The Phase Problem

Lewis & Clark Workshop
Macromolecular Crystallography

The Phase Problem

- Data collection → |F|
- Map calculation requires vector **F**
  - direction or phase offset
- Phases can not be measured directly

Methods to be covered

- Direct methods - briefly
- *Ab initio* - skip
- Molecular Replacement
- Isomorphous Replacement
- Multi-wavelength Anomalous Diffraction

Phase determination – Direct Methods

- Statistical interdependence of structure factors
  - $P(\alpha_h) = f\{|F_{h2}|, |F_{h3}|, \ldots\}$
- Apply constraints
  - E.g. atomicity
    - Spheres uniform density
    - Separated by vacuum
- Nobel Prize
  - Hauptman & Karle
- Applies to "small" molecules
  - Salts
  - Organic molecules
  - Small proteins
    - "Shake-N-Bake"
    - Hauptman & Weeks; Sheldrick
    - < 1000 atoms
- Heavy atom "sub-structures"
  - Derivatives
  - SeMet

Part 2:

MOLECULAR REPLACEMENT
WHEN RELATED STRUCTURE KNOWN

Overview

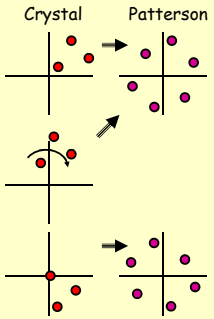
- Quickest method
- When related "probe" structure is known
- Requirement
  - Know how to superimpose probe structure
  - On unknown structure
  - In a different unit cell
  - (Before unknown structure is known)
  - How to:
    - Orient – 3 angles – "Rotation Function"
    - Place – 3 position vector components – "Translation function"
- Method not without its difficulties

How related must the probe structure be?

- No hard & fast rules – but empirical bottom line
- To get an interpretable map
  - > 70% structure needs to be approximated
    - Atoms say w/in 2 Å
- Sometimes can combine probes, sum → > 70%
  - Difficult to figure orientation / translation
  - Methods improving…

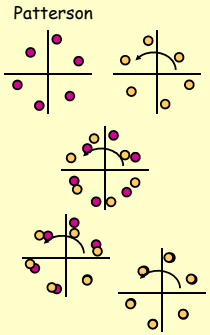
Determination of the Orientation

- Patterson synthesis
  - $P(\mathbf{x}) = \Sigma_h |\mathbf{F}_h|^2 \cos 2\pi(\mathbf{hx})$
  - No phases
  - Auto-correlation
  - Vectors between atoms
- Compare
  - Vectors w/in molecule
  - Not between molecules
- "Self-vectors" shorter
  - Patterson depends on molecular orientation



Orientation from Patterson Overlap

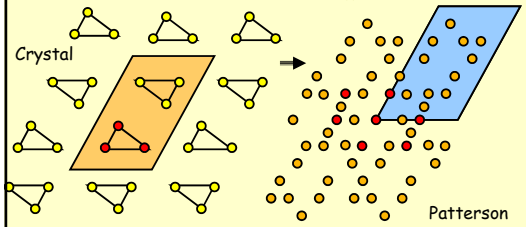
- Rotate Probe model coordinates
  - Calculate Patterson
  - Assess overlap
- Compare to observed Patterson
- Step over 3 angles
- At which orientations are observed and calculated Pattersons well correlated?



Challenges of Rotation Function

- Many solutions look ~equally good.
- The highest scoring is not always correct
- Correct could be 30th… or worse

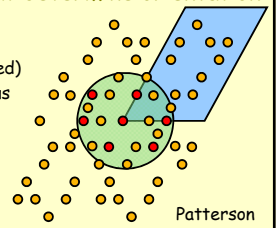
Patterson vectors that determine orientation



- Patterson contains
  - Peaks for all molecules
  - Peaks between neighbors – w/in & between unit cells
- Red Patterson peaks are from single molecule

Patterson vectors that determine orientation

- If consider only peaks close to origin
  - More are self peaks (red)
- Less likely to have spurious solution
- "Integration radius"
- Impossible to completely separate
  - Self vs. cross peaks
  - →Noise in rotation function
    - →perhaps some spurious solutions



$$R([C]) = \int_v P_1(\mathbf{u}) P_0([C]\mathbf{u})\, d\mathbf{u}$$

Care needed with rotation functions

- Most sensitive to…
  - Large reflections – $|F|^2$
    - make sure all large F have been measured
  - Higher resolution data – say 3 to 5 Å
    - Check that RF not sensitive to exact limits
- Very noisy
  - Rank according to signal / noise
  - Correct solution is often the 5th, sometimes the 30th peak.
    - Continue structure determination with several solutions – which works out best?

