Protein Structure Determination using NMR Restraints

BCMB/CHEM 8190

Programs for NMR Based Structure Determination

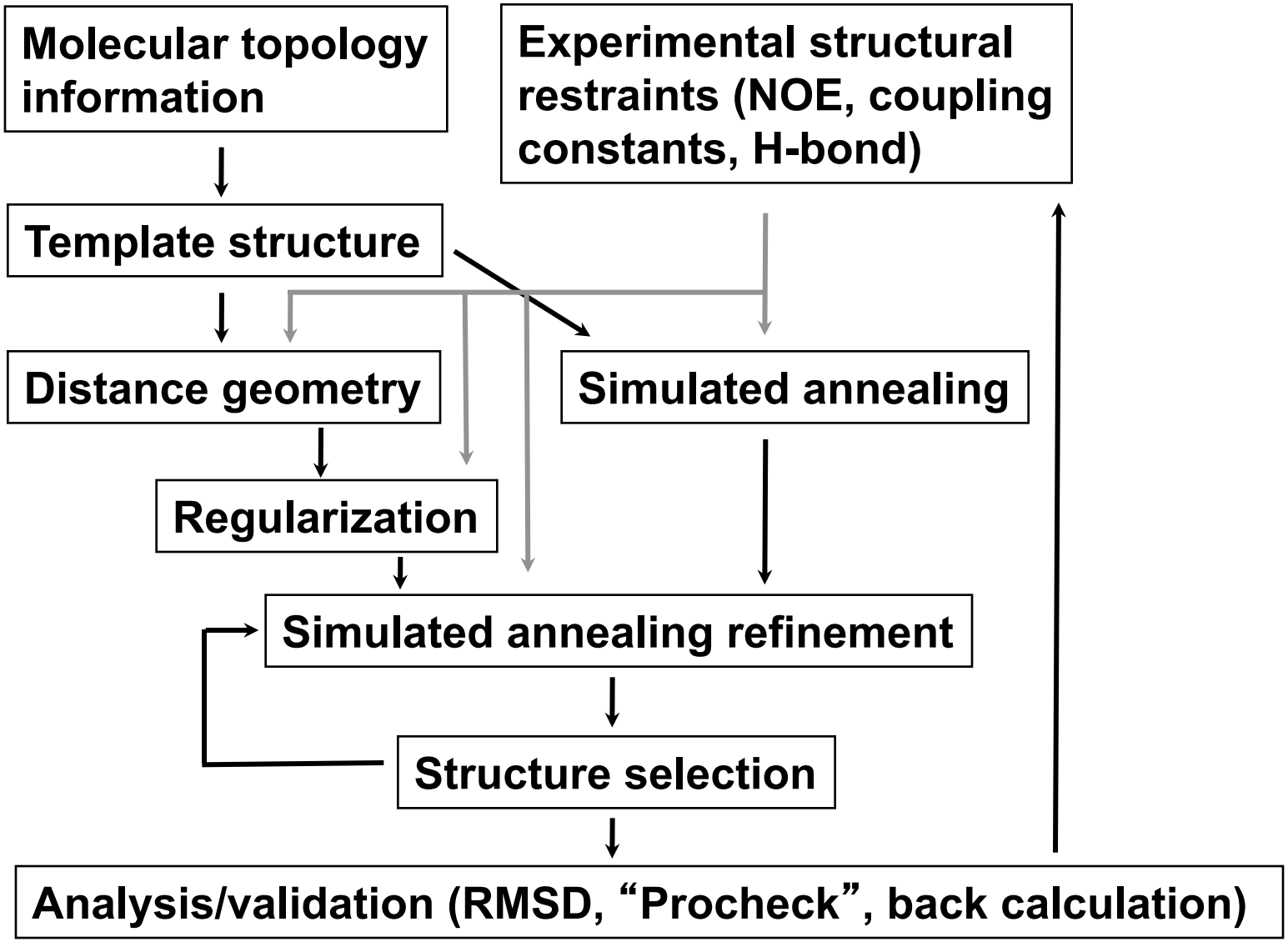
- CNS - Brünger, A. T.; Adams, P. D.; Clore, G. M.; DeLano, W. L.; Gros, P.; Grosse-Kunstleve, R. W.; Jiang, J. S.; Kuszewski, J.; Nilges, M.;Pannu, N. S.; Read, R. J.; Rice, L. M.; Simonson, T.; Warren, G. L. *Acta Cryst. D* **1998**, 54, 905.
  - http://cns-online.org/v1.3 (also older versions: 1.2, 1.1)

- X-PLOR-NIH - Schwieters, C. D.; Kuszewski, J. J.; Tjandra, N.; Clore, G. M. J.Magn.Reson. **2003**, 160, 65.
  - http://nmr.cit.nih.gov/xplor-nih/

- DYANA/CYANA - Güntert, P.; Mumenthaler, C.; Wüthrich, K. *J. Mol. Biol*. **1997**, 273, 283 and Güntert, P. *Prog. NMR Spectrosc.* **2003**, 43, 105-125.
  - http://www.las.jp/english/products/cyana.html
  - http://www.cyana.org/wiki/index.php/Main_Page

- ARIA - Linge, J. P.; Habeck, M.; Rieping, W., et al. *Bioinformatics* **2003**, 19, 315-316.
  - http://aria.pasteur.fr/

Overview of Structure Calculations



Adapted from Brünger, A. T., "X-PLOR Version 3.1, A System For X-ray Crystallography and NMR"

Molecular Topology

- Definitions of the covalent structure of the amino acids, and related info
- The empirical energy function ("force field") is defined for the amino acids
- In CNS/X-PLOR, "parameter" files and "topology" files
  - parameter files: energy constants, standard values
  - topology files: atom names/types/charges masses/connectivities for each amino acid type

```
residue ALA
  group
    atom N   type=NH1 charge=-0.36 end
    atom HN  type=H   charge= 0.26 end
    atom CA  type=CH1E  charge= 0.00 end
    atom HA  type=HA  charge= 0.10 end
    atom CB  type=CH3E  charge=-0.30 end
    atom HB1 type=HA  charge= 0.10 end
         …etc…

  bond N  HN
  bond N  CA    bond CA  HA
  bond CA  CB   bond CB  HB1   bond CB  HB2     bond CB  HB3
  bond CA  C
  bond C   O
         …etc…
```

