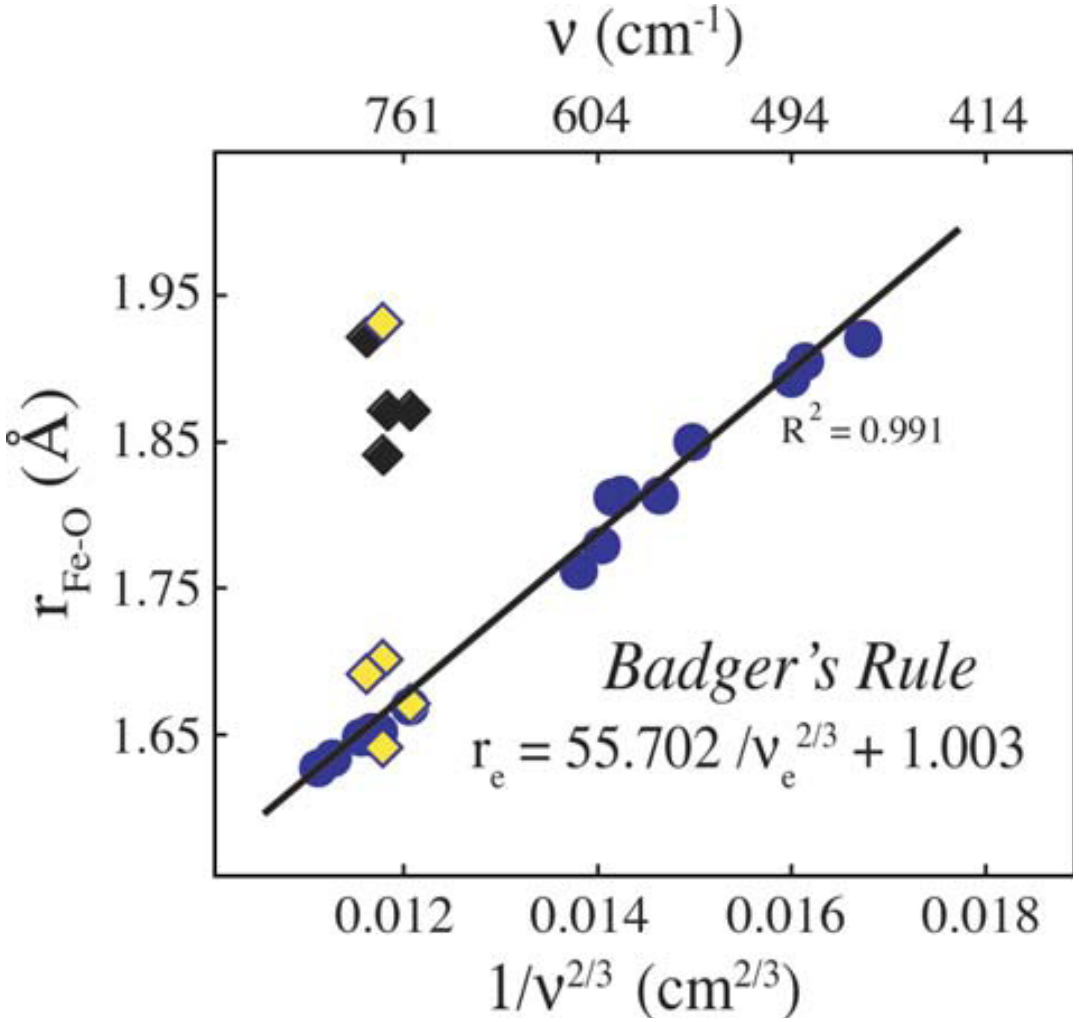


1.85 Å

Fig. 3. Compound I with an O–H bond and a bond length of Fe-O of ca. 1.85 Å.