Translation functions

- Position w/in unit cell *when orientation known*
- Greatest challenge of Molecular Replacement
- What position most consistent w/ diffraction data?
- Translation function: $T(t) = \int_V P_{1,2}(\mathbf{u},\mathbf{t})\, P(\mathbf{u})\, d\mathbf{u}$
  - $P_{1,2}$ are Patterson vectors between molecules related by crystal symmetry
  - P(u) is observed Patterson
- Patterson Correlation, Corr(t) =

$$\frac{\sum_h (F_o^2 - \langle F_o^2\rangle)(F_c^2 - \langle F_c^2\rangle)}{\left\{\sum_h (F_o^2 - \langle F_o^2\rangle)^2 \sum_h (F_c^2 - \langle F_c^2\rangle)^2\right\}^{1/2}}$$

Translation Functions are Challenging

- Patterson functions intrinsically noisy
- Translation functions sensitive to exact orientation
  - Slight orientational error →
  - May miss correct position
- Techniques to improve your chances
  - Combine with other information
    - Packing analysis – molecules overlap?
  - Refine orientation – Patterson correlation function

Solving Molecular Replacement

- Two steps: (a) Orientation (RF); Position (TF)
- Several packages that combine them
  - Explore several possible RF solutions
  - Reduce errors due to differing conventions
- Programs: Phaser (Max. likelihood); AMoRe; GLRF
- Model → $\underline{\mathbf{F}}_{calc}$; ($|F_{calc}|$, φ_{calc})
  - Combine w/ data: ($|F_{obs}|$, φ_{calc}) → hybrid map
  - Remodel → better φ_{calc} → better map → model…
- Success judged by agreement between F_{calc} & F_{obs}.
  - … and ability to improve it with refinement
  - Expected (new) features in map, e.g. sequence
  - Need for caution

Part 3:

ISOMORPHOUS REPLACEMENT

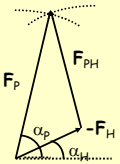
CLASSIC APPROACH W/O RELATED STRUCTURE

Confusing Names

- Uses Heavy Atoms, but *not* "Heavy Atom Method"
- Adds atoms rather than *replacing* them
  - Historically – based on methods where replaced
- Isomorphous – protein must remain in same conformation after heavy atoms added
  - or almost so

Phase Det. – Isomorphous Replacement

1. Collect "native" data set: $|F_P|A$
2. Attach heavy atom(s) to protein
3. Collect "derivative" data set: $|F_{PH}|$
4. Solve heavy atom positions from ($F_{PH} - F_P$)
   - Like small molecule structure
   - Calculate $\mathbf{F}_H$ (vector)
5. Vector relationship: $\mathbf{F}_{PH} = \mathbf{F}_P + \mathbf{F}_H$.
6. Triangulation even w/o α_{PH}, α_P.
7. Solve for α_P.



Heavy Metals

- Few atoms bound
  - Need to be able to solve as small molecule
- Need to be able to detect
  - High atomic number – $f^2 = \sum_i Z^2$.
    - Contribution $\propto Z^2$.
- Hg, Pt, Pb, Au, U…
  - > 200 reagents, e.g.: K_2PtCl_4, $HgAc_2$, *p*-chloromurcuribenzoic acid, $UO_2(NO_3)_2$, $PbAc_2$
  - Try a wide selection

Heavy Metal - Chemistry

- Hg binds covalently to Cys
  - Great if works
  - Sometimes reduces essential disulfides
    - Denatures protein
- Covalent binding to 1° amines:
  - K_2PtCl_4, K_2AuCl_4…
  - Charged interaction also possible, e.g. K_2AuCl_2
- Electrostatic binding
  - E.g. $PbAc_2$, uranyl acetate & carboxylates

Why particular reagents may not work

- Conformational change
  - Denaturing
  - Subtler non-isomorphism
- Binds at too many sites (to determine positions)
- No binding sites – reactive sites occluded
- Buffer interactions
  - $PtCl_4^{2-}$, $AuCl_4^{2-}$ react w/ amino "Good" buffers
  - Reagent precipitated
    - Buffers containing PO_4, SO_4 precipitate Hg^+, Hg^{++}, Pb^{++} etc..

Searching for derivatives

- Typically have to test dozens of reagents
  - Sometimes hundreds
  - Each at several concentrations
- Excellent guidelines for efficient searches:
  - Petsko, G. Methods in Enzymology **114**
  - Blundell & Johnson, "Protein Crystallography", 1976.
- Chemical series – try most reactive, then least
  - E.g. $PtCl_4^{2-}$, $AuCl_4^{2-}$
- But… Differ in "hardness", lability
  - Ionic vs. covalent interactions
- Try examples of "soft" & "hard" species

Derivatives – the bottom line

- Diffraction / phasing power
- Days of work, each test
- Data set
  - Quality of diffraction
  - Are the intensities changed?
  - Determine sites
  - Phases – good enough?