- example: topology file entry for alanine

Experimental Restraints

- NOE data from 2D and 3D experiments are a primary source of information

$$I_{cp} = C\{\exp(-\rho T) \bullet (1 - \exp(-2\sigma T)\}$$

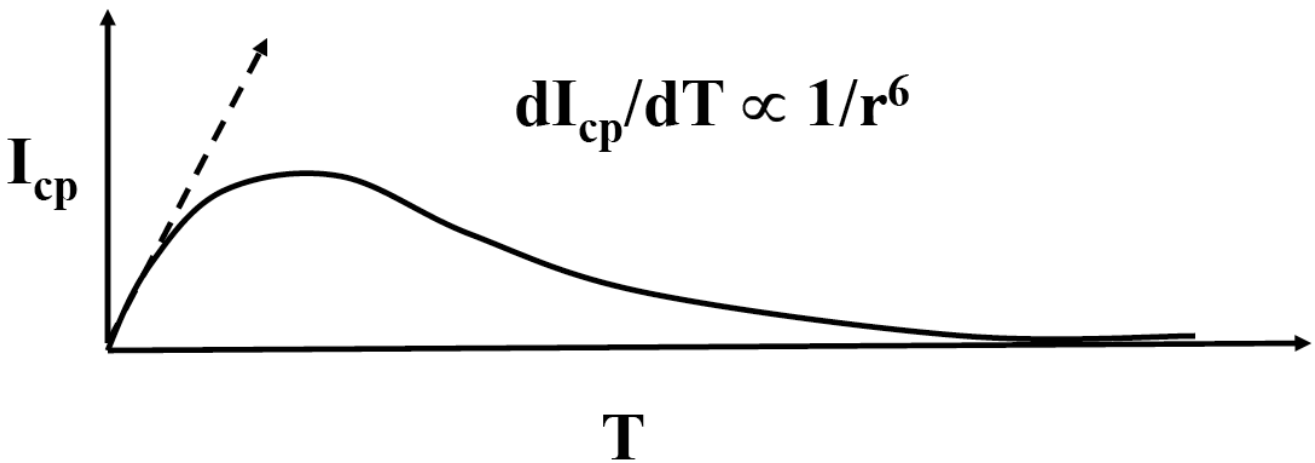
$$\rho = 2W_1 + W_2 + W_0$$

ρ: direct spin-lattice relaxation rate

$$\sigma = (W_2 - W_0)$$

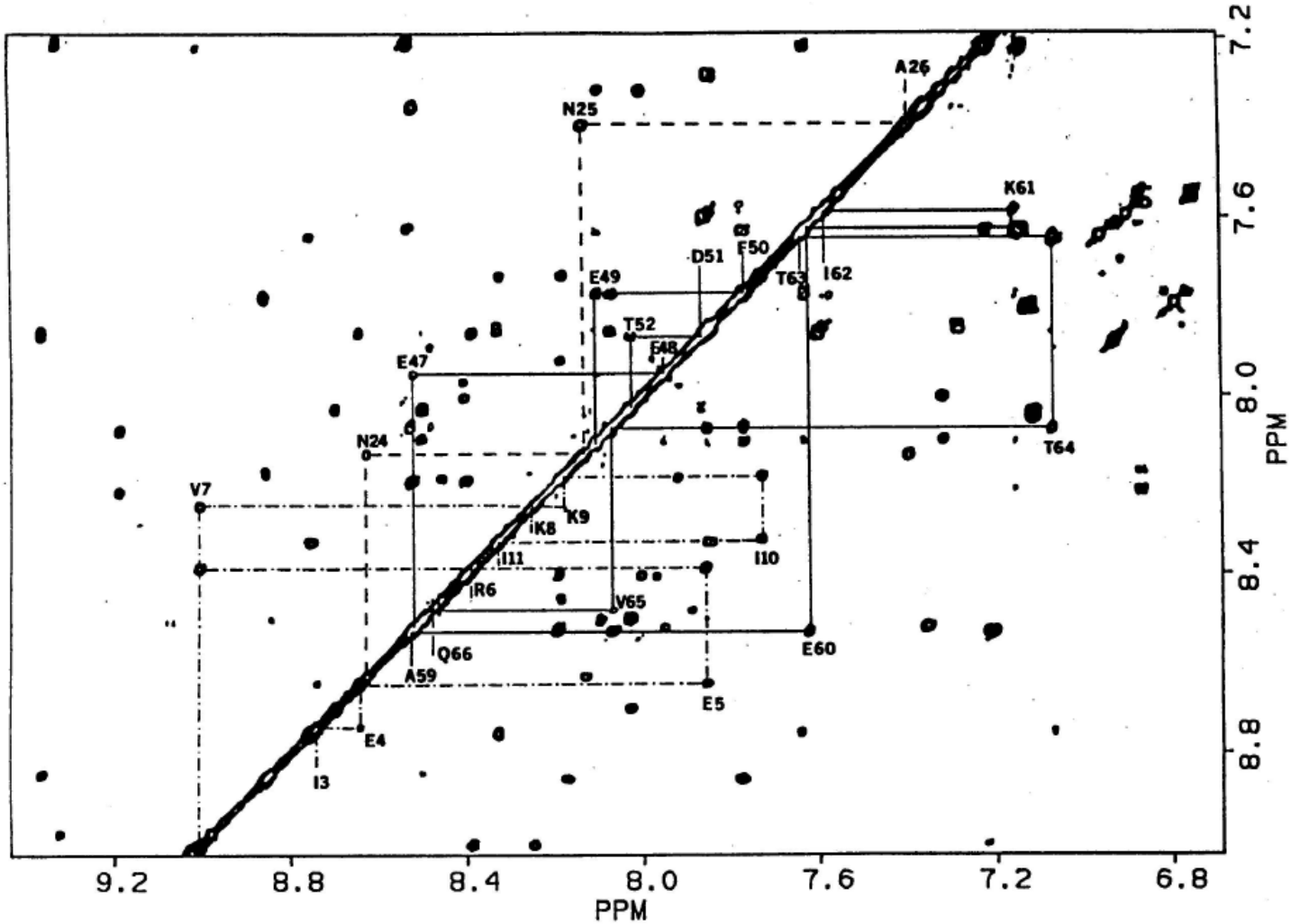
σ: cross-relaxation rate

- crosspeak intensity proportional to $1/r^6$ for short mixing times

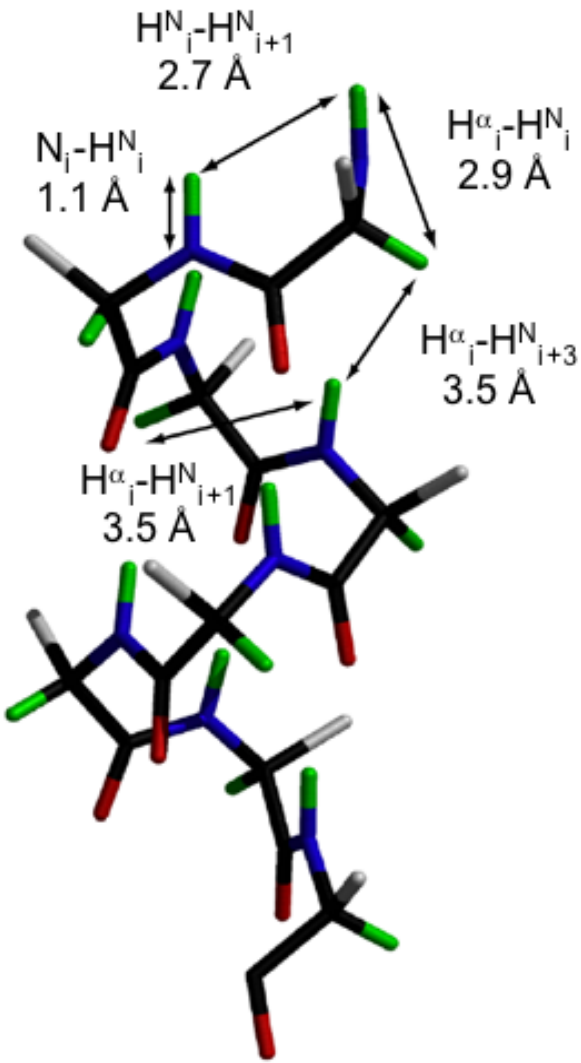


NOESY Spectrum of Acyl Carrier Protein (ACP)

- Crosspeaks in NOE spectra give ^1H-^1H distances
  - crosspeak intensities are approximately proportional to $1/r^6$
  - big crosspeaks, short distances, small crosspeaks, larger distances (up to approximately 5 or 6 Å or so)



Experimental Restraints



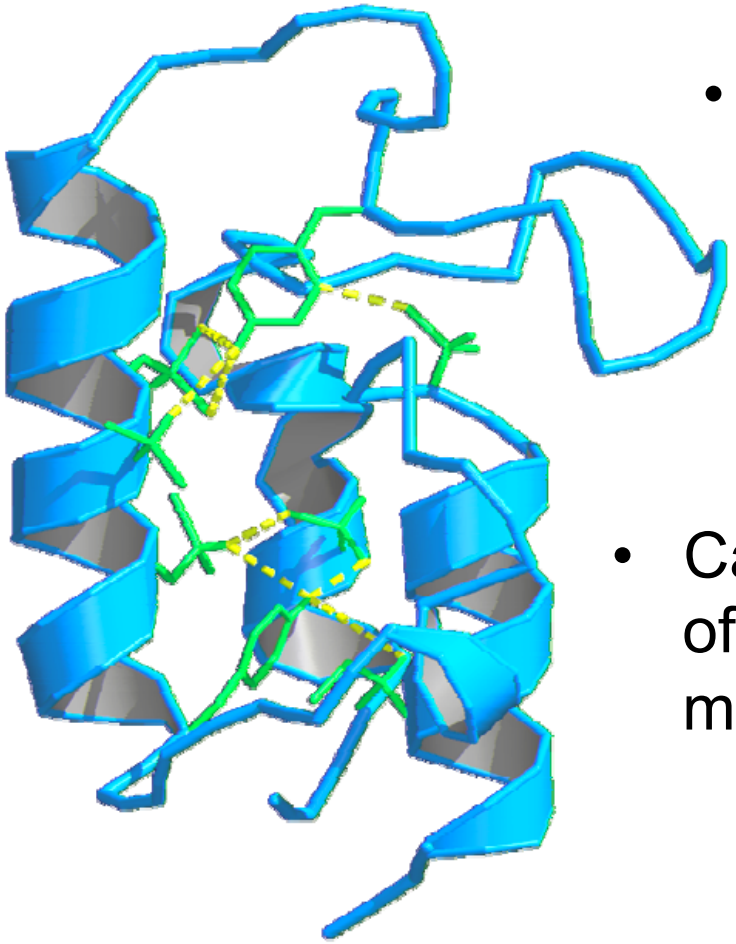
NOE interactions in an idealized α-helix

- Sequential NOEs (NOEs between neighboring residues) define secondary structure
- Short, well-defined ^{1}H-^{1}H distances can be used to calibrate NOE intensities
- In proteins, NOE intensities are usually converted to approximate distance ranges
  - "strong" 1.8-2.7 Å
  - "medium" 1.8-3.3 Å
  - "weak" 1.8-5.0 Å
  - "very weak" 1.8-6.0 Å

  (lower bound is sum of van der Waals radii for two protons)

Experimental Restraints

- Long range NOEs (side chain to side chain) are among the most important in structure determination
  - these are between nuclei in amino acids far from one another in the primary sequence, but close in distance



- Provide important conformational restraints for structural elements in distant sections of the sequence (i.e. tertiary structure)

- Can provide proper relative orientation of structural elements (if enough are measured and properly assigned)

