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Oxidation of selenomethionine: some MADness in the method!

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Since it was first reported, the multiwavelength anomalous diffraction (MAD) technique for the determination of protein structures has become widely accepted and increasingly popular. Here, it is demonstrated that the anomalous signal from selenomethione (SeMet) substituted proteins can be significantly enhanced by oxidation.

1. Introduction

Recent advances in gene cloning and expression techniques, coupled with the increase in the number and 'quality' of wavelength-tuneable synchrotron beamlines, improved techniques and software for data processing and analysis, have led to a concomitant rise in the use of MAD. It is increasingly being used as the initial means of structure determination where molecular replacement is not possible. The growing number of protein structures solved using MAD that have been submitted to the PDB in recent years attests to this.

A wide range of atoms can be used in MAD, provided they have a major absorption edge within the wavelength range of a synchrotron beamline. However, by far the most commonly used anomalous scatterer is selenium, specifically in the form of selenomethionine.

2. MADness

The theoretical basis of the MAD method to solve the phase problem in crystallography is well established (Hendrickson & Ogata, 1997) and the improvements in hardware, methodology and software make MAD experiments a routine process. In common with isomorphous replacement techniques, MAD perturbs the intensity of the observed 'native' structure-factor amplitudes, thus allowing solution of the phase problem. MAD has the advantage over isomorphous replacement methods that all of the data can be collected from a single crystal, thus eliminating problems arising from crystal non-isomorphism.

Critically, the strength of the anomalous signal in MAD will be somewhat smaller than the corresponding isomorphous difference between derivative and native crystals. The magnitude of the MAD phasing signal may be estimated from the ratio of the expected anomalous or dispersive differences arising from the anomalous scattering centres to the expected total scattering from the macromolecule (Crick & Magdoff, 1956; Smith, 1997). As an example, for a typical protein of 400 residues with eight SeMet residues, the expected anomalous signal will be no more than ~4% and the dispersive signal ~3%. This signal is near the noise level of moderate-quality diffraction data, as opposed to the corresponding isomorphous difference signal, which in this example would be ~8%. Consequently, collection of MAD data needs to be precise and accurate.

Given the average noise level in a typical data set, the minimum number of anomalous scattering centres required to give a measurable signal for a given mass of protein can be estimated. In the case of selenium, the generally accepted minimum is one Se atom for every 14 000 Da. This corresponds to an anomalous signal of ~3%. The outcome depends on a combination of factors, including the redox state of the anomalous scattering centres, their order within the lattice, the overall order of the crystal lattice, the strength and coherence of the diffraction and the accuracy of the data.

What happens in the situation where the calculated strength of the anomalous signal is sufficient for MAD to succeed, but one or more of the stated factors conspire to compromise the data? The order of the crystal lattice and the strength of diffraction are functions of the crystal and need to be approached by optimization of the crystallization conditions. The accuracy of data collection is largely a matter of experimental technique.

The redox state of the anomalous scatterer can make a large difference to the size and integrity of the anomalous signal. In the case of selenomethionine, the redox state of the selenium affects both the magnitude and energy of the anomalous signal, both of which can be determined directly by an X-ray fluorescence scan around the selenium *K* absorption edge.

Smith & Thompson (1998) demonstrated in experiments with selenium metal, inorganic selenium salts and free aqueous selenomethionine that oxidation of the selenium leads to a significant increase in the magnitude of the absorption at the *K* edge. There is also a small but significant shift of the absorption edge to higher energy.

One of the key implications of this shift in energy of the absorption edge is the requirement to ensure homogeneity of the redox state of the anomalous scatterer within the crystal lattice. Heterogeneity would 'smear' out the absorption profile of the scatterer, critically reducing the size of the MAD signal and making precise determination of the anomalous peak and inflection points difficult, thus impairing accurate measurement.

Current thinking, as shown in the literature (Smith & Thompson, 1998), is that selenomethionine-substituted proteins should be purified and crystallized in the presence of β-mercaptoethanol or other reducing agents to ensure that the selenomethionine is kept in the reduced form. The rationale is that selenomethionine is much more unstable and more readily oxidizable than methionine. We demonstrate that in certain circumstances there may be advantages in fully oxidizing the incorporated selenomethionine to optimize the MAD signal.