Journal of Inorganic Biochemistry 100 (2006) 448–459

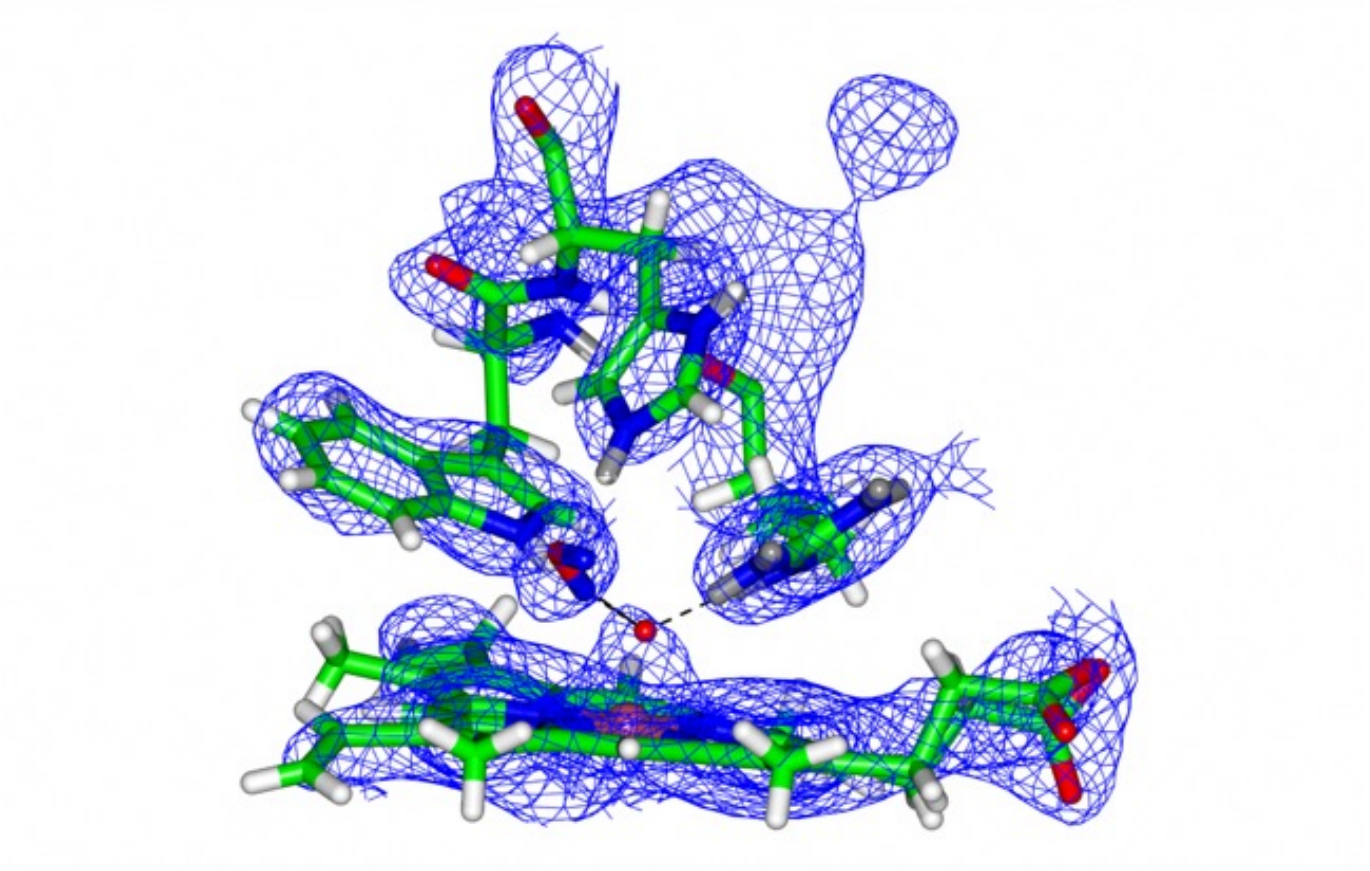
No method so far could unambigiously show the nature of the iron-oxide bond



Plot of computed stretching frequency vs Fe–O bond distance. **Yellow diamonds** are from resonance Raman or EXAFS and the solid diamonds from X-ray crystal structures. The **blue circles** are from calculations.