Experimental Restraints

- Example of a CNS/X-PLOR/X-PLOR-NIH input file for NOE-based distance restraints (restraint file)
  - depending on the protein size, such a file may have hundreds or thousands of lines/restraints

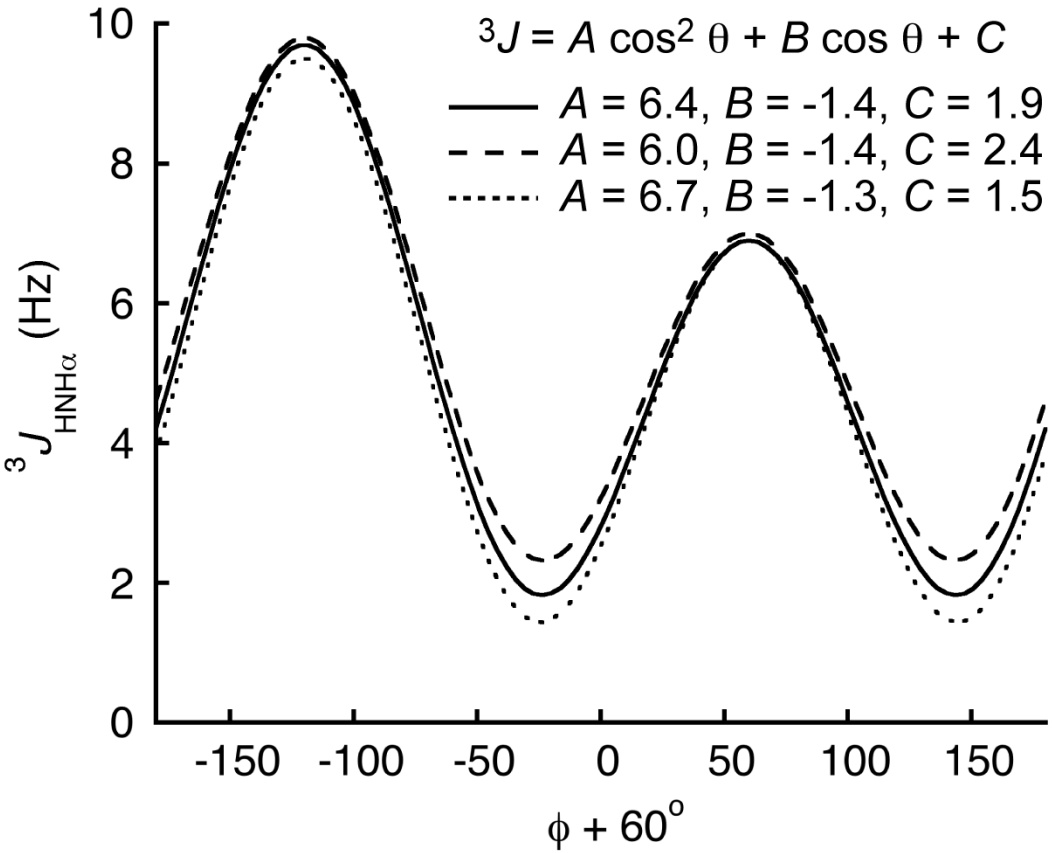
```
!V2
assign (resid 2 and name HG2#) (resid 3 and name HN) 4.0 2.2 1.5      !#A 762  2.78e+05
assign (resid 2 and name HB) (resid 3 and name HN) 4.0 2.2 1.0        !#A 760  2.82e+05
assign (resid 2 and name HA) (resid 3 and name HN) 2.5 0.7 0.4        !#A 34   2.36e+06
assign (resid 2 and name HG1#) (resid 3 and name HN) 2.5 0.7 0.9      !#A 23   1.27e+06
assign (resid 2 and name HG2#) (resid 46 and name HN) 4.0 2.2 1.5     !#A 637  1.85e+05
assign (resid 2 and name HG1#) (resid 56 and name HN) 3.0 1.2 1.2     !#A 348  8.33e+05

!K3
assign (resid 3 and name HB#) (resid 3 and name HN) 2.5 0.7 0.4       !#A 22  1.45e+06
assign (resid 3 and name HA) (resid 3 and name HN) 3.0 1.2 0.5        !#A 21  7.75e+05
assign (resid 3 and name HB#) (resid 4 and name HN) 4.0 2.2 1.0       !#A 74  3.87e+05
assign (resid 3 and name HB1) (resid 4 and name HN) 4.0 2.2 1.0       !#A 37  3.87e+05
assign (resid 3 and name HN) (resid 4 and name HN) 4.0 2.2 1.0        !#A 763 2.01e+05
assign (resid 3 and name HN) (resid 4 and name HA) 4.0 2.2 1.0        !#A 32  6.64e+05
assign (resid 3 and name HG#) (resid 4 and name HN) 4.0 2.2 1.0       !#A 55  2.57e+05
!assign (resid 3 and name HG2) (resid 4 and name HN) 4.0 2.2 1.0      !#A 54  3.32e+05
        …..etc…..

!Q4
assign (resid 4 and name HG#) (resid 4 and name HE2#) 4.0 2.2 1.0     !#A 694  4.75e+05
assign (resid 4 and name HG#) (resid 4 and name HE2#) 3.0 1.2 1.0     !#A 693  6.40e+05
        ……etc……
```

Experimental Restraints

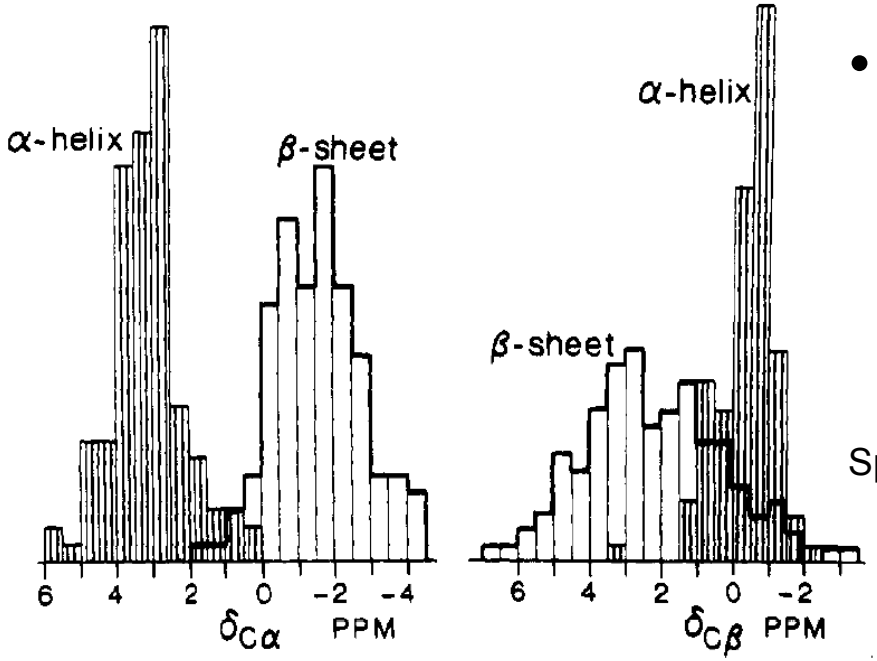
- Coupling constants can be used to restrain main chain ϕ an ψ angles (and side chain χ_1, χ_2, etc.) via Karplus relationships



- example: HNHA experiment for estimating ϕ
- excellent, widely used experiment

Vuister and Bax (1993) *J. Am. Chem. Soc.* 115, 7772-7777.
Wang and Bax (1996) *J. Am. Chem. Soc.* 118, 2483-2494.

Experimental Restraints



- Chemical shift deviations from random coil values provide information on secondary structure and hence ϕ and ψ

Spera and Bax (1991) *J. Am. Chem. Soc.* 113, 5490-5492.

- Combined with database information (ϕ, ψ, and corresponding chemical shifts), good quantitative predictions for ϕ and ψ from chemical shifts can be made, as can uncertainties in the predictions ("Talos" program and others).

"Talos": Cornilescu, Delaglio and Bax (1999) *J. Biomol. NMR* 13, 289-302.
"Talos+": Shen, Delaglio, Cornilescu and Bax (2009) *J. Biomol. NMR* 44, 213-223.