Screening tests – eliminate candidates

- Does it precipitate?
  - Mother liquor – no need to waste protein!
- Does it react?
  - Colored compounds
    - Some change color w/ valency e.g. Pt(II) → Pt(IV)
      - E.g. $PtCl_4^{2-}$, $AuCl_4^{2-}$
    - Others – color should concentrate in crystal
  - Non-colored
    - Does overdose crack a crystal?
      - No: probably not reacting
      - Yes: reacting or osmotic shock?
  - Does it change the diffraction pattern?

How much should the diffraction be changed?

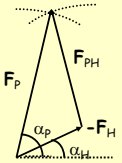
- Maximize heavy atom signal w/o changing protein
- Measure $\Delta F = \Sigma|F_{PH} - F_P| \; / \; \Sigma F_P$.
  - Above 30% - usually non-isomorphous
  - Below 12% - barely detectable
    - Note both F_{PH} & F_P likely have 6% random error
- Want
  - Small number of binding sites (1 to 6)
  - Complete reaction at these sites
    - Full "occupancy"
- Check w/ Patterson or Difference Fourier (later)
- Usually need to optimize concentration, soak time

Frustrations of Screening

- Can fail at a number of stages
- Final tests require substantial investment of work
  - Careful preliminary tests!
- May need to try many compounds
- May need to transfer to more favorable buffer
- Will need ~ three derivatives
  - Couple of months → a year or two

From heavy atoms to phases... (overview)

- For each reflection...
- Solve for α_P by triangulating: $\mathbf{F}_{PH} = \mathbf{F}_P + \mathbf{F}_H$.
- Need α_H, calculated from positions in unit cell.
- Determination of positions
  - Difference Fourier if preliminary phases
  - Difference Patterson w/o phases



Meaning of the Patterson

- $P(\mathbf{u}) = \int_u \rho(\mathbf{x})\rho(\mathbf{x}-\mathbf{u})d\mathbf{x} = \Sigma_h |\mathbf{F}_h|^2 \cos 2\pi(\mathbf{hx})$
- Let $\rho(\mathbf{x}) = 0$, except at atom positions
- $P(\mathbf{u})$ is zero except when $\mathbf{x}$ & $\mathbf{x}-\mathbf{u}$ are atoms
- Peaks in $P(\mathbf{u})$
  - When $\mathbf{u}$ is an inter-atomic vector
  - Height = ρ(atom1) x ρ(atom2) = $Z_1 \times Z_2$.
  - Number = N^2, N at origin
    - Blurred according to resolution - overlapped
- Interatomic vectors → solve small structure
  - Large structure – Patterson too complicated
- Difference Patterson $|F_{PH}-F_P|$ approx heavy atoms

Patterson → Atom positions: Harker Sections

- Patterson peaks a.k.a. "vectors"
- Crystal symmetry → concentration in planes
- Example 2-fold along b:
  - (x,y,z) = (-x, y, -z) → vector = (2x, 0, 2z)
  - Harker section (u,v,w) v=0; u=2x; w=2z
- Example 2_1 along b:
  - (x,y,z) = (-x, y+ ½, -z) → vector = (2x, ½ , 2z)
  - Harker section (u,v,w) v= ½ ; u=2x; w=2z

1. Search (Harker sections) for peaks
2. Find (x,y,z) consistent w/ peaks
   - Educated guesswork
   - *Systematic computational searches*

Difference Pattersons Full of Error

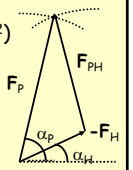
- Crude approximation
  - Heavy atom vectors: $\Sigma_h|\mathbf{F}_{PH,h}-\mathbf{F}_{P,h}|^2\cos 2\pi(\mathbf{hx})$
    - "P" for protein; "PH" for protein + heavy atom
  - Can only calculate: $\Sigma_h(|F_{PH,h}|-|F_{P,h}|)^2\cos 2\pi(\mathbf{hx})$
  - Many background peaks
- Small (20%) difference between 2 exptl values
- Then squaring the difference!
- Very sensitive to
  - Errors in intensity data
  - Missing reflections
- Some prove intractable

What to do when Patterson insoluble?

- Put aside
- Find another derivative
- Use 2nd derivative to calculate approx phases
- Calculate difference Fourier using 1st derivative amplitudes and 2nd derivative phases
  - $\rho(\mathbf{x}) = 1/V\ \Sigma_{\mathbf{h}}\ (|\mathbf{F}_{PH,\mathbf{h}}| - |\mathbf{F}_{P,\mathbf{h}}|)\ \exp\{-2\pi i\mathbf{h}.\mathbf{u}\}$
  - Coefficients are not squared – less error
  - N peaks for N sites