Figure taken from: Journal of Inorganic Biochemistry 100 (2006) 448–459

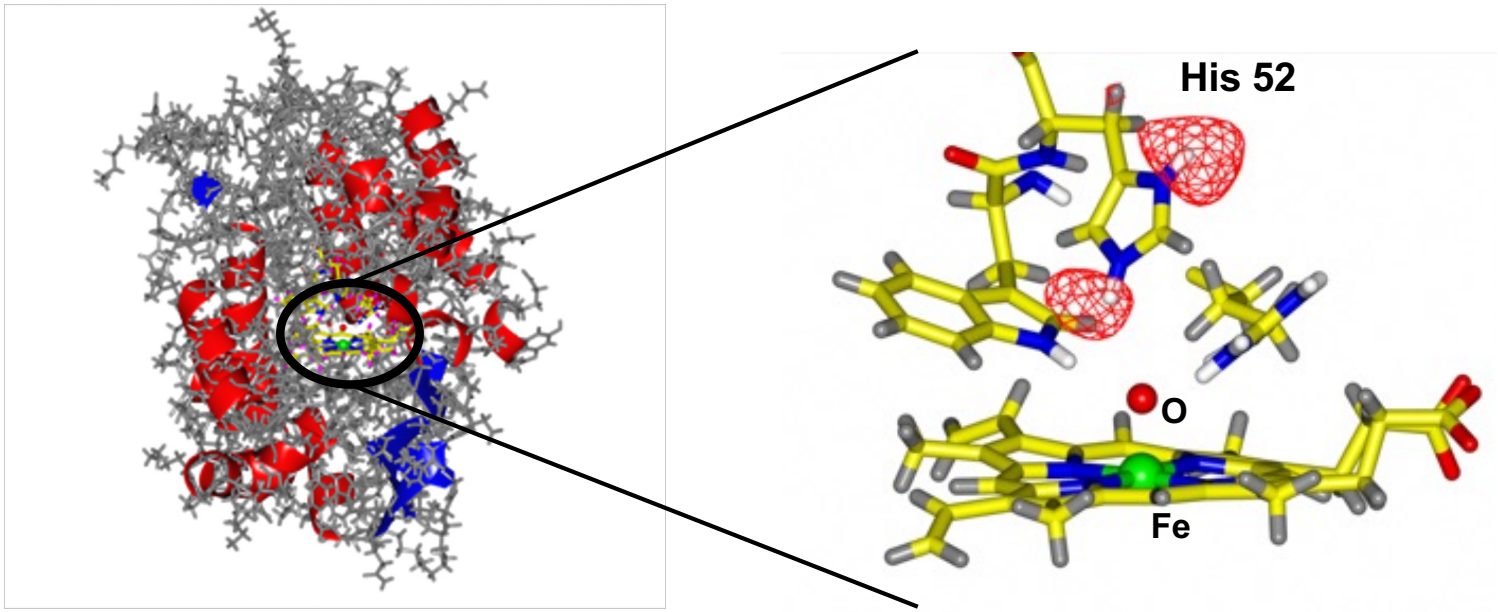
Compound I of Cytochrome c Peroxidase



Cecilia M. Casadei, Andrea Gumiero, Clive L. Metcalfe, Emma J. Murphy, Jaswir Basran, Maria Grazia Concilio, Susana C. M. Teixeira, Tobias E. Schrader, Alistair J. Fielding, Andreas Ostermann, Matthew P. Blakeley, Emma L. Raven, Peter C. E. Moody, Science 2014;345:193-197

Omit-Map for the two exchangable hydrogen atoms at His52

Cytochrome-c-Peroxidase, Compound I at 100 K:



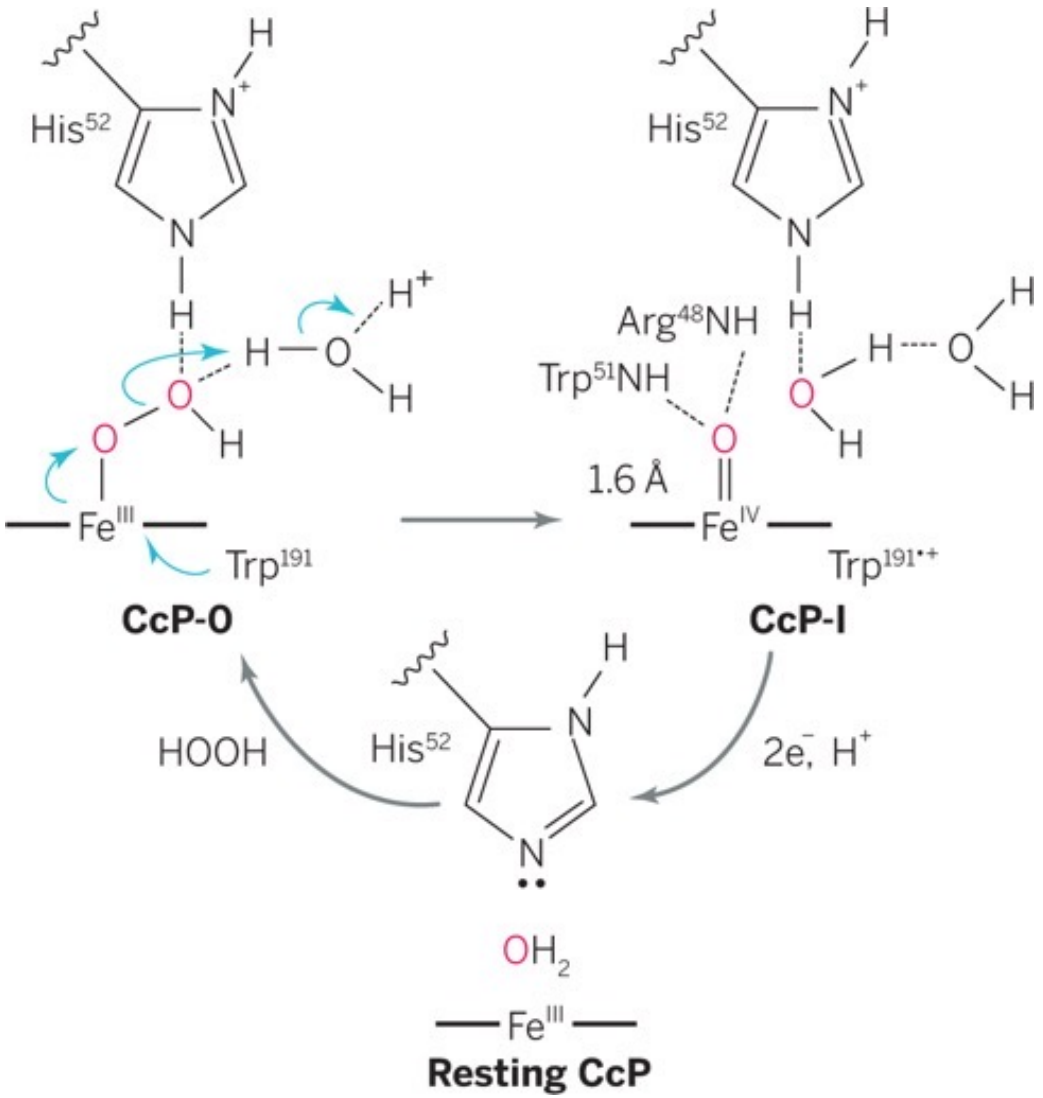
→ The oxygen atom bound to iron is not protonated.