Experimental Restraints

- As with distance restraints, dihedral angle restraints are provided as generous ranges of values
- Example of a CNS/X-PLOR/X-PLOR-NIH input file for ϕ and ψ restraints

```
!remark phi angle constraints

  !!    v2
 assign (resid  1  and name c ) (resid  2  and name n )
        (resid  2  and name ca) (resid  2  and name c )   1.0  -125.0 25.0 2
  !!    k3
 assign (resid  2  and name c ) (resid  3  and name n )
        (resid  3  and name ca) (resid  3  and name c )   1.0  -152.0 20.0 2
  !!    q4
 assign (resid  3  and name c ) (resid  4  and name n )
        (resid  4  and name ca) (resid  4  and name c )   1.0  -95.0 20.0 2
                     …etc…
!remark psi angles constraints

  !!    m1
 assign (resid 1 and name n ) (resid 1 and name ca)
        (resid 1 and name c ) (resid 2 and name n )   1.0  180.0 50.0 2
  !!    2
 assign (resid 2 and name n ) (resid 2 and name ca)
        (resid 2 and name c ) (resid 3 and name n )   1.0  180.0 50.0 2
  !!    k3
 assign (resid 3 and name n ) (resid 3 and name ca)
        (resid 3 and name c ) (resid 4 and name n )   1.0  120.0 50.0 2
                     …etc…
```

Experimental Restraints

- Hydrogen bond restraints can be determined from direct NMR observation or from other physical data (hydrogen/ deuterium exchange)

- Example of a CNS/X-PLOR/X-PLOR-NIH input file for hydrogen bond restraints for a well-defined α-helical region

```
!  hydrogen bond
!
assign   (segid AS1 and resid  10  and name O    )  (segid AS1 and resid  14  and name HN    )  1.9  0.1  0.1
assign   (segid AS1 and resid  10  and name O    )  (segid AS1 and resid  14  and name N     )  2.85 0.15 0.15
assign   (segid AS1 and resid  11  and name O    )  (segid AS1 and resid  15  and name HN    )  1.9  0.1  0.1
assign   (segid AS1 and resid  11  and name O    )  (segid AS1 and resid  15  and name N     )  2.85 0.15 0.15
assign   (segid AS1 and resid  12  and name O    )  (segid AS1 and resid  16  and name HN    )  1.9  0.1  0.1
assign   (segid AS1 and resid  12  and name O    )  (segid AS1 and resid  16  and name N     )  2.85 0.15 0.15
assign   (segid AS1 and resid  13  and name O    )  (segid AS1 and resid  17  and name HN    )  1.9  0.1  0.1
assign   (segid AS1 and resid  13  and name O    )  (segid AS1 and resid  17  and name N     )  2.85 0.15 0.15
assign   (segid AS1 and resid  14  and name O    )  (segid AS1 and resid  18  and name HN    )  1.9  0.1  0.1
assign   (segid AS1 and resid  14  and name O    )  (segid AS1 and resid  18  and name N     )  2.85 0.15 0.15
assign   (segid AS1 and resid  15  and name O    )  (segid AS1 and resid  19  and name HN    )  1.9  0.1  0.1
assign   (segid AS1 and resid  15  and name O    )  (segid AS1 and resid  19  and name N     )  2.85 0.15 0.15
assign   (segid AS1 and resid  16  and name O    )  (segid AS1 and resid  20  and name HN    )  1.9  0.1  0.1
assign   (segid AS1 and resid  16  and name O    )  (segid AS1 and resid  20  and name N     )  2.85 0.15 0.15
assign   (segid AS1 and resid  17  and name O    )  (segid AS1 and resid  21  and name HN    )  1.9  0.1  0.1
assign   (segid AS1 and resid  17  and name O    )  (segid AS1 and resid  21  and name N     )  2.85 0.15 0.15
```

Generating Initial Structures: Distance Geometry

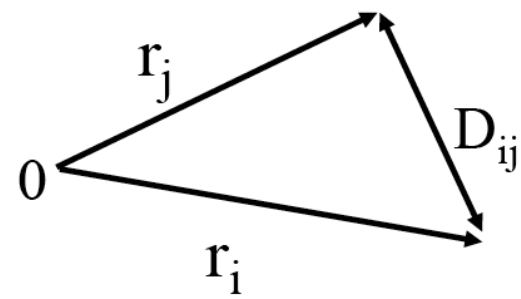
Braun, W. (1987) *Quart. Rev. Biophys.* 19, 115-157
Crippen and Havel (1988) *Distance Geometry and Molecular Conformation*

- Calculate Cartesian coordinates directly from known (covalent structure) and experimental distances

- First generate the "metric matrix"
  - write $n \times n$ matrix of distances
  - calculate $n \times n$ metric matrix of vector products

$M =$

	$r_i \bullet r_j$		



$r_i \bullet r_j$ can be written in terms of distances

$$D^2_{ij} = r_i^2 + r_j^2 - 2 r_i \bullet r_j$$

Generating Initial Structures: Distance Geometry

- Then solve for positions in Cartesian space:
  - diagonalize M; $|\lambda| = |A|\,|M|\,|A^{-1}|$; $|M| = |A^{-1}|\,|\lambda|\,|A|$
    - the diagonal matrix corresponds to vectors in real space
  - only 3 eigenvalues should be finite ($r_i \bullet r_i$ finite only for $x \bullet x$, etc)
    - corresponding eigenvectors contain Cartesian coordinates

$$r_i \bullet r_i = \Sigma_k \lambda_k A_{ik}^{-1} A_{jk} = A_{j1}^{-1} A_{i1} + A_{j2}^{-1} A_{i2} + A_{j3}^{-1} A_{i3} = x_i x_i + y_i y_i + z_i z_i$$

  - hence, elements of A are x,y,z coordinates of atoms

- Problems
  - incomplete distance matrix (actually, a sparse matrix)
  - experimental distances are not exact
    - in practice, use upper and lower bounds and fill in matrix by random number selection within bounds
  - solution is approximate
    - experimental distances are often significantly different than calculated distances and must be "regularized"

Detailed tutorial: http://www.colby.edu/chemistry/CompChem/MMtutor.pdf

Generating Initial Structures: *Simulated Annealing* and Error Functions

- Define potential energy function:

$$E = E_{bond} + E_{vdw} + E_{angle} + \ldots\ldots + \mathbf{E_{NMR}}$$

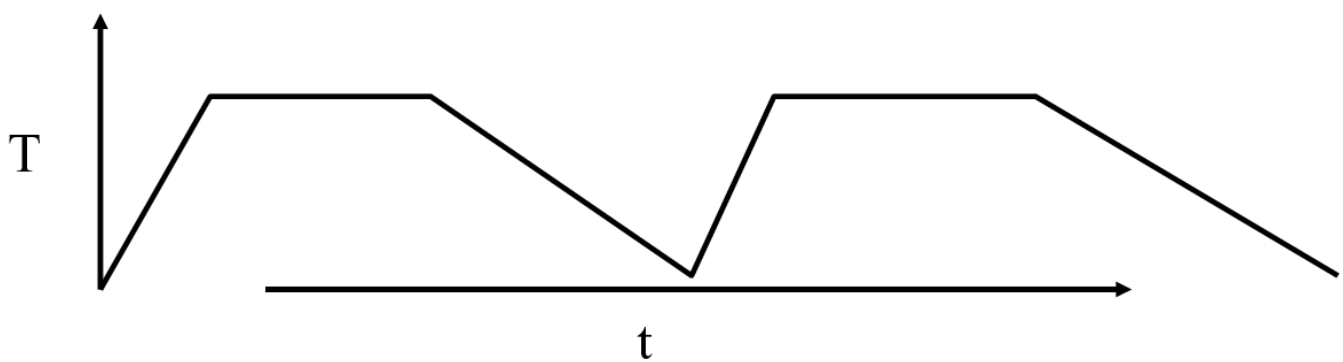
- Include a term for NMR data/restraints:

$$\mathbf{E_{NMR}} = \Sigma_l\, (r_{obs} - r_{trial})_l^2 \ldots \text{(or use } r_{min,max} \text{ for } r_{obs})$$