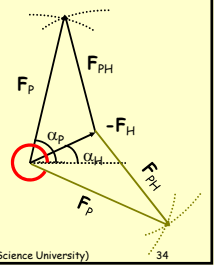
Using heavy atom positions…

- From Difference Patterson / Fourier
- Calculate $\mathbf{F}_H$ vector = $\Sigma f_h \exp\{2\pi i\mathbf{h}.\mathbf{x}\}$
- W/ measured $|F_P|$ & $|F_{PH}|$ amplitudes
  - Using cosine rule:
  - $|F_{PH}|^2 = |F_P|^2 + |F_H|^2 + 2|F_P||F_{PH}|\cos(\alpha_P-\alpha_H)$
  - $\alpha_P = \alpha_H + \cos^{-1}\{((|F_{PH}|^2 = |F_P|^2 + |F_H|^2) / 2|F_P||F_{PH}|\}$

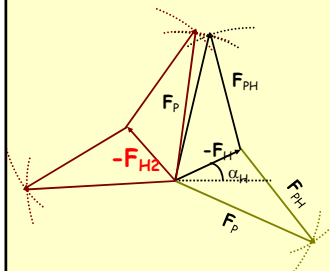


Single Isomorphous Replacement Phase Ambiguity

- $\alpha_P = \alpha_H + \cos^{-1}\{((|F_{PH}|^2 - |F_P|^2 - |F_H|^2) / 2|F_P||F_{PH}|\}$
  - Symmetry of cosine: 2 angles have same cosine
  - $\alpha_P = \alpha_H \pm$ something
- Two phase angles are equally probable
- (Note convention of plotting negative $\mathbf{F}_H$)



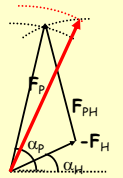
Multiple Isomorphous Replacement (MIR) to Resolve this Ambiguity

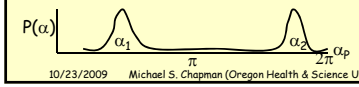


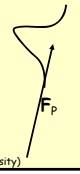
- 2nd derivative w/ heavy atoms in different places
- Different F_H
- Only one solution same for both derivatives
- Or nearly so…

Effect of Errors

- Consider small error in $|F_P|$:
  - Changes intersection point
  - Changes protein phase
- Measure particular $|F_P|$
  - "Real" value + random error
  - $P(|F_P|)$ is distribution
  - → Distribution of α_P.
  - "***Phase probability distribution***"
- Remember 2 possible phases
  - → Bi-lobed distribution

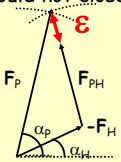




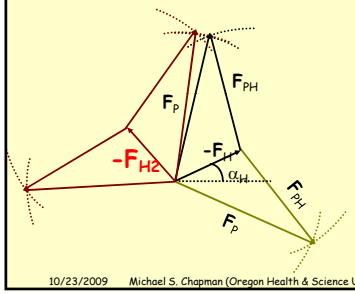


Types of Errors

- $|F_P|$, $|F_{PH}|$ experimental measurement error
- $|F_H|$ if heavy atom model is incomplete/inaccurate
  - Heavy atom refinement methods
  - Maximum Likelihood *vs.* Least-Squares
- Lack of closure, ε
  - Errors → triangle $\mathbf{F}_{PH} = \mathbf{F}_P + \mathbf{F}_H$ should not close
- Other errors contribute to ε
  - Non-isomorphism
  - Protein changed
  - Derivative not protein + heavy atoms



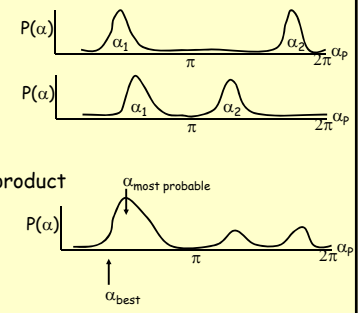
MIR & Phase Probability Distributions



- Each derivative → probability distribution
- How to combine the information?

MIR Phase probability distributions

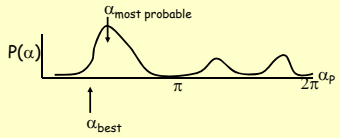
- Derivative 1
- Derivative 2
- Derivative 3…
- Combined by product



Use of Phase Probabilities

- Updated as new phase information added
- Modified according to constraints
  - Non-crystallographic symmetry
  - Solvent flattening, etc..
- Map calculation
  - One phase for each reflection
  - Which one?

