

## 2 Crystallisation

### 2.1 Crystallisation of Macromolecules

In the process of crystallisation, molecules become ordered resulting in a periodically arranged pattern in all three dimensions. The periodic arrangement of molecules in the crystalline state allows us to observe the phenomenon of diffraction. The scattering contribution of a number of identical molecules in the three dimensional space takes an observable value at some points and approaches zero elsewhere. The intensity of the X-ray diffraction pattern of a crystal increases with the diffracting volume of the crystal.

The rotational and translational degrees of freedom of the molecules in solution coincides with a relatively low entropy of the system. The loss of these degrees of freedom during the ordering process of crystallisation increases the entropy. However, by forming new interactions (chemical, steric, electrostatic, hydrophobic, Van der Waals) between the molecules, the free energy of the system is lowered providing the driving force for crystallisation. Biological macromolecules usually exist in an aqueous environment and hence experience a free energy minimum when they are fully solvated. During crystallisation, the system is subtly driven towards a state where the macromolecules have lower solubility such that the macromolecules come out of solution in the form of crystals. The supersaturation point at which crystals form depends on a number of parameters which affect protein solubility, including precipitants, protein concentration, ionic strength, temperature, the presence of organic

solvent, the pH and the binding of counter ions to the protein (McPherson, 1982).

Various techniques for crystallising macromolecules have developed. These include the batch crystallisation, where precipitating agent is added directly to the protein solution to bring the solution to a state of low supersaturation; the “hot box” technique which exploits the temperature gradient of solubility; equilibrium dialysis, in which a semipermeable diaphragm allows equilibration of the solvent and small ions but not macromolecules (Theorell, 1932), and evaporation and vapour diffusion techniques, where the concentration of the solution is altered by evaporation of solvent or by vapour diffusion. In the latter technique protein solution containing a salt solution below the concentration needed for precipitation can be equilibrated by vapour diffusion with a large volume of more concentrated salt solution (Blundell & Johnson, 1976). The most common technique for protein crystallisation is vapour diffusion. There are various approaches: hanging drop and sitting drop being the most popular. In these experiments, the concentration of a precipitant is altered towards the value at which the protein crystallisation is nucleated. Common precipitants are polyethylene glycol (of various molecular weights), lithium sulfate, sodium potassium tartrate, sodium acetate, 2-propanol, 2-methyl-2,4-pentanediol, sodium formate, magnesium formate, ammonium phosphate, ammonium sulfate or sodium citrate. Alternatively, “salting-in” and “salting-out” procedures can be exploited. “Salting-in” occurs using salt at very low ionic strength in which the solubility of the protein increases as the ionic strength

increases and is also used as a purification tool. “Salting-out” occurs as the concentration of salt increases and involves anions (McPherson, 1982).

In the hanging drop vapour diffusion method (McPherson, 1976), a microdroplet of mother liquor is placed on a microscope cover slip, which is then inverted and placed over a small well containing about 1 ml of the reservoir solution which differs in precipitant concentration and is protein free. Silicising the cover slip helps to prevent spreading of the droplet. The solvent gradually vaporises from the protein solution to dilute the more concentrated reservoir solution until the two are in equilibrium (Davies & Segal, 1971). The sitting drop vapour diffusion method is a slight variation of this technique where a say 50  $\mu$ l drop of mother liquor is placed in a well sitting in a sealed plastic Petri dish containing about 10 ml of the precipitating solution. It is often possible to grow bigger crystals using the sitting drop technique but more protein is necessary for this approach compared to the hanging drop method.

## 2.2 Crystallisation of *N*-acetylneuraminase Lyase

*N*-acetylneuraminase lyase was expressed, isolated and purified as reported previously (Lilley *et al.*, 1992; Ohta *et al.*, 1991; Aisaka *et al.*, 1991; Kawakami *et al.*, 1986) by Glenn Lilley. The *E. coli* gene encoding Neu5Ac lyase was isolated by the polymerase chain reaction, cloned into the inducible expression vector pTTQ18 and overexpressed in *E. coli* by Neva Ivancic. The recombinant enzyme was then purified to homogeneity.

Crystals of lyase were originally grown by Robyn Malby. The conditions used were by equilibrating either citrate/phosphate buffer (30 mM/60 mM respectively, pH 6.8–7.2) or citrate buffer (30 mM, pH 5.4), and ammonium sulfate (40% v/v of saturated solution) against 60% v/v saturated ammonium sulfate. For the work described here Neu5Ac lyase crystals were grown at room temperature by using the hanging and sitting drop vapour diffusion method. The reservoir solution contained saturated ammonium sulfate (SAS) and 75 mM sodium phosphate buffer at pH 6.9. Equal volumes of reservoir solution and a  $17 \frac{mg}{ml}$  protein solution in 0.1 M sodium chloride, 0.02% sodium azide and 25 mM Tris-HCl (pH 7.5) were mixed to give 14  $\mu$ l drops. Bipyramidal crystals of Neu5Ac lyase (Figure 3) grew to sizes suitable for X-ray diffraction experiments (maximum dimensions 0.7 mm x 0.7 mm x 1.3 mm) within one week. An artificial mother liquor in which the crystals could be kept stable contained 65% SAS and 37.5 mM sodium phosphate buffer at pH 6.9.