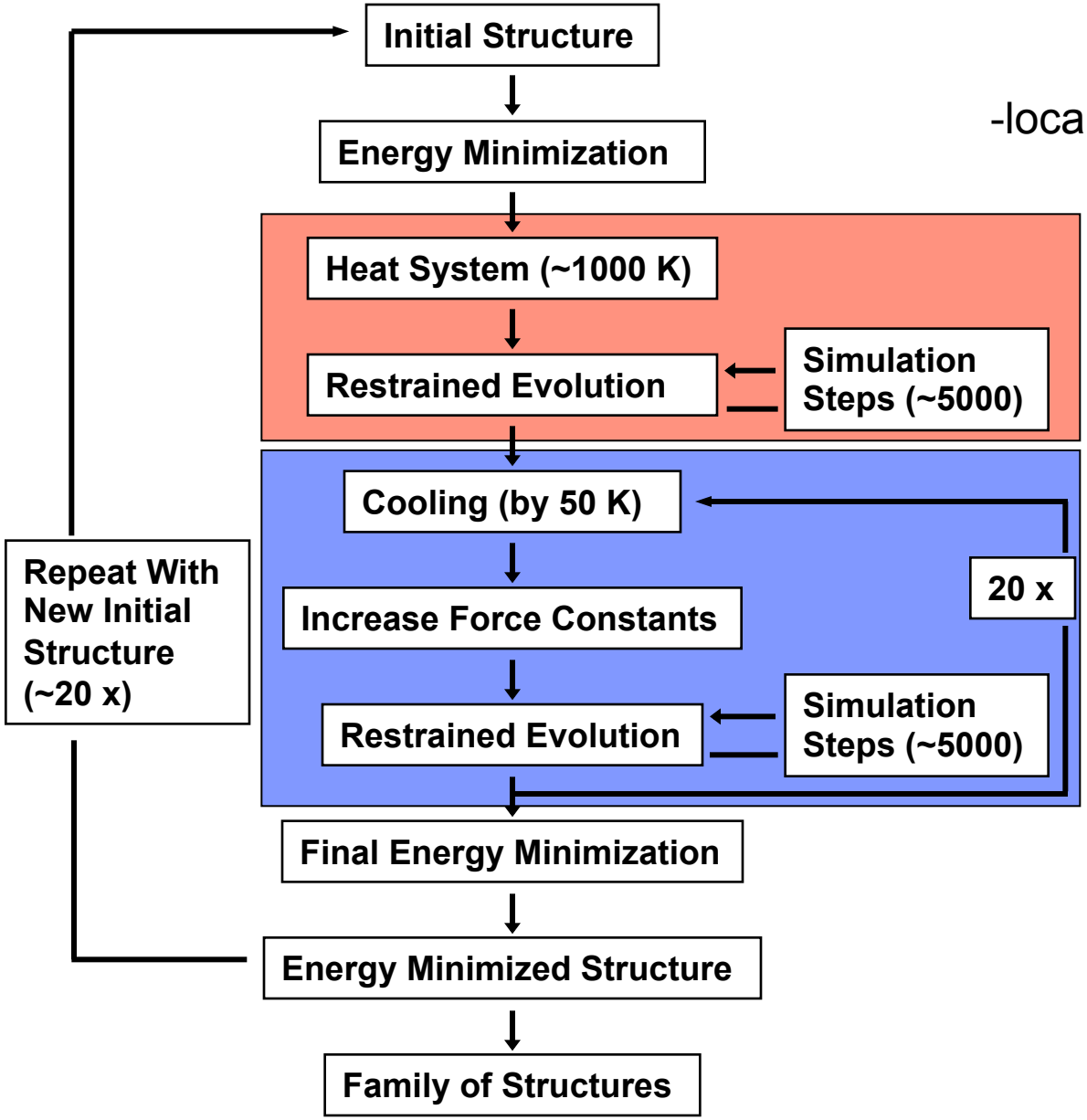
- Molecular dynamics: solve for the motion of the atoms with time using the potential energies
  - integrating Newton's equations to give velocities and positions of atoms as a function of time

$$x_{new} = x_{old} + t \bullet v_x = x_{old} + t \bullet \int a_x dt, \qquad y_{new} = \ldots$$

$$a_x = F_x/m = -(1/m) \bullet dE/dx + a_{rand}(T), \qquad a_y = \ldots$$



Simulated Annealing



-local energy minimum reached

-heat system to cross local energy barriers
-atomic positions determined from velocities/accelerations

-slow reduction of atomic velocities (many steps)
-increase weights of experimental restraints

-global energy minimum?

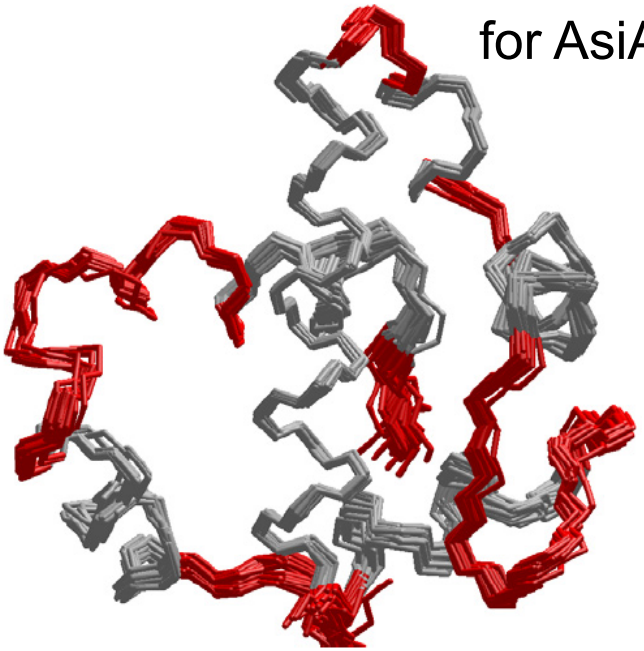
Based on figure from Horst Joachim Schirra Max-Planck Institute for Biochemistry
http://www.cryst.bbk.ac.uk/pps2/projects/schirra/html/home.htm

Structure Refinement

- Simulated annealing methods can be used to refine structures
- Refinement can include additional restraints, changing weights or force constants for restraints, using NOE intensities directly, etc.
- Ultimately, ensembles of structures are calculated and compared

20 NMR structures for DNA J

25 NMR structures for AsiA



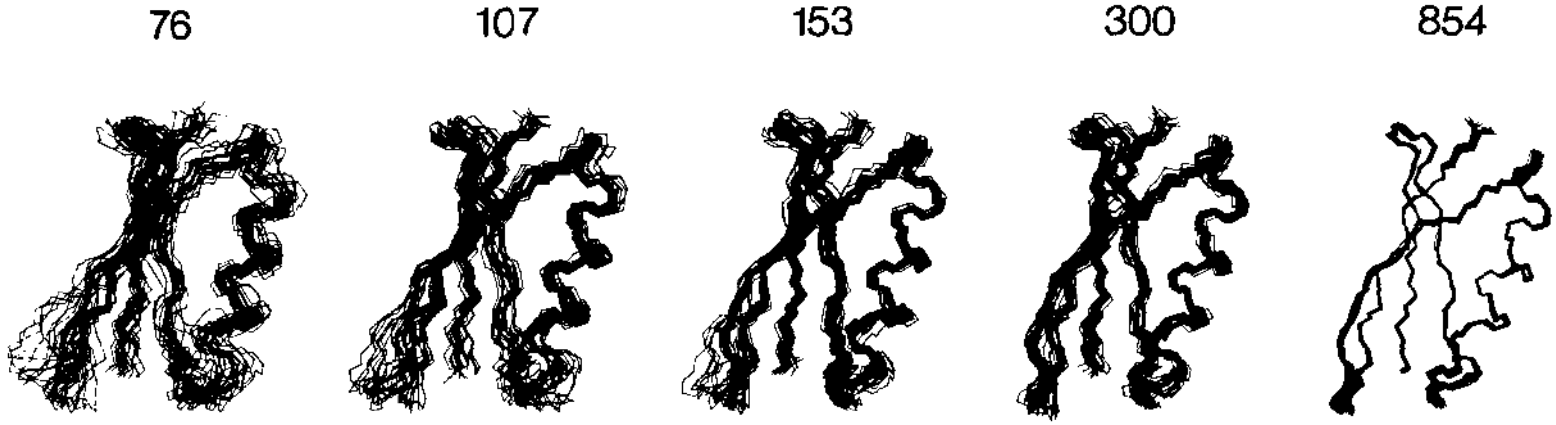
Validation of Structures

- Important to analyze 'final' structures and ensembles for characteristics that typically indicate good structures
  - R factor for NOEs: n ~ 1/6
  - $R = \Sigma_{NOEs}[(I_{obs})^n - (I_{calc})^n] / \Sigma_{NOEs}[(I_{obs})^n$
  - Other statistics: RMSD of backbone and all atoms
  - NOE violations (any experimental restraint violations)
  - Molecular energy
  - “Procheck” output
  - Protein Structure Validation Software Suite (PSVS)
    http://psvs-1_5-dev.nesg.org

Validation of Structures

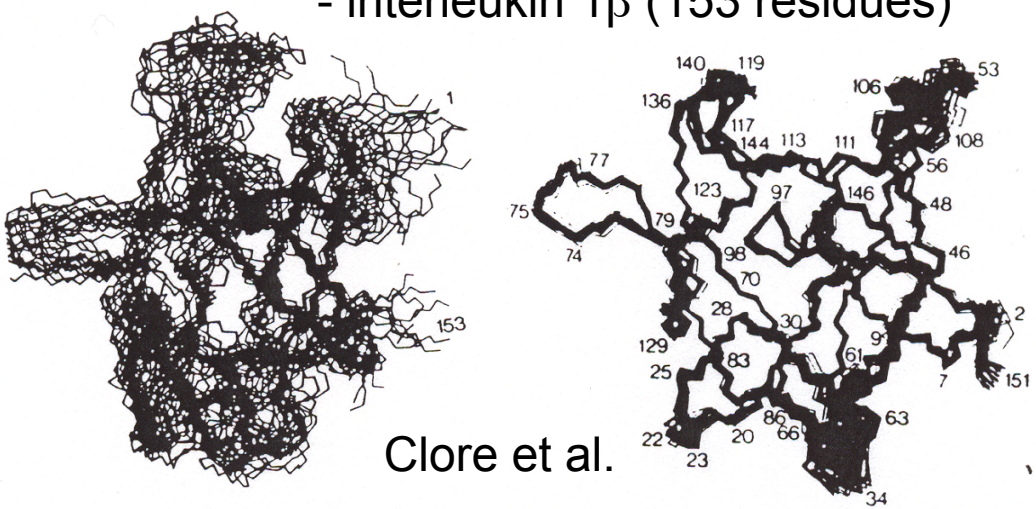
- Example: RMSD improves with number of (NOE) restraints

  - IgG binding domain of streptococcal protein G (56 residues)



Clore, G. M. et al. (1993) *J. Mol. Biol.* 231, 82-102

  - interleukin 1β (153 residues)



Clore et al.