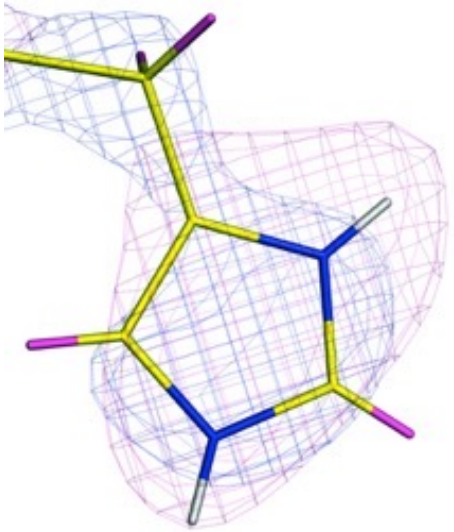
→ The amino-acid His52 is doubly protonated

↷ The reacton mechanism has to be thought over again!

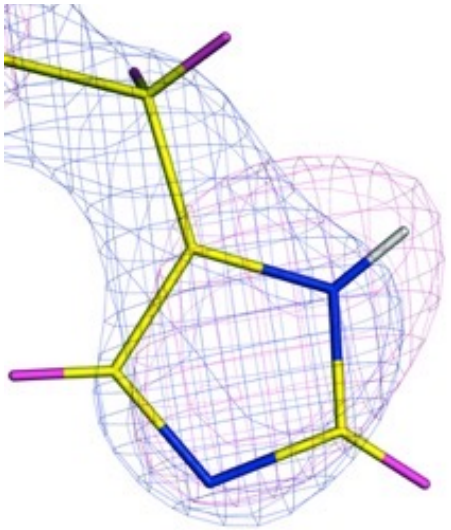
Casadei et al. Science **345**, 193 (2014)

Proton-mediated mechanism.Reaction of ferric CcP with H2O2 first gives CcP-0, followed by O-O bond scission driven by external protonation to afford CcP-I.