3. Experimental procedure and data analysis

The system used is the transporter TolC (Koronakis *et al.*, 1997; Thanabalu *et al.*, 1998), an *Escherichia coli* integral outer membrane protein, expressed in its natural host organism. The protein was overexpressed, purified and successfully crystallized in the presence of detergents. Large crystals grew within one week and belong to space group *R*3, with unit-cell parameters $a = b = 265$, $c = 95$ Å, $\alpha = \beta = 90$, $\gamma = 120^\circ$. A trimer occupies the asymmetric unit. Following cryoprotection and freezing to 100 K, these crystals diffracted to 2.5 Å, although thermal diffuse scatter was a constant problem which we were unable to eliminate.

After a lack of success in finding conventional heavy-atom derivatives, SeMet MAD was tried. The protein was retransformed into a *met*$^-$ strain and successfully expressed from modified Le Masters' medium (LeMaster & Richards, 1985) in the presence of selenomethionine. The protein was purified in the presence of 20 m*M* β-mercaptoethanol and crystallized with 5 m*M* β-mercaptoethanol. Crystals were isomorphous to native crystals and diffracted to similar resolution limits. Amino-acid composition analysis indicated 80% or better substitution of methionine with selenomethionine. There are five methionine residues per monomer (total 428 amino acids), giving a ratio of one Se atom per 9 kDa.

Initial tests and data were collected at the SRS at Daresbury on station 9.5. Fluorescence scans varied with preparation, but all exhibited a very broad edge and, in particular, showed little evidence of a white line at the peak. The most likely explanation for this seemed that despite extensive precautions, the preparation contained a heterogeneous population of reduced and oxidized selenomethionine. The measured anomalous signal was very low and unsurprisingly location of the positions of the Se atoms using a variety of different procedures and structure solution were unsuccessful.

Crystals derived from two further preparations, collected on station BM14 at the ESRF, showed a sharp edge and a good white line in their fluorescence spectra. Their fine structure clearly indicated that the selenomethionine was most likely to be all reduced (Fig. 1). Nevertheless, despite careful data collection to ~3.0 Å (with a final merging *R* of 7.3%), the measured anomalous signal was again low and structure solution was similarly unsuccessful.

Further optimization of crystallization conditions and careful data collection yielded little improvement, leaving both the redox state of the selenomethionine and insufficient measurable anomalous signal, possibly owing to measurement errors arising from the thermal diffuse scattering, as the most likely reasons for the failure of MAD.

The inability of high concentrations of β-mercaptoethanol to reproducibly keep all of the selenomethionine residues in the reduced state indicated that some of these residues had become oxidized, most likely during extrusion of the protein through the periplasm and insertion into the outer membrane. Once folded, these residues are buried and thus protected from reduction by β-mercaptoethanol. Logically, those selenomethionine residues that were reduced are presumably accessible and thus susceptible to oxidation.

Smith & Thompson (1998) carried out experiments with free aqueous selenomethionine, keeping it reduced with β-mercaptoethanol and also deliberately oxidizing it by treatment with 0.15% hydrogen peroxide for 1 h. They showed that although the identity of the oxidation product was not clear, oxidation was successful and complete. Furthermore, the fluorescence scan of the oxidized selenomethionine showed a 30–40% increase in magnitude of the absorption edge over reduced selenomethionine (Fig. 1). They further speculated that it may be possible to briefly treat selenomethionine-substituted protein crystals with peroxide immediately prior to freezing in order to oxidize the selenomethionine and thus afford a similar increase in the magnitude of the anomalous signal.

In this case, rather than treat the crystals, the protein in solution has been treated. Prior to crystallization, all traces of β-mercaptoethanol were removed and the protein was treated with hydrogen peroxide. The protein was put into a dialysis bag and hydrogen peroxide was added to a final concentration of 0.1%. After around 10 s exposure, the dialysis bag was sealed and immediately placed in dialysis buffer to remove the peroxide from the protein. No precipitation or other adverse affects were observed following addition of the peroxide. After dialysis, the protein was concentrated and crystallization trials were set up under normal conditions, but without addition of any reducing agents. Crystals grew within a week and were visually indistinguishable from native or β-mercaptoethanol-treated SeMet-substituted crystals. They were isomorphous to 'unoxidized' crystals and although they still showed some thermal diffuse scattering, they diffracted slightly more strongly.