Mother liquors containing phosphate and ammonium sulfate could have

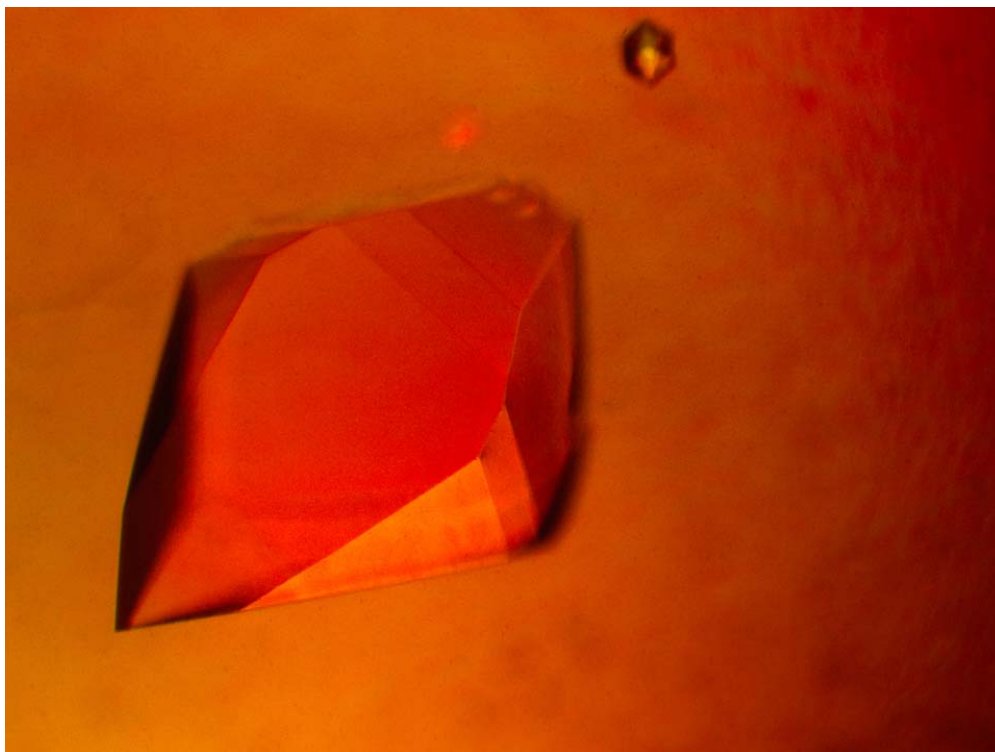


Figure 3: Bipyramidal crystal of Neu5Ac lyase grown from a SAS/NaP solution suitable for X-ray diffraction experiments. The colour is due to the use of polarising filters. The magnification used was 40x.

presented some difficulties when it comes to preparing isomorphous heavy-atom derivative crystals. The cations of suitable “A” metals (such as alkali metals, the alkaline earths, the lanthanides, some actinides and group III A, IV A and V A transition metals) can bind to water molecules and acetate, citrate or phosphate of the buffer medium in addition to the hydroxyl and carboxylate groups of the protein. Phosphate is therefore a poor mother liquor for attempting protein derivatisation with uranium and rare-earth metals (Petsko, 1985). On the other hand, the heavy metals at the end of the transition series, such as platinum, gold or mercury, will tend to bind methionine, cysteine, cystine, imidazole or amino groups of the protein as well as chloride and ammonia in the buffer solution. Thus, ammonium sulfate is a poor mother liquor constituent to achieve heavy-atom binding to the protein at pH greater than 6, due to the production of the nucleophile  $NH_3$  (Petsko, 1985; Sigler & Blow, 1965). Efforts were therefore made to crystallise Neu5Ac lyase in buffers other than sodium phosphate and with precipitants other than saturated ammonium sulfate. Neu5Ac lyase crystals could not be obtained from various precipitants at different concentrations such as polyethylene glycol (PEG) of various molecular weights, lithium sulfate (by itself without the addition of ammonium sulfate), sodium potassium tartrate, sodium acetate, 2-propanol, 2-methyl-2,4-pentanediol, sodium formate or magnesium formate. However, crystals were obtained by the hanging and sitting drop vapour diffusion method mixing equal volumes of reservoir solution containing saturated ammonium sulfate and 375 mM lithium sulfate with the protein solution. They are isomorphous with those reported above. It was not necessary to include data from

these crystals in the structure determination or refinement as an isomorphous derivative was successfully obtained from the crystals grown from saturated ammonium sulfate and 37.5 mM sodium phosphate. The crystals diffract to better than 2 Å resolution.