His 52
Compound I

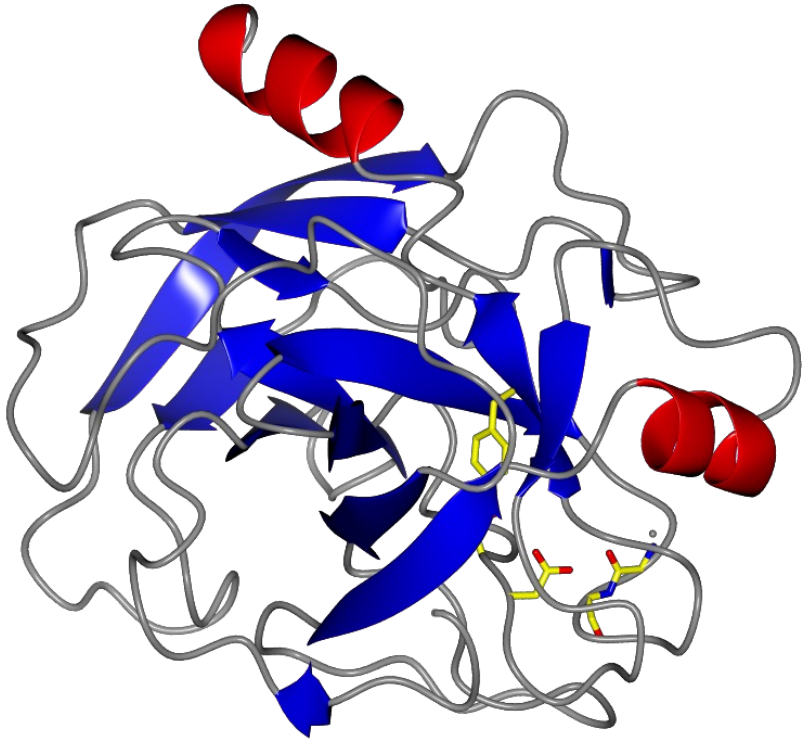


His 52 ferric
(resting)

J T Groves, and N C Boaz Science 2014;345:142-143

The Trypsin model enzyme

Bindung von Inhibitoren an das aktive Zentrum von Trypsin: Ladungen verschieben Protonierungszustände

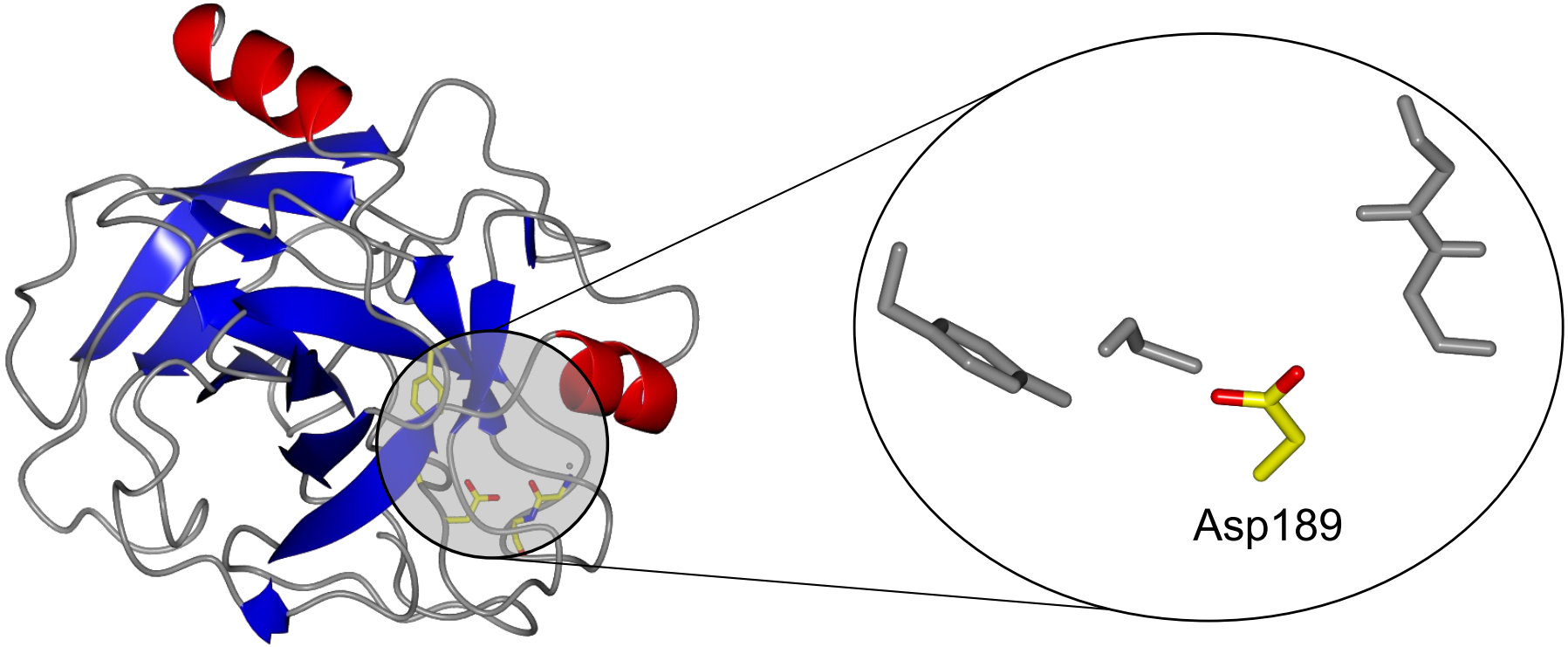


- Trypsin ist ein Modellprotein für die Klasse der Serinproteasen
- Es schneidet Proteine, die basische Aminosäuren enthalten wie zum Beispiel Arginin oder Lysin

Group of Prof. G. Klebe (Univ. Marburg)