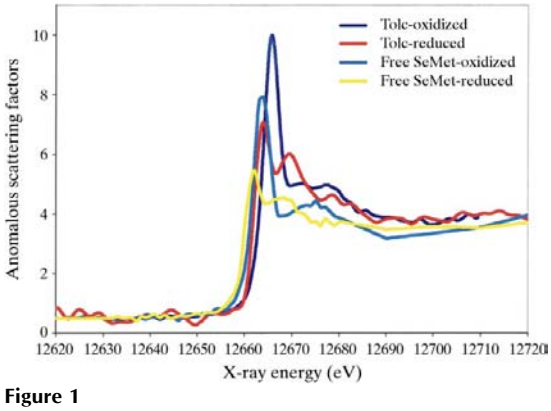

Figure 1
Anomalous scattering-factor spectra for crystals of oxidized and reduced TolC SeMet, and oxidized and reduced aqueous SeMet (data provided courtesy of Janet Smith). All data collected on station BM14 at the ESRF.

Fluorescence scans measured on station BM14 at the ESRF showed a dramatic difference from scans of the reduced form measured on the same beamline (Fig. 1). The scans show a very sharp absorption edge and pronounced white line. The peak is shifted slightly to higher energy with respect to the scan for the reduced crystal and, more significantly, the magnitude of the edge has increased by more than 40% over the reduced form. The scans of the reduced and oxidized crystals almost exactly match Smith & Thompson's results for free aqueous selenomethionine (Fig. 1). Comparison of the scan of the oxidized TolC with that of the oxidized SeMet clearly indicates that oxidation of the selenomethionine in the protein was complete. Fig. 2 shows Kramers–Kronig transformations of the scans for the reduced and oxidized crystals. This indicates that oxidation of the selenium has increased the anomalous signal from 7 e to 10 e, a rise of over 40%. The dispersive signal, on the other hand, from the inflection point against the high-energy remote (at 14 keV; data not shown) has only risen from 6.5 e to 7.5 e, an increase of ∼15%.

A full three-wavelength MAD data collection was measured to 2.8 Å from a single crystal on station BM14. Following data reduction and scaling with *HKL* (Otwinowski & Minor, 1996), 14 of the expected 15 Se atoms were successfully located with *SOLVE* (Terwilliger & Berendzen, 1999). The structure was successfully determined from this data and has now been extended to 2.1 Å with further data collected at the APS in Chicago. Full details of the structure will be presented elsewhere.

The product of peroxide oxidation of selenomethionine is unknown, although Klayman & Gunther (1973) suggest that it may be the result of a four-electron oxidation to the selenone ($-SeO_2$). The electron density for the 15 selenomethionine residues is inconclusive about the chemical structure (Fig. 3), although it does not seem to suggest that the selenone has formed. Chemical analysis will most likely be required to test this more thoroughly.

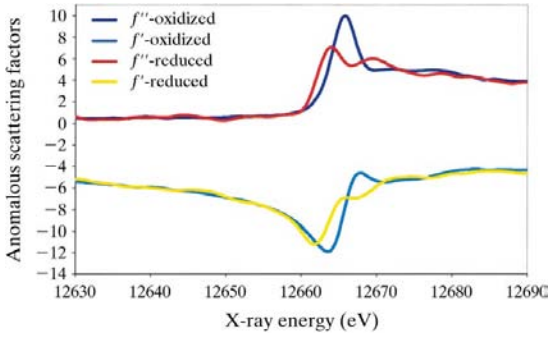

Figure 2
Kramers–Kronig transform of XAFS scans for oxidized and reduced TolC SeMet crystals. Data collected on station BM14 at the ESRF.