Best & Most Probable phases



Most probable
- Obvious choice
- Sometimes used

Best
- Other peaks:
  - Small chance of different phases
- Weighted average
- Statistically best phase to use
- Usually used

Uncertainty in the Best Phase

- More confident of phase if
  - One peak dominates P(α)
  - Peak is sharp
- Different reflections may have phases determined w/ more or less confidence
- Can we use this information to give maps of minimal error?
- More emphasis to well-determined reflections.
- Weights – a.k.a. "figure of merit"

MIR - Conclusion

Advantages

- Prior structure not required
- Requires only standard laboratory x-ray equipment
- Errors are random not systematic

Disadvantages

- A lot of work
- Large random errors

McPherson Cpts 6 & 7

Drenth: Cpt 7

- Use other methods when appropriate
- MIR is Robust method of last resort

Part 4:

ANOMALOUS DIFFRACTION - MAD PHASING

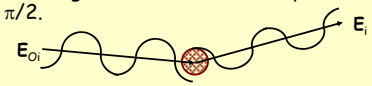
Anomalous Diffraction

- **SIR**AS – A way of resolving the phase ambiguity
  - Sometimes
- Multiwavelength Anomalous Diffraction (**MAD**)
  - Powerful new method → accurate phases

Drenth: §§7.8-9 Cpt 9

Review - Scattering by a Free Electron.

- Electomagnetic radiation = oscillating field.
- Field accelerates a charged particle with frequency ν.
- At max (or min) of field, $\mathbf{E}_i$...
  - Force on charged particle is greatest
  - Acceleration is greatest:
    - e- passes through node of oscillation
  - Electron displacement $\pi/2$ from $\mathbf{E}_i$.
- The accelerating orbital electron initiates a second electromagnetic wave with a 2nd phase change of $\pi/2$.



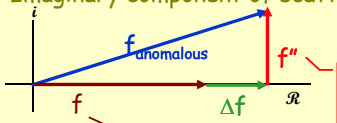
When an Electron is Not Free

- As nucleus becomes larger & more +ve...
- Electrons increasingly tethered
- Scattering from dipoles w/ natural oscillation frequency υ_n.
- Compared to a free electron, scattering is

$$f_n = \upsilon^2 / \{\upsilon^2 - \upsilon_n^2 - i\kappa_n\upsilon\}$$

  - Forced, damped oscillator
  - υ = frequency of incident radiation
  - Changes magnitude
  - Note also complex
  - Phase lag, dependent on damping constant, κ_n.
  - Phase difference (scattered-incident) > 2π.

Imaginary component of scattering factors



Imaginary component – always rotates f anticlockwise

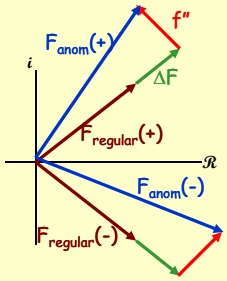
Free electron scattering (non anomalous)

Bound electrons – change in real component a.ka. f'

- f' used more than Δf, but also used for f + Δf.
  - Δf will be used to avoid confusion
- $f_{anom} = f + \Delta f + f''$

Effect on Heavy Atom Structure Factors

- Imaginary f" rotates structure factor anti-clockwise
- $\mathbf{F_H(+h) \neq F_H(-h)}$
  - Different directions
- $\mathbf{F_{PH}(+) = F_P + F_H(+)}$ $\neq \mathbf{F_{PH}(-) = F_P + F_H(-)}$
  - Friedel's law breaks
- Can use $|F_{PH}(+)|$, $|F_{PH}(-)|$ as 2 derivatives
  - As slightly different
  - As know $\alpha_P(+) = -\alpha_P(-)$



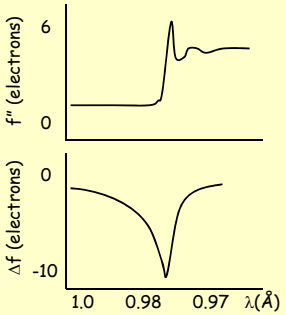
Precise Data Needed

- Anomalous scattering is small
  - ~ 6% for Hg atom & $Cu_{K\alpha}$ radiation
  - Can increase by changing λ
    - Needs synchrotron source w/ tunable wavelength
- Precisely measured data to be able to detect anomalous signal

When are Anomalous Effects Significant?

- $f_n = \upsilon^2 / \{\upsilon^2 - \upsilon_n^2 - i\kappa_n\upsilon\}$
- Limit: $\upsilon \gg \upsilon_n \rightarrow f_n = 1$
  - Scattering from free electron
- Limit: $\upsilon \ll \upsilon_n \rightarrow f_n = 0$
  - No Scattering
- Significant when $\upsilon \approx \upsilon_n$
  - υ_n are the absorption edges: K, L …

Anomalous scattering Nr absorption edge



- Se K edge
- 30 electrons
  - Max 30% change

Two Strategies for Phasing with Anomalous Diffraction

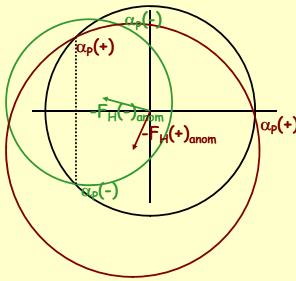
With tunable x-ray source

- MAD method
- Collect at 3 wavelengths
  - Maximize $|\Delta F|$ - λ_1
  - Maximize f" - λ_2
  - Far from edge - λ_3
- Treat $F(\lambda_3)$ as ~ native
  - No need for another crystal
- $F(\lambda_1)$, $F(\lambda_2)$ like 2 derivatives