Schiebel J. et al., Angewandte Chemie Int. Edition (2017), 56 (17):4887-4890

Bindung von Inhibitoren an das aktive Zentrum von Trypsin: Ladungen verschieben Protonierungszustände

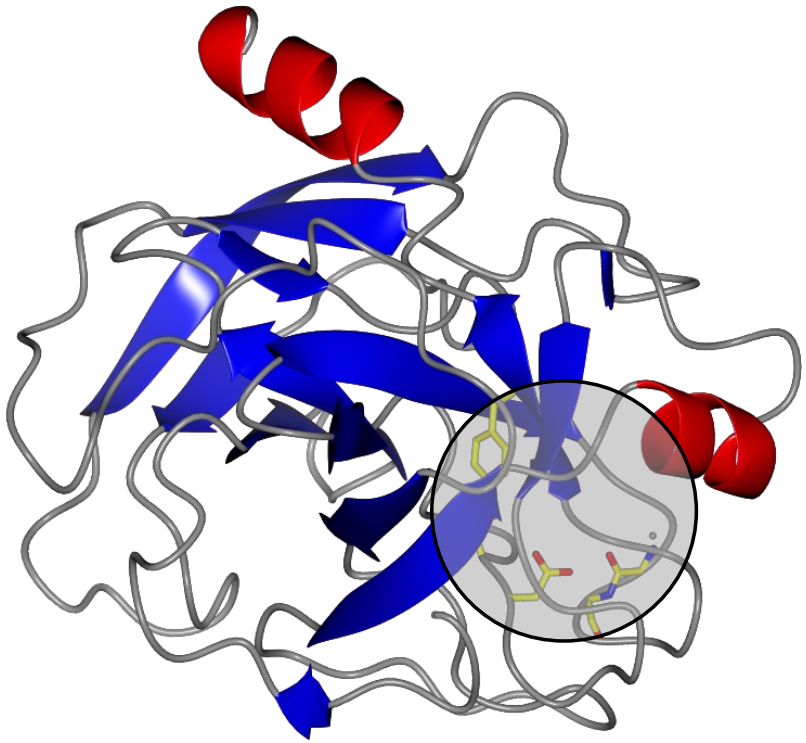


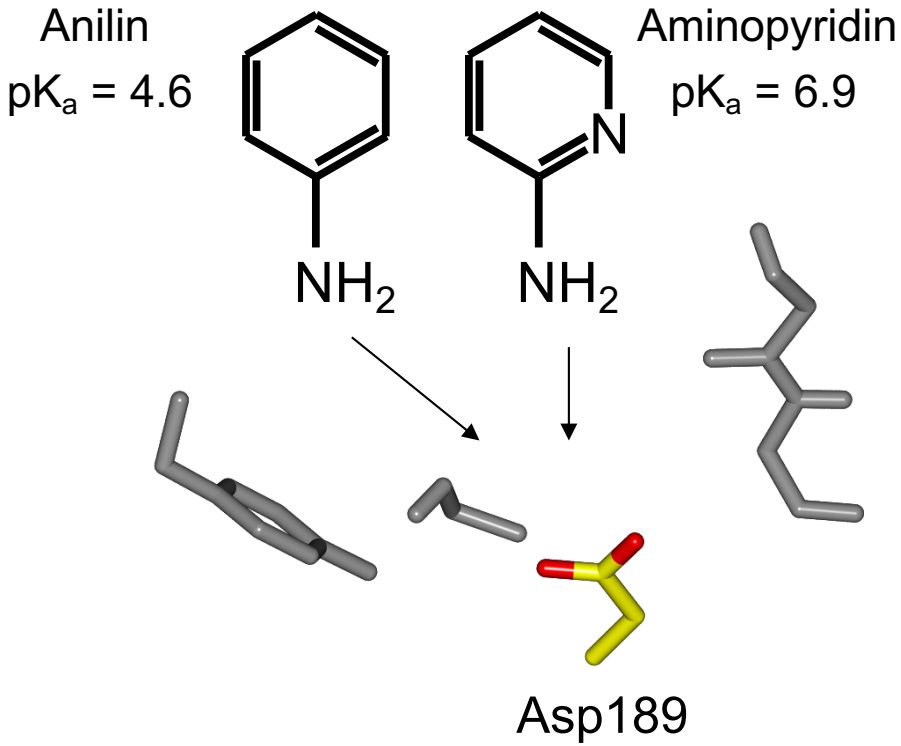
➡ Asp189 ist verantwortlich für die Substratspezifität

Gruppe von Prof. G. Klebe (Univ. Marburg)

Schiebel J. et al., Angewandte Chemie Int. Edition (2017), 56 (17):4887-4890

Bindung von Inhibitoren an das aktive Zentrum von Trypsin: Ladungen verschieben Protonierungszustände