### 2.3 Heavy-Atom Compound Soaking Experiments

Isomorphous replacement is a method used to determine phases of Bragg reflections of macromolecular structures (Watenpaugh, 1985). In the ideal isomorphous replacement the heavy-atom compound binds with the protein in a limited number of places without disturbing the crystal packing and is sufficiently electron dense to provide observable changes in the intensities of the X-ray diffraction pattern. In order to obtain an isomorphous derivative crystal a heavy-atom must be introduced into the native crystal such that it causes minimal conformational changes in the protein molecules and hence the only changes in electron density between it and the native crystal are at the sites of heavy-atom substitution. In other words, heavy-atom binding must be specific and not cause changes in the crystal structure. An isomorphous derivative can be obtained by binding of a heavy-atom to the surface of the protein molecule so that it does not disturb the molecular structure but occupies a position in the solvent channels in the protein crystal. Heavy-atom soaking methods involve diffusing a heavy-atom compound into an existing native crystal. Here, intermolecular contacts in the crystal lattice will reduce the number of potentially accessible reactive groups of the protein available for binding (Petsko, 1985).

Determination of free sulfhydryl ( $-SH$ ) groups was performed (by Glenn Lilley) following Ellman's method (Ellman, 1959) and showed that the four cysteine residues per protomer are not involved in disulfide-bridges and are therefore available for heavy-atom binding. The sulfur ion of cysteine undergoes nu-

cleophilic reaction with mercurial or organomercurials at pH 7 or higher (Pet-sko, 1985). Mercury compound soaking experiments were therefore carried out using *p*-chloro-mercuribenzoic acid (PCMB), dimercuriacetate (DMA), tetra-kis(acetoxymercuri)methan (TAMM), potassium mercuric iodide, methylmercury chloride, mercuric chloride or Baker's dimercurial (BDM). It appears that the crystals are very sensitive to mercurials.

Many of the heavy-atom derivative data sets exhibited non-isomorphism without any significant change in the cell dimensions. These showed poor scaling statistics and their mean fractional isomorphous difference ( $R_{iso} = \frac{\sum ||F_{PH}| - |F_P||}{\sum |F_P|}$ ) increased with resolution. This could be due to movement of the protein to allow heavy-atom binding. Better isomorphism was achieved by backsoaking both native and derivative crystals in artificial mother liquor before data collection.

Four cysteine residues per protomer would generate twelve or sixteen binding sites per asymmetric unit (assuming either a trimer or a tetramer in the asymmetric unit). Multiple heavy-atom binding sites are difficult to interpret because the Patterson function which needs to be deconvoluted to obtain the heavy-atom positions increases in difficulty and complexity with the square of the number of heavy-atoms. In an attempt to avoid multiple binding sites, I-mannosamine, which is an analogue of one of the substrates (*N*-acetyl-mannosamine) was synthesised (by Mark von Itzstein). It was hoped that this would only bind to the active site, but unfortunately this compound did not seem to bind to the protein. Uchida *et al.* (1984) showed PCMB to be an

inhibitor of Neu5Ac lyase hence use of this inhibitor could potentially result in a single site derivative. However it was not possible to soak this mercury compound into the crystals without destroying them (see above).

A heavy-atom contribution to the diffraction intensities which is strong compared to the scattering factors of the large number of atoms in the asymmetric unit (1188 residues) is achieved by use of very electron-dense compounds. Clusters of heavy-atom compounds which have a high total atomic number (such as Baker's dimercurial  $[(CH_3CO_2HgCH_2CH(OCH_3))_2]$ , dimercuriacetate  $[(NO_3Hg)_2CH(CO_2)]$  di $\mu$ iodobis(ethylenediamine)diplatinum(II)-nitrate  $[Pt_2I_2(H_2NC_2H_4NH_2)_2(NO_3)_2]$  or tetrakis(acetoxymercuri)methan  $[C(CH_3CO_2Hg)_4]$  with molecular weights  $M_r$  of 635, 625, 888 and 1051 respectively) were used for soaking experiments, but they often dissociate into single heavy-atom functional groups when dissolved in the mother liquor. Heavy-atom compounds which are not as electron dense [such as potassium hexachloroiridate(IV) ( $K_2IrCl_6$ ), potassium ruthenate ( $K_2RuO_4 \cdot H_2O$ ), sodium tungstate ( $Na_2WO_4$ ) or potassium hexachloroiridate(IV) ( $K_2IrCl_6$ )] showed a very low mean fractional isomorphous difference, resulting from either lack of binding to the protein or their low atomic number. The average change of intensity for acentric reflections due to addition of heavy-atoms is  $\sqrt{\frac{2N_H}{N_P}} \cdot \frac{f_H}{f_P}$  (Crick & Magdoff, 1956) with  $N_H$  heavy and  $N_P$  light atoms of scattering factor  $f_H$  and  $f_P$  respectively. This means that the average change of intensity for a heavy-atom addition decreases with the size of protein. The soaking experiments with various heavy-atom compounds are summarised in