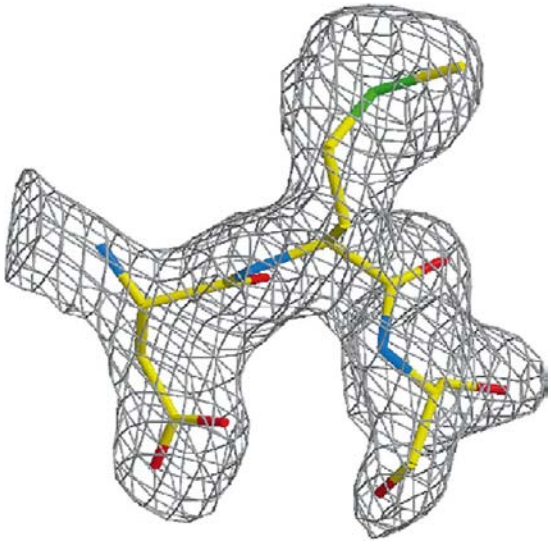

Figure 3
2.1 Å σ_A-weighted $2F_o - F_c$ electron-density map for a representative SeMet residue from TolC, contoured at 1.2σ.

4. Discussion

Oxidation of the selenomethionine in this protein by treatment with hydrogen peroxide has clearly had a dramatic effect on the phasing power. Peroxide is quite a powerful and non-discriminating oxidizing agent; however, concerns over possible breakdown or other instability of the selenomethionine owing to oxidation have proved unfounded. The fluorescence scans suggest that the oxidation process was successful and proceeded to completion, giving a homogeneous population, after a very brief exposure to peroxide. The other main concerns over the use of peroxide were oxidation of other amino-acid residues, possibly leading to structural changes or damage. In this case, peroxide does not seem to have had any detectable deleterious effects. The protein did not show adverse reaction to peroxide treatment and crystallized under identical conditions to untreated protein. After methionine/selenomethionine, another amino-acid residue susceptible to oxidation is cysteine. Fortunately, there are no cysteine residues in TolC. Furthermore, analysis of the electron density for other susceptible residues, such as tyrosine, tryptophan and histidine, showed no sign of any other oxidation. It has not been possible to determine whether peroxide treatment, or indeed selenomethionine substitution itself, has had any affect on function. However, that the crystals are all isomorphous to native crystals and refinement against reduced native data shows no significant deviation in structure, and similar R values to refinement against the oxidized SeMet data indicates that oxidation by peroxide treatment has not caused any appreciable structural changes.

In solving this problem, a method has been found which might find general use in solving structures using selenium MAD, where the ratio of selenium to protein is low, or where keeping all of the selenomethionine reduced is difficult or where the measured anomalous signal is insufficient. The range of applicability of this procedure would have to be established on a case-by-case basis. Clearly, peroxide treatment is unlikely to be applicable in enzymes or other proteins with catalytic cysteines or other oxidation-susceptible residues playing key catalytic or structural roles. However, in many other cases, this method may be useful where selenium MAD is used, in order to increase the strength of the anomalous signal and thus improve the chances of structure solution. Treating crystals directly, as suggested by Smith & Thompson (1998), has not been tried but there appears no reason why this should not work as well as pre-treating the protein and may be better in cases where treating the protein in solution is undesirable.

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References

Crick, F. H. C. & Magdoff, B. S. (1956). *Acta Cryst.* **9**, 901–908.
Hendrickson, W. A. & Ogata, C. M. (1997). *Methods Enzymol.* **276**, 494–523.

Klayman, D. L. & Gunther, W. H. H. (1973). *Organic Selenium Compounds: Their Chemistry and Biology*. New York: Wiley–Interscience.
Koronakis, V., Li, J., Koronakis, E. & Stauffer, K. (1997). *Mol. Microbiol.* **23**, 617–626.
LeMaster, D. M. & Richards, F. M. (1985). *Biochemistry*, **24**, 7263–7268.
Otwinowski, Z. & Minor, W. (1996). *Methods Enzymol.* **276**, 307–326.
Smith, J. L. (1997). *Proceedings of the CCP4 Study Weekend. Recent Advances In Phasing*, edited by K. S. Wilson, G. Davies, A. W. Ashton & S. Bailey, pp. 25–39. Warrington: Daresbury Laboratory.
Smith, J. L. & Thompson, A. (1998). *Structure*, **6**, 815–819.
Terwilliger, T. C. & Berendzen, J. (1999). *Acta Cryst.* D**55**, 849–861.
Thanabalu, T., Koronakis, E., Hughes, C. & Koronakis, V. (1998). *EMBO J.* **17**, 6487–6496.