With Fixed wavelength

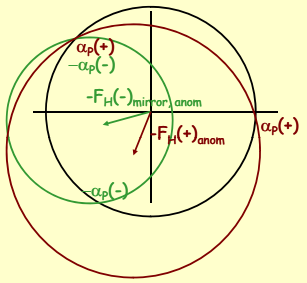
- SIRAS / MIRAS
- Collect native + derivative
  - Primary phasing from SIR / MIR
- Collect both F(+), F(-)
- Differences in $F_H(+)$, $F_H(-)$
  - Supplementary phase information
  - Breaks ambiguity
  - (Determines hand)

Theory – Anomalous Diffraction → Phases



- Know $\alpha_P(+) = -\alpha_P(-)$
- Correct solutions must be mirror images about the Real axis
- (Dotted line)

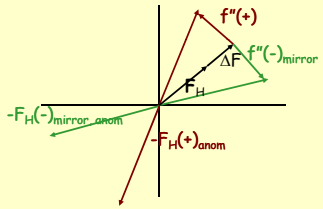
A Trick to Simplify



- Plot mirror of $F_H(-)$
- Solutions now $-\alpha_P(-) = \alpha_P(+)$
- Correct solutions now superimpose

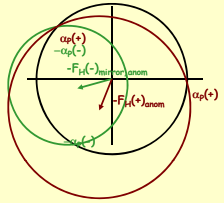
Mirror image changes direction of rotation

- f″ rotates $\mathbf{F}_H$ anticlockwise
- f''_{mirror} rotates $\mathbf{F}_H$ clockwise



SIRAS

- Resolve phase ambiguity with single derivative
- Based ~ 6% differences between $F_{PH}(+)$ & $F_{PH}(-)$
- Can only be exploited w/ excellent data
- $\alpha_P(+)$ and $-\alpha_P(-)$…
  - Likely not exactly the same
  - Approximately at best
- Maps rarely interpretable until phases refined



SIRAS & MIRAS

- SIRAS
  - Modest supplement to SIR phasing
- MIRAS
  - Modest supplement to MIR phasing

Multiwavelength Anomalous Diffraction

MAD Phasing

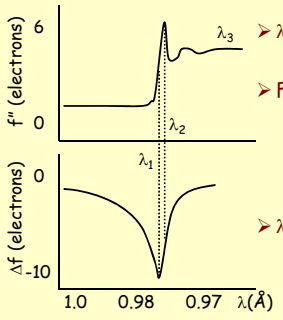
MAD

- Principles exactly the same as SIRAS
- but… Tune λ to maximize the anomalous effects
- Change λ to mimic isomorphous replacement
  - MIR: Change protein & collect diffraction
  - MAD: Same protein & change wavelength
    - Protein must contain an anomalous scatterer
    - "Derivative" is isomorphous – by definition
      - Eliminate major source of error
        - MAD can → very precise phases

Anomalous Scatterers

- Natural atom
  - Fe proteins etc..
- Isomorphous atom substitution
  - Lanthanide for Ca^{++}, etc..
  - Se for S
    - Express in bacteria that require Met.
    - Replace Met in media by seleno-Met.
    - Expression can be a challenge.
- When all else fails:
  - Make derivative – solve derivative not native

Picking wavelengths



- λ_1 absorption edge
  - Max Δf
- λ_3 far from edge
  - Little effect
- $F(\lambda_1) - F(\lambda_3)$:
  - Large change in $|F_H|$ magnitude
  - Little change in direction (f")
- λ_2 --Max f"
  - Max Bijvoet difference: $||F_{PH}(+)| - |F_{PH}(-)||$

Processing MAD Data

- Start as in SIR – determine heavy atom sites
- Then calculate phases…
- Several methods
  - All fundamentally like MIRAS
  - Where do the magnitudes of $F(\lambda_1)$, $F(\lambda_2)$… intersect?
  - Known Magnitudes and directions for
    - $F_A = F_H$, Δf, f"

MAD Algorithms

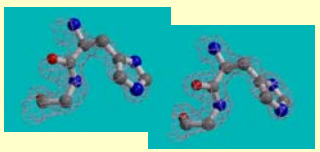
- Hendrickson & Smith – deterministic method
  - Calculate F_A, Δf, f" from 1st principles
  - Phase determined geometrically
    - 2 wavelengths enough (if no exptl. error)
    - 3rd → Least squares → best solution
- *Pseudo* MIR – pretend each λ is a derivative
  - Statistics through phase probability distributions
- Now – Maximum likelihood methods
  - SHARP: Maximum likelihood refinement of MIR / MAD parameters (Bricogne & Colleagues)
  - SOLVE / RESOLVE: Maximum likelihood MAD → auto-building (Terwilliger & Colleagues)