Table 1: Heavy-atom soaking trials. The  $R_{iso}^1$  of only five data sets lie within 0.12 and 0.20 (see chapter 7.1, page 58, for explanation of these values) and the third PIP derivative (which could be used successfully for phasing) has the lowest  $R_{merge}^2$  out of these. The heavy-atom soaking experiments of the last six compounds listed here were performed by Albert Van Donkelaar.

<i>heavy-atom compound</i>	<i>Concentration</i>	<i>Soaking time</i>	$R_{merge}^2$	$R_{iso}^1$
BDM <sup>a</sup>	0.5 mM	24 h	0.073	0.111
BDM <sup>a</sup>	0.5 mM	5 h	0.132	0.160
BDM <sup>a</sup>	0.5 mM	7 h	0.073	0.110
PbAc <sup>b</sup>	15 mM	19 d	0.091	0.261
PbAc <sup>b</sup>	12 mM	21 d	0.042	0.275
PbAc <sup>b</sup>	12 mM	7 d	0.071	0.111
PbAc <sup>b</sup>	12 mM	11 d	0.048	0.115
PbAc <sup>b</sup>	12 mM	7 d	0.096 <sup>3</sup>	0.109
PIP <sup>c</sup>	1 mM	11 h	0.250	0.271
PIP <sup>c</sup>	1 mM	11 d	0.066	0.270
PIP <sup>c</sup>	1 mM	46 h	0.045	0.143
PIP <sup>c</sup>	0.1 mM	2 d	0.117 <sup>3</sup>	0.168
EMTS <sup>d</sup>	0.2 mM	7 d	0.277	0.333
EMTS <sup>d</sup>	0.2 mM	66 h	0.082	0.217
EMTS <sup>d</sup>	1 mM	24 h	0.069	0.280
EMTS <sup>d</sup>	0.1 mM	25 h	0.063	0.418
IMan <sup>e</sup>	20 mM	cocrystallised	0.082 <sup>3</sup>	0.083
IMan <sup>e</sup>	20 mM	cocrystallised	0.084 <sup>3</sup>	0.144
TlCl	2 mM	3 h	0.160 <sup>3</sup>	0.384
DANP <sup>f</sup>	saturated solution		0.121 <sup>3</sup>	0.191
K <sub>2</sub> PtCl <sub>4</sub>			0.109 <sup>3</sup>	0.368
K <sub>2</sub> PtCl <sub>6</sub>			0.054	0.178
K <sub>2</sub> RuO <sub>4</sub>			0.104 <sup>3</sup>	0.097
K <sub>2</sub> IrCl <sub>6</sub>			0.079 <sup>3</sup>	0.078
Na <sub>2</sub> WO <sub>4</sub>			0.106 <sup>3</sup>	0.109

$$^1 \text{Mean fractional isomorphous difference } R_{iso} = \frac{\sum ||F_{PH}| - |F_P||}{\sum |F_P|}$$

(including data between 20 and 4.5 Å resolution)

<sup>2</sup> see equation 2 (chapter 5, page 45) for definition

<sup>3</sup>Photon Factory data at high resolution

<sup>a</sup>Baker's dimercurial;  $[CH_3CO_2HgCH_2CH(OCH_3)]_2$

<sup>b</sup>Trimethyllead acetate;  $(CH_3)_3PbCO_2CH_3$

<sup>c</sup>Diiodobis(ethylenediamine)diplatinum(II)nitrate,  $[C_2H_4(NH_2)_2PtI]_2$

<sup>d</sup>Thiomersalate;  $CH_3CH_2HgSC_6H_4CO_2Na$

<sup>e</sup>I-Mannosamine ( $\alpha$ -Mannosamine with the iodine in position C<sup>6</sup>)

<sup>f</sup>Diamino, dinitro platinum;  $(NH_3)_2Pt(NO_2)_2$

Table 1.1. The PIP derivative (soaked for 46 hours at a concentration of 1 mM PIP) resulted in a successful isomorphous derivative and initial phases could be obtained from the determination of its heavy-atom binding sites.