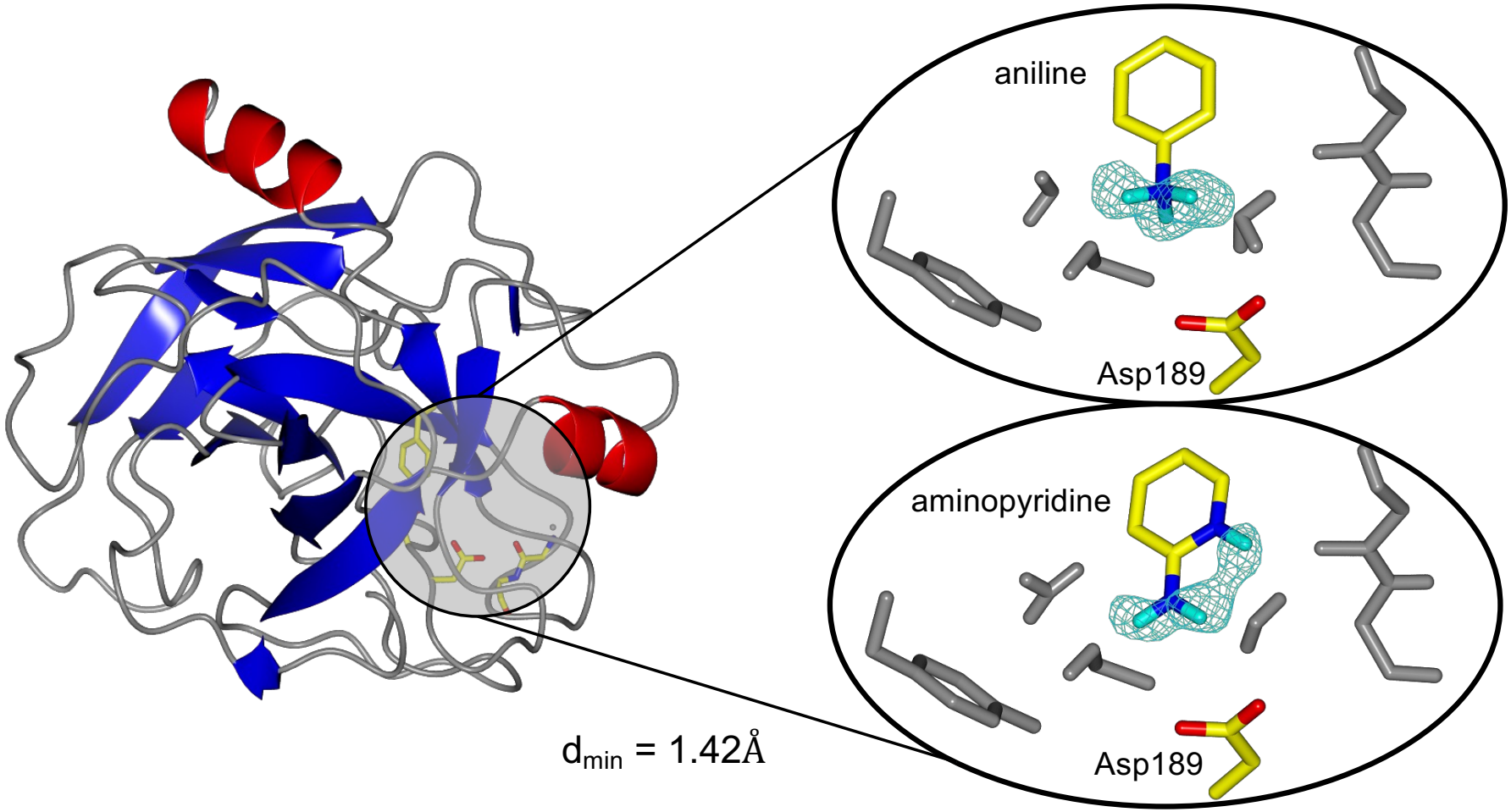


➡ Wie binden weniger basische Fragmente? Werden auch sie protoniert?

Gruppe von Prof. G. Klebe (Univ. Marburg)

Schiebel J. et al., Angewandte Chemie Int. Edition (2017), 56 (17):4887-4890

Die Antwort: Ja, basische Fragmente werden protoniert! Und auch Tautomere sind bei der Bindung zu berücksichtigen!