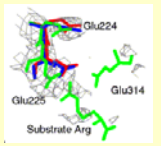
Isomorphism in MAD

- All data from one crystal
  - "Native" + "Derivative"
- Data sets are isomorphous by definition
- Eliminate big source of error in phasing
- Surprising how much one can do w/ a little anomalous signal
  - If perfectly isomorphous

Why's everyone MAD about MAD

- No derivatives required
  - Seleno-Met expression or metalloprotein
- At most one derivative required
- Most accurate experimental phases possible
  - If strong anomalous scatterer
  - Mannose Binding Protein A / Ho^{3+} (Burling & Brünger)





AK – typical of MIR

Part 5:

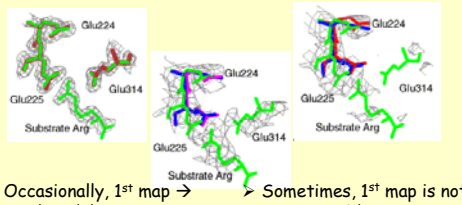
PHASE REFINEMENT

NOT TO BE CONFUSED W/ ATOMIC REFINEMENT

Phase Determination → Phase Refinement

- Phase determination is approximate
  - Molecular replacement:
    - known model is not unknown structure
  - Isomorphous replacement:
    - Small differences between F_{PH} & F_P.
    - Assumes heavy metals do not change protein structure
- Phases may need refining
- Maps will have much error

Role of Phase Refinement



- Occasionally, 1st map → good model
- Atomic refinement converges easily
- Little/no need for phase refinement
- Sometimes, 1st map is not interpretable
  - Some can be modeled
  - None can be modeled
- Phase refinement attempts to improve it

Information that can be used

- Partial model
- Constraint that two identical subunits should have same electron density
  - When not related by crystallographic symmetry
- Map features common to all protein crystals
  - Solvent regions flatter
  - Expected shape of density
    - Histogram of density levels

Density Modification and More

- Averaging, solvent flattening are examples of "Density modification"
- Something gained by merely modifying map
  - Symmetry averaging reduces noise
- More gained by requiring phases to be consistent with the constraint

Phase changes

- Consider:
  - Fourier transform: F, ϕ → map
  - Inverse transform: map → same F, ϕ.
    (Not doing anything)
- Now Consider:
  - Fourier transform: F, ϕ → map
  - Modify map → *map'* (symmetry, solvent flatten)
  - Inverse transform: *map'* → *F', ϕ'*. (changed)
    - FT again: *F', ϕ'* → *map'*
    - Map would fit constraints exactly
    - (But actually, can do a lot better…)
  - Note that both F & ϕ have changed
- Expected ϕ to change
- F was observed – probably should not be changing

Phase combination

- New Regime:
  - Fourier transform: F, ϕ → map
  - Modify map → *map'* (symmetry, solvent flatten)
  - Inverse transform: *map'* → *F', ϕ'*. (changed)
  - Discard F'.
  - Use original |F| w/ modified ϕ'.
  - FT: |F|, ϕ'. → ***map"***
    - Fits constraints better than map, but not like *map'*.
  - Inverse transform again: ***map"*** → ***F", ϕ'***.
  - Have further improved the phases
- Cycle until no further change in phases

End Point of Phase Refinement

- Map consistent with:
  - Constraints
    - Symmetry, solvent flattening, partial model…
  - Observed amplitudes

Phase Refinement by Density Modification

Constraints that are commonly imposed:

- Solvent flattening / flipping
- (Histogram matching)
- Symmetry averaging

Density modification 1 – Solvent Flattening

- Solvent molecules more motile
  - Smeared at high resolution
- Solvent regions should be ~ featureless = "flat".
- Phase errors → errors in all parts of map
  - Solvent regions may not start flat
  - How can we change phases to maximize the flatness?

Solvent Flattening B.-C. Wang implementation

- Determine solvent region in map
- Change density to average
- FT-invert map → $|F_{map}|$, ϕ_{map}
- Discard $|F_{map}|$; Combine ϕ_{map} with $|F_o|$, $\phi_{experimental}$
- Calculate a new map
  - Flatter, but not flat
- Repeat the process

How to determine solvent region -- Premise

- Need to know which areas to flatten.
- Solvent electron density
  - Few features
  - Some density everywhere
  - Low average value
- Protein regions
  - Very High where protein atoms
  - Lower than solvent between protein atoms
  - Average higher than solvent

Determination of Protein-Solvent Boundary

Wang (1985)
- User defines "solvent fraction", S.
- Locally average density
  - Weighted average
  - Smeared over 10Å radius
- Designate lowest S fraction as solvent

Leslie (1987)
- Smearing density is a convolution w/ weighting function.
- Scalar product in reciprocal space.
- Weighting function is centrosymmetric
  - Convolution is scalar multiplication – simple
- Attenuate |F|'s
- FT → smeared map
- Then like Wang (1985)

Solvent Flattening - Summary

- Can be applied to all proteins
- Sometimes ambiguous map → interpretable.