	Left	Right
# distance restraints	536	2780
RMSD (backbone)	2.0	0.4

Validation of Structures

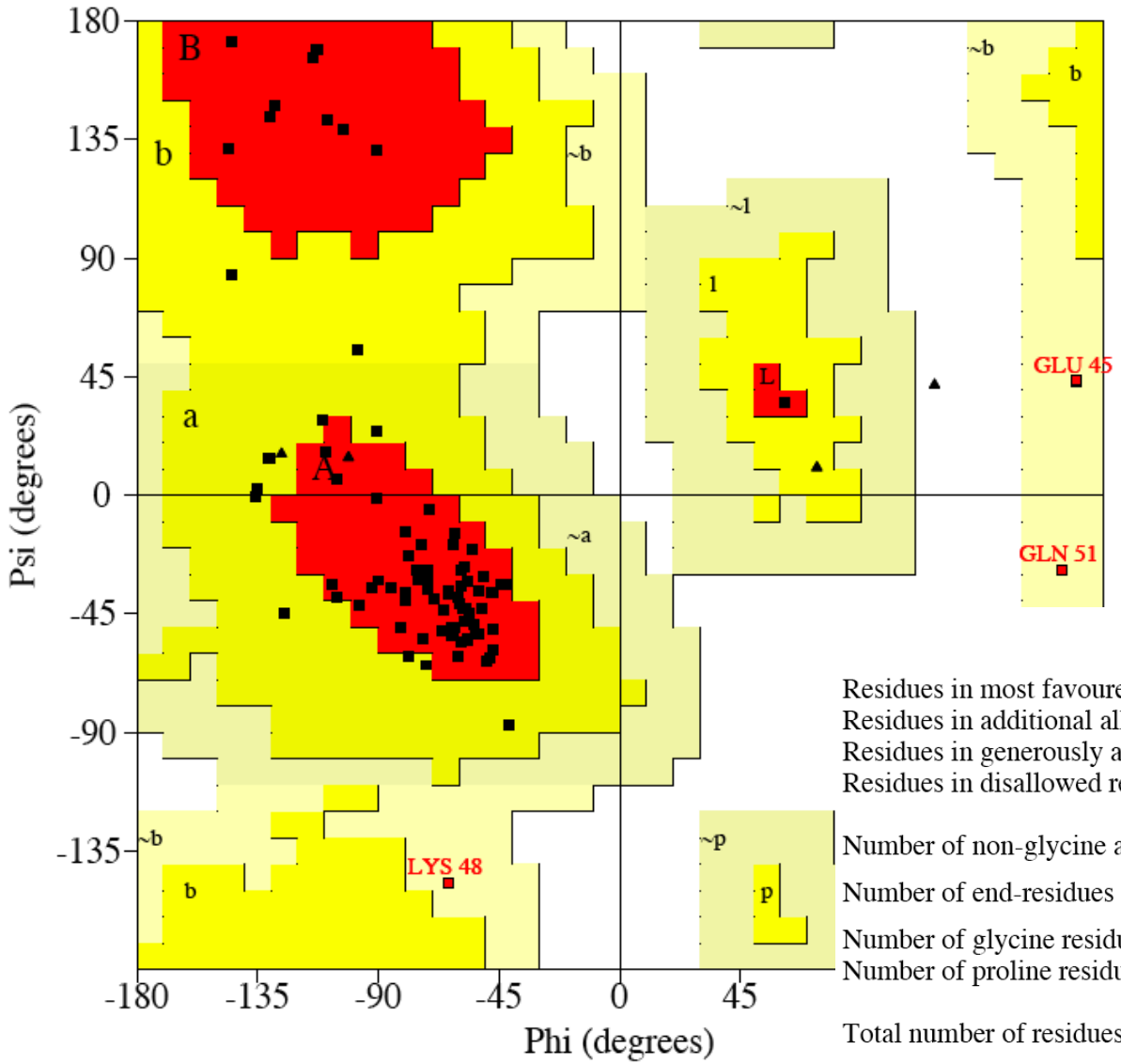
- “Procheck” and "Procheck-NMR": performs a number of checks of structural quality

  - covalent geometry
  - dihedral angles
  - non-bonded interactions
  - main chain hydrogen bonds
  - stereochemical parameters
  - residue-by residue analyses
  - other parameter comparisons
  - planarity
  - chirality
  - disulfide bonds

- Laskowski R A, MacArthur M W, Moss D S & Thornton J M (1993) *J. Appl. Cryst.*, 26, 283-291
- Morris A L, MacArthur M W, Hutchinson E G & Thornton J M (1992) *Proteins*, 12, 345-364
- https://www.ebi.ac.uk/thornton-srv/software/PROCHECK/

Procheck Example

- Distribution of phi-psi angles (Ramachandran plot)



- Most ϕ, ψ pairs should fall in favoured or allowed regions

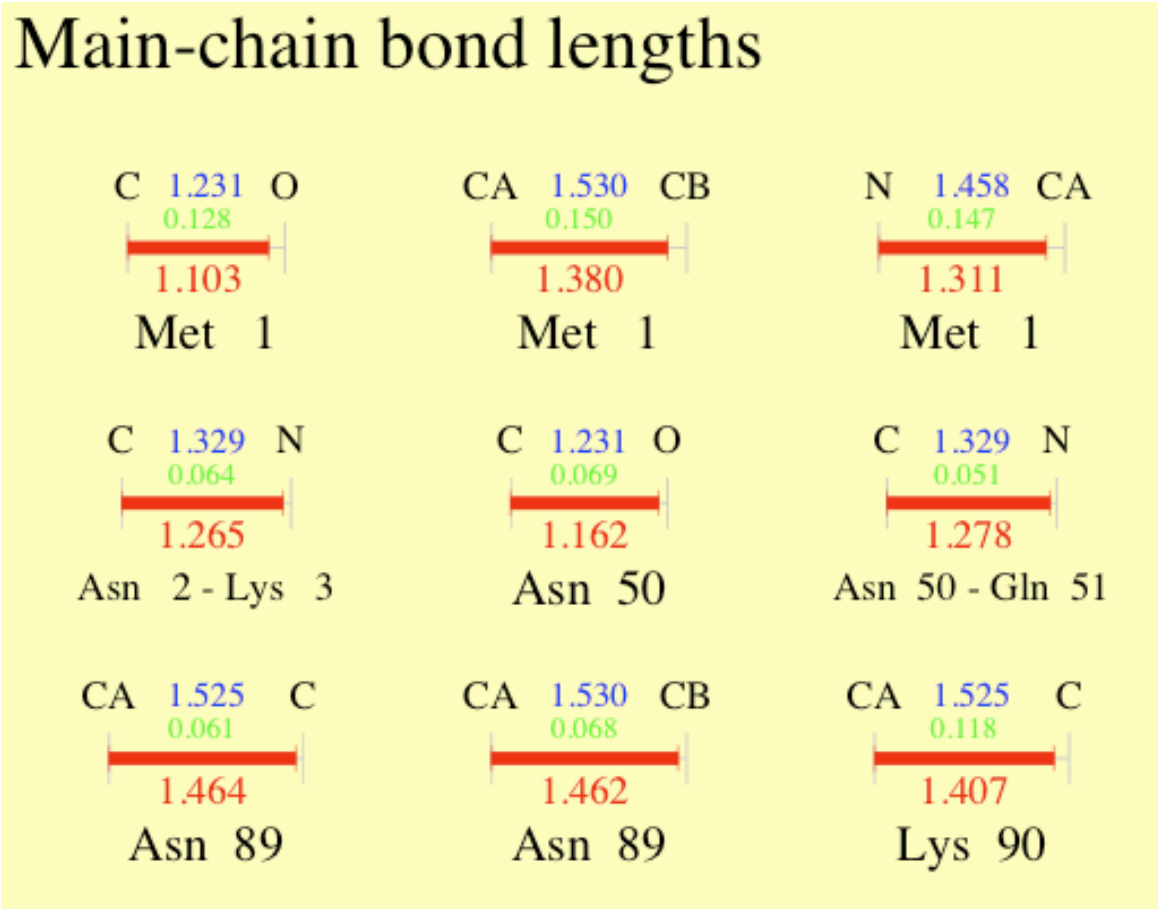
Plot statistics

Residues in most favoured regions [A,B,L]	140	83.3%
Residues in additional allowed regions [a,b,l,p]	22	13.1%
Residues in generously allowed regions [~a,~b,~l,~p]	6	3.6%
Residues in disallowed regions	0	0.0%
	---	------
Number of non-glycine and non-proline residues	168	100.0%
Number of end-residues (excl. Gly and Pro)	4	
Number of glycine residues (shown as triangles)	8	
Number of proline residues	0	