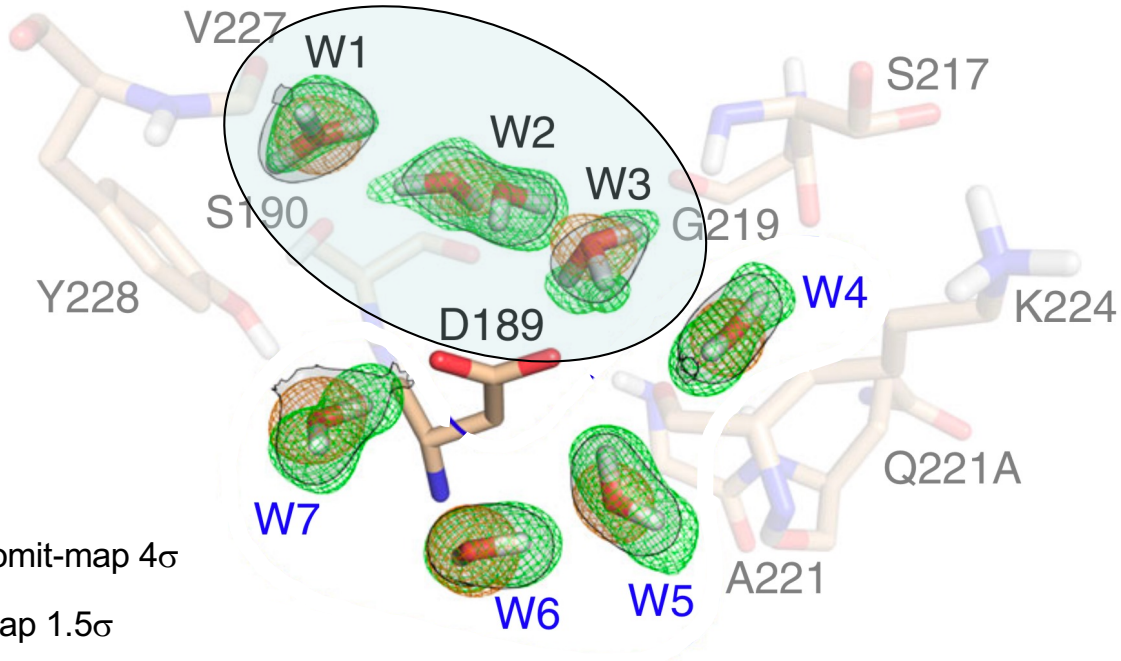
Gruppe von Prof. G. Klebe (Univ. Marburg)

Fo-Fc omit-map; +3.5σ

Schiebel J. et al., Angewandte Chemie Int. Edition (2017), 56 (17):4887-4890

Wasserstuktur im Protein Trypsin ohne gebundenes Substrat:

d_{min} = 1.45Å



➡ Wenige Wasserstoffbrückenbindungen, hohe Beweglichkeit der Wassermoleküle

Gruppe von Prof. G. Klebe (Univ. Marburg)

Schiebel J. et al., Nat. Commun. (2018), 9:3559

Zusammenfassung

- Neutronen geben wichtige Hinweise auf die Ladungen im aktiven Zentrum von wichtigen Enzymen. Das ermöglicht die Entwicklung von nebenwirkungsfreien Medikamenten gegen viele Krankheiten, auch solche gegen die es noch keine Medikamente gibt.

Zum Beispiel beim Enzym Trypsin, einer Serinprotease, wurde gefunden:

- Anilin ist wider erwarten protoniert
- 2-Aminopyridin bindet als Tautomer mit dem Proton am Pyridinring.
- Aktives Zentrum des Trypsin ist gefüllt durch Wasser mit hoher Beweglichkeit

- Die Konkurrenz durch die modernen Transmissions-Elektronen-mikroskope muss die Neutronenproteinkristallographie nicht fürchten, da die relevanten Protonierungszustände von dieser Technik auch nicht gut erfasst werden können.

Was man sich merken kann….

- Enzyme sind eine Untergruppe von den Proteinen, die durch die DNA kodiert werden. Sie sind die chemische Fabrik des Lebens. Sie sparen Energie durch Erniedrigung der Aktivierungsenergie und sie sind sehr substratspezifisch und produzieren wenige bis keine Nebenprodukte.
- Das Studium der Struktur dieser Enzyme gibt uns Aufschluss über mögliche Optimierungsmöglichkeiten und lässt uns auch bessere Medikamente entwickeln, die gezielt in den Stoffwechsel des Menschen eingreifen können und so auch weniger Nebenwirkungen haben.
- Das Instrument BioDiff kann den Wasserstoff in diesen Enzymen sichtbar machen, was nur NMR-Spektroskopie auch kann, aber nur bis zu einer gewissen Größe des Enzymes bzw. Anzahl von Aminosäuren.

Der Dank geht an unsere Messgäste und an das BioDiff-Team:

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Dieter Richter

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Hanna Kwon
Peter Moody
Andreas Heine
Johannes Schiebel
Gerhard Klebe

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