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Symmetry Averaging

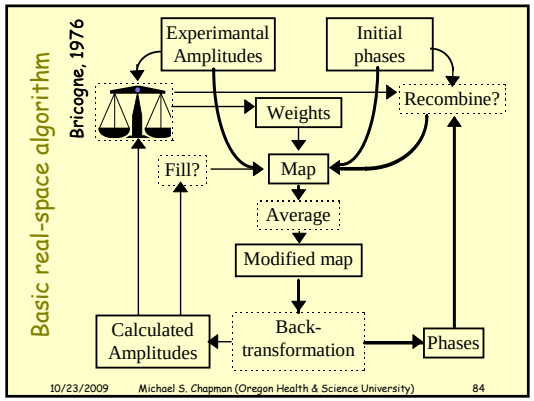
A powerful form of density modification

Source of the Information - Redundancy!

- Diffraction = continuous molecular transform sampled at lattice points
- ½ information to reconstruct - missing phases
- 2nd crystal:
  - Transform sampled @ different pts.
  - Information to calculate phases
    - in principle
  - Multiple crystals ≈ internal symmetry
  - Multiple copies of molecule in crystal a.u.:
    - Unit cell bigger ⇒ more reflections
    - Same information needed to solve unique part

History

- Reciprocal space methods developed by Rossmann, Blow, Crowther, Main *et al.*, 1960's
- Potential realized when a real-space equivalent was formulated (Bricogne, 1976)
- Slow realization - multiple copies advantageous
  - 1980's: more structures determined w/ NCS
- 1990's: many determinations w/ multiple crystals



Averaging Prerequisites

- Initial phases
- "Envelope" – which part of unit cell to average
- Orientation of the symmetry
- Position (origin) about which to rotate
- Usual methods
  - Rotation and Translation functions

Nomenclature

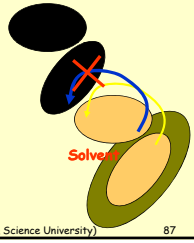
- Due to central importance of Rotation & Translation functions, often see reference to
  - "Phase refinement by Molecular Replacement"
- Confusing! - Prefer
  - "Molecular replacement" for
    - use of homologous known structure for phasing
  - "Symmetry averaging" for
    - Use of symmetry redundancy for phase improvement

Envelope defines regions to average

- Average protein w/ same bit of protein
  - Not solvent, some other part of protein…
- General case – define individual protein

Too large & overlapped neighbor might be "averaged" w/ solvent

Or wrong protein, perhaps from a different unit cell:

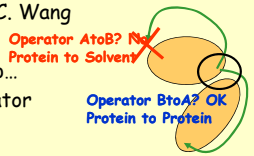


The Envelope Challenge

- Requires electron density map
- May start very poor
- Recognizing solvent protein boundary not trivial
  - Solvent flattening methods may help
- Distinguishing proteins near guess-work
- Need good enough guess to start
  - Structure determination often blocked by poor starting envelope – envelope definition is often the most challenging step in structure determination.

Automatic Envelope Determination

- Solvent boundary à la B.C. Wang
- Trial & error
  - For each region in map…
  - Apply symmetry operator
  - If density not similar, might not be protein
- Smoothing, Overlap trimming
- Programs use one or more of these tricks
  - MAMA (Kleywegt & Jones, 1993), Envelope (Rossmann et al., 1992), DM (Cowtan & Main, 1993), Solomon (Abrahams & Leslie, 1996)…
- May be able to improve envelope after some initial cycles of averaging

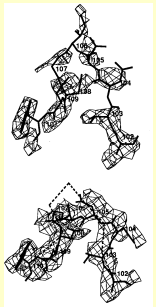


Current Programs do more

- Rave, DM, Solomon, Squash, Solve/Resolve
  - 2nd generation programs
- Important aspects more & more similar
- User-friendliness, portability
  - Averaging, FT's phase combination all in one program
- Incorporation of…
  - Other density modification, *e.g.* solvent flattening
  - Multiple crystal forms
  - Sophisticated envelopes

Power of Symmetry Averaging

- Most powerful type of phase refinement.
  - Final maps can be excellent
- Power $\propto\sqrt{\#}$ equivalents
- Phase Extension
  - Generate phases for reflections that have no phase
  - When many equivalents
  - Phases for reflections near those already phased
    - 1 or 2 lattice units
  - Extend very slowly in resolution



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Summary

- Phase refinement is often required to get an interpretable map
- Maps are also improved with phases calculated from a preliminary model, but
  - 1st have to be able to build a model
  - Will consider "ϕ_{calc}" maps later
- Next workshop – building an initial model