Total number of residues	180	

Procheck Example

- Bond length and bond angle distortions



- Bond length and bond angle variations from normal values can signify potential structural distortions

Bond lengths (red) differing (differences in green) by > 0.05 Å from small-molecule values (blue)

Ambiguous Distance Restraints (ADRs)

- Ambiguous NOEs are those for which more than one assignment is possible

$$V_{total} = \sum_{a=1}^{N_\delta} V_a$$

- The volumes (intensities) of these can be treated as sums of possible contributions…..

$$V_{calc} \approx \sum_{a=1}^{N_\delta} d_a^{-6}$$

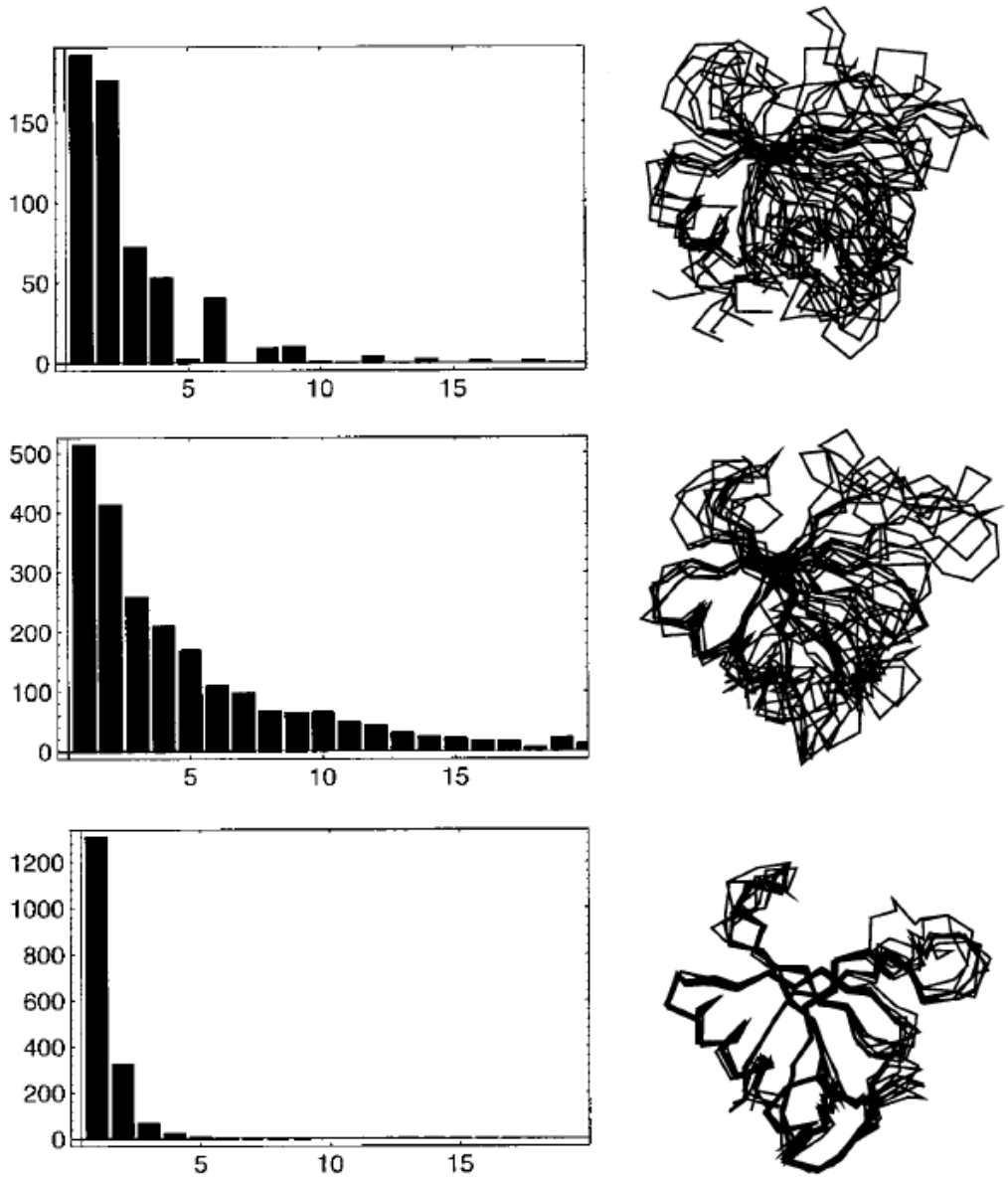
- ….. and can be approximated with a 6th power law

$$\overline{D} \equiv \left(\sum_{a=1}^{N_\delta} d_a^{-6} \right)^{-1/6}$$

- Ambiguous distance restraint: an effective or summed "distance" between more than two points

- Ambiguous NOEs can be used in iterative procedures for simultaneous structure calculation and NOE assignment

Ambiguous Distance Restraints (ADRs)



Nilges et al., (1997) *J. Mol. Biol.* 269, *408-422*

- ARIA: Ambiguous Restraints for Iterative Assignment.

  - ambiguous restraints are assigned as structure calculations proceed.

  - number of NOEs assigned uniquely increases in subsequent iterations coupled with improved RMSD

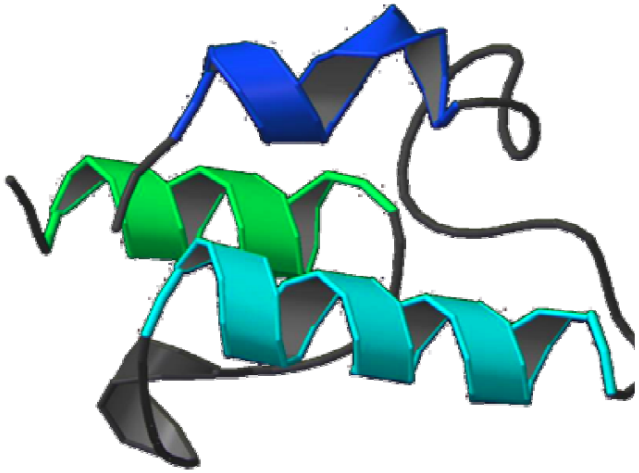
- Are routines in X-PLOR-NIH and CYANA that perform similarly

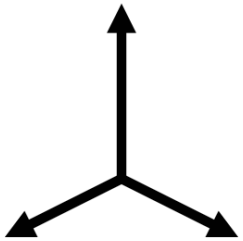
Structure Refinement Using RDCs

- Write RDCs in principal alignment frame:
  $D = (D_a/r^3)\{(3\cos^2\theta-1)/r^3 + (3/2)R\sin^2\theta\cos(2\phi)\}$

- Write error function in terms of D_{meas} and D_{calc}
  $E_{RDC} = (D_{meas} - D_{calc})^2$

- Seek minimum in E_{RDC} to refine structure
  - need to float alignment axes during search





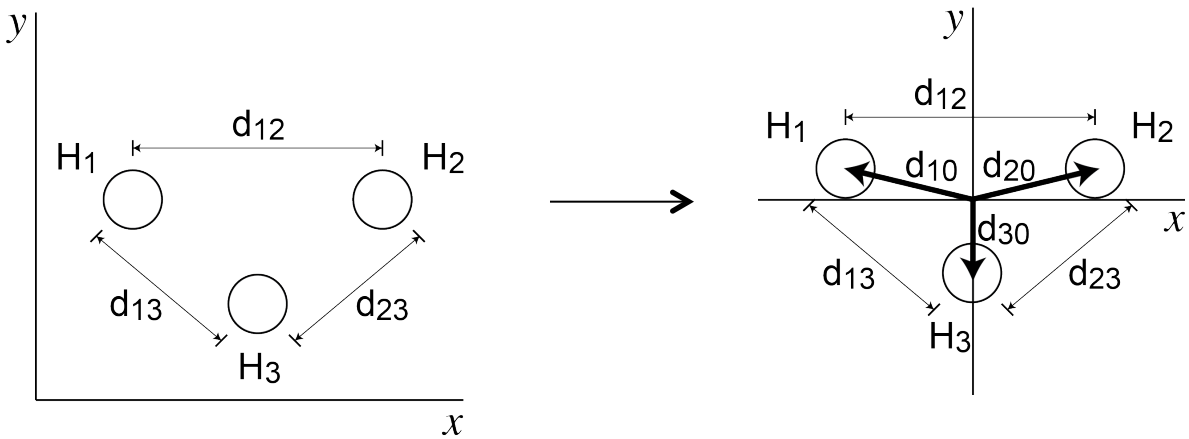
REsidual Dipolar Coupling Analysis Tool (REDCAT)

Valafar, H., & Prestegard, J. H. (2004) *J. Magn. Reson.* 167, 228-241
Dosset, Hus, Marion & Blackledge (2001) *J. Biomol. NMR* 20, 223-231

- Given a proposed structure and RDCs, calculates order tensor solutions.
- Finds best order tensor solution.
- Gives principal elements and Euler angles.
- Back-calculates RDCs.
- Estimates errors and helps identify problematic data

Appendix: Distance Geometry Example

- Assume three hydrogen atoms, H_1, H_2, and H_3
- From nuclear Overhauser effect (NOE) measurements, the distances between them (d_{12}, d_{13}, d_{23}) are known
- The goal is to determine the Cartesian coordinates of H_1 (x_1, y_1, z_1), H_2 (x_2, y_2, z_2) and H_3 (x_3, y_3, z_3)



- First move the coordinate system so the origin is the geometric center (centroid) of the molecule:

$$\text{for } N \text{ atoms}: \sum_{i=1}^{N} x_i = 0, \ \sum_{i=1}^{N} y_i = 0, \ \sum_{i=1}^{N} z_i = 0$$

- the latter condition already met as H_1, H_2, and H_3 lie in the x-y plane

- Atom positions are now defined by vectors from the origin
  - the magnitudes of these vectors are d_{10}, d_{20}, and d_{30}

Appendix: Distance Geometry Example

- The next goal is to construct the metrix matrix, [*M*]
  - this is a square, symmetrical matrix of dot products of the vectors that define the positions of the atoms relative to the origin

$$m_{ij} = \sum_{i=1}^{N}\left(x_i x_j + y_i y_j + z_i z_j\right) = \left\|d_{i0}\right\|\left\|d_{j0}\right\|\cos\theta = 1/2\left(d_{i0}^2 + d_{j0}^2 - d_{ij}^2\right)$$

- The following geometric relationship permits the vector magnitudes to be computed from the interatomic distances

$$d_{i0}^2 = \frac{1}{N}\sum_{j\neq i}^{N} d_{ij}^2 - \frac{1}{N^2}\sum_{j=1}^{N}\sum_{k>j} d_{jk}^2$$

- Diagonalizing the matrix

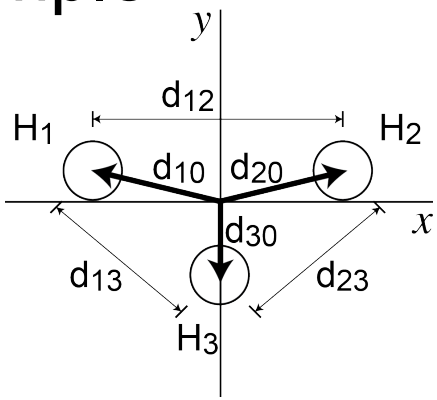
$$[\lambda] = [A]^{-1}[M][A] \qquad [M] = [A][\lambda][A]^{-1}$$

- The eigenvalues (from [λ]) and eigenvectors (column vectors in [A]) give the atomic positions (cartesian coordinates)

$$x_i = \lambda_1^{1/2}\vec{A}_{i1} \qquad y_i = \lambda_2^{1/2}\vec{A}_{i2} \qquad z_i = \lambda_3^{1/2}\vec{A}_{i3}$$

Appendix: Distance Geometry Example

- Example: d_{12} = 4.0 Å, d_{13} = d_{23} = 2.5 Å
  - what are the cartesian coordinates for H_1, H_2 and H_3?
- First, populate metrix matrix, [*M*]



$$m_{ij} = 1/2\left(d_{i0}^2 + d_{j0}^2 - d_{ij}^2\right) \qquad d_{i0}^2 = \frac{1}{N}\sum_{j\neq i}^{N} d_{ij}^2 - \frac{1}{N^2}\sum_{j=1}^{N}\sum_{k>j} d_{jk}^2$$

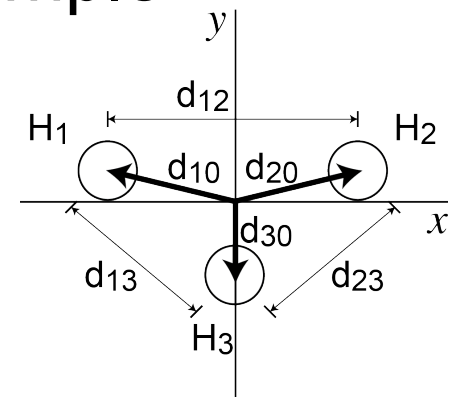
$$d_{10}^2 = \frac{1}{N}\left(d_{12}^2 + d_{13}^2\right) - \frac{1}{N^2}\left(d_{12}^2 + d_{13}^2 + d_{23}^2\right) = \frac{1}{3}\left(4.0^2 + 2.5^2\right) - \frac{1}{3^2}\left(4.0^2 + 2.5^2 + 2.5^2\right) = 4.25 \quad d_{10} = 2.06155$$

$$d_{20}^2 = \frac{1}{N}\left(d_{21}^2 + d_{23}^2\right) - \frac{1}{N^2}\left(d_{12}^2 + d_{13}^2 + d_{23}^2\right) = \frac{1}{3}\left(4.0^2 + 2.5^2\right) - \frac{1}{3^2}\left(4.0^2 + 2.5^2 + 2.5^2\right) = 4.25 \quad d_{20} = 2.06155$$

$$d_{30}^2 = \frac{1}{N}\left(d_{31}^2 + d_{32}^2\right) - \frac{1}{N^2}\left(d_{12}^2 + d_{13}^2 + d_{23}^2\right) = \frac{1}{3}\left(2.5^2 + 2.5^2\right) - \frac{1}{3^2}\left(4.0^2 + 2.5^2 + 2.5^2\right) = 1.00 \quad d_{30} = 1.00000$$

$$M = 1/2\begin{bmatrix} d_{10}^2 + d_{10}^2 - d_{11}^2 & d_{10}^2 + d_{20}^2 - d_{12}^2 & d_{10}^2 + d_{30}^2 - d_{13}^2 \\ d_{20}^2 + d_{10}^2 - d_{21}^2 & d_{20}^2 + d_{20}^2 - d_{22}^2 & d_{20}^2 + d_{30}^2 - d_{23}^2 \\ d_{30}^2 + d_{10}^2 - d_{31}^2 & d_{30}^2 + d_{20}^2 - d_{32}^2 & d_{30}^2 + d_{30}^2 - d_{33}^2 \end{bmatrix} = 1/2\begin{bmatrix} 8.5 & -7.5 & -1 \\ -7.5 & 8.5 & -1 \\ -1 & -1 & 2 \end{bmatrix}$$

Appendix: Distance Geometry Example



- Diagonalize the metrix matrix

$$M = 1/2\begin{bmatrix} 8.5 & -7.5 & -1 \\ -7.5 & 8.5 & -1 \\ -1 & -1 & 2 \end{bmatrix} = \begin{bmatrix} 4.25 & -3.75 & -0.5 \\ -3.75 & 4.25 & -0.5 \\ -0.5 & -0.5 & 1 \end{bmatrix}$$

$$M = [A][\lambda][A]^{-1}$$

$$\begin{bmatrix} 4.25 & -3.75 & -0.5 \\ -3.75 & 4.25 & -0.5 \\ -0.5 & -0.5 & 1 \end{bmatrix} = \begin{bmatrix} 0.707 & 0.408 & 0.577 \\ -0.707 & 0.408 & 0.577 \\ 0 & -0.816 & 0.577 \end{bmatrix}\begin{bmatrix} 8.0 & 0 & 0 \\ 0 & 1.5 & 0 \\ 0 & 0 & 0 \end{bmatrix}\begin{bmatrix} 0.707 & -0.707 & 0 \\ 0.408 & 0.408 & -0.816 \\ 0.577 & 0.577 & 0.577 \end{bmatrix}$$

- Calculate the coordinates

$$x_i = \lambda_1^{1/2}\vec{A}_{i1} \qquad y_i = \lambda_2^{1/2}\vec{A}_{i2} \qquad z_i = \lambda_3^{1/2}\vec{A}_{i3}$$

atom H_1: $x_1 = \lambda_1^{1/2}\vec{A}_{11} = 8^{1/2} \times 0.707 = 2.0$ $\quad y_1 = \lambda_2^{1/2}\vec{A}_{12} = 1.5^{1/2} \times 0.408 = 0.5$ $\quad z_1 = \lambda_3^{1/2}\vec{A}_{13} = 0$

atom H_2: $x_2 = \lambda_1^{1/2}\vec{A}_{21} = 8^{1/2} \times -0.707 = -2.0$ $\quad y_2 = \lambda_2^{1/2}\vec{A}_{22} = 1.5^{1/2} \times 0.408 = 0.5$ $\quad z_2 = \lambda_3^{1/2}\vec{A}_{23} = 0$

atom H_3: $x_3 = \lambda_1^{1/2}\vec{A}_{31} = 8^{1/2} \times 0 = 0$ $\quad y_3 = \lambda_2^{1/2}\vec{A}_{32} = 1.5^{1/2} \times -0.816 = -1.0$ $\quad z_3 = \lambda_3^{1/2}\vec{A}_{33} = 0$

- here H_1 and H_2 have the 'wrong' signs for the x coordinates; see if you can find a mistake that would lead to this