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Application of CW and pulsed EPR, MoSophe and DFT calculations in unravelling the electronic structure of the molybdenum(V) centre in dimethylsulfoxide reductase

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Dimethylsulfoxide reductase, a bacterial molybdenum oxotransferase, belongs to the Type-III Clade of the dimethylsulfoxide (DMSO) reductase family of molybdenum enzymes and catalyses the conversion of DMSO to dimethylsulfide (DMS) with an accompanying two electron transfer. The molybdenum cofactor within DMSO reductase contains an organic component known as molybdopterin (MGD) which is a modified pterin providing an ene-dithiolene side chain responsible for ligating the Mo. The active site of the DMSO reductase contains two MGD ligands, a single oxo group and the amino acid ligand Serine in a trigonal prismatic geometry.

A continuous wave (CW) EPR spectrum attributable to the Low-g Type-I Mo(V) species and a sulfur centered radical were observed upon dithionite reduction of dimethylsulfoxide reductase from the photosynthetic bacterium *Rhodobacter capsulatus* (Lan *et al.,* 2007a&b), of the naturally abundant and ^{95}Mo enriched Low-g Type-I Mo(V) CW EPR spectrum reveals that while the magnitudes of the principal components of the g and A matrices resemble the Slow Mo(V) center found in desulfo xanthine oxidase, their orientation is quite different and the largest ^{95}Mo hyperfine component is associated with the smallest g value rather than the largest g value. The coordination sphere of the Low-g Type-I Mo(V) species consists of an an ene-dithiolene (P-MGD), Ser-147 and a protonated oxo group, which form the base of a square pyramid. In conjunction with the results obtained from a multifrequency CW EPR and density functional theory (ORCA) of a series of thiomolybdenyl complexes (Drew *et al.*, 2007a&b), the g and triclinic A(^{95}Mo) matrices are consistent with the unpaired electron located in a $|d_{x^2-y^2}>$ ground state molecular orbital in which the x and y axes are located between Mo-ligand bonds. In addition to the Low-g Type-I Mo(V) species, the CW EPR spectrum exhibits an orthorhombic signal (g_z = 2.0545, g_y = 2.0182, g_x = 1.999) with small ^{95}Mo ($A_2 = 5.0\times10^{-4}$ cm^{-1}) hyperfine coupling on the g_y resonance. Both 3-pulse ESEEM and HYSCORE spectra revealed the presence of one or more weakly coupled protons and isotropic hyperfine coupling ($A_{iso}(^{14}N)$ = 6.7 MHz) to a single nitrogen nucleus. The CW- and pulsed-EPR results are consistent with an unpaired electron centered on sulfur atom (S1) of Q-MGD which is delocalized onto the pyranopterin ring system. These results implicate sulfur centered radicals in the stabilization of the charge on the molybdenum ion in DMSO reductase and/or electron transfer between the native electron donor DorC and the Mo center via the Q-MGD.

Analysis of the continuous wave and pulsed electron paramagnetic resonance spectra and EPR potentiometric titration experiments reveal that the Mo(V) High-g Unsplit Type-2 species is the intermediate species formed during the catalytic reduction of DMSO reductase from Rhodobacter capsulatus. The spin Hamiltonian parameters for the Mo(V) High-g Unsplit Type-2 species obtained from naturally abundant and ^{95}Mo enriched DMSO reductase reveal that the unpaired electron is present in a $|d_{z^2}>$ ground state molecular orbital and that the geometry of the active site Mo centre is trigonal prismatic. Since the tigonal prismatic geometry of the Mo centre is retained upon reduction of the resting (Mo(VI)), to Mo(V) and Mo(IV) the active site within DMSO reductase is an example of an entatic state. The Mo(V) High-g Split species, previously proposed to be catalytically relevant in the reductive direction, has been shown to be involved in the oxidative half reaction.

Lane I, Hanson GR, McEwan AG, Noble CJ & Pilbrow JR.(2007a) *Journal of the American Chemical Society*, Submitted.

Lane I, Noble CJ, Ridge J, Benson N, McEwan AG & Hanson GR. (2007b) *Journal of the American Chemical Society*, Submitted.

Hanson GR, Noble CJ, Benson S.(2007) *Biological Magnetic Resonance*, **28**, In Press.

Drew SC, Hill JP, Lane I, Hanson GR, Gable RW & Young CG. (2007a) *Inorganic Chemistry*, **46:** 2373-87.

Drew SC, Young CG & Hanson GR. (2007b) *Inorganic Chemistry*, **46:** 2388-97.

Using NMR to study kinase regulation

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Histidine kinases (HKs) are ubiquitous molecular sensors that are used by bacteria to detect and respond to an impressive variety of environmental cues. In recent years, HKs have become attractive antimicrobial targets due to their roles in mediating virulence and antibiotic resistance in bacterial pathogens. Unfortunately, attempts to develop therapeutically useful HK inhibitors have proved unsuccessful, primarily due to our limited understanding of the way HKs function as well as the mechanisms by which their activity is regulated. We have used a variety of NMR methods to probe the way in which kinase function is regulated by both the HK sensor domain as well as endogenous antikinases. Moreover, we have developed a unique NMR-based approach that can be used to: (i) search for the molecular signals that activate or repress HKs; (ii) screen small molecule libraries for ligands that might serve as leads for rational development of HK inhibitors. These NMR methodologies have been developed using a model histidine kinase that regulates sporulation in the soil bacterium *Bacillus subtilis* and its pathogenic relative *Bacillus anthracis*.

Boltzmann-statistics analysis of solid state NMR experiments

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A data analysis approach employing Boltzmann-statistics variation of Maximum Entropy is introduced to determine distances using solid-state NMR data from rotational-echo double-resonance (REDOR) experiments [Gehman *et al.*, 2007]. Using structural studies of amyloid-beta peptide from Alzheimer's disease, we demonstrate that this approach can provide the means to determine an unconstrained population distribution of fractional contributions for some data sets governed by amenable complex analytic forms. These examples illustrate the general utility of the Boltzmann statistics approach, as the fitting of nonlinear physical data is often a hazardous affair. As in our examples, frequently an analytical model governing a measured process is firmly grounded in theory, and the measured signal is a sum of contributions from one or more fractional components. One needs to fix the number of components used in a fit, thereby constraining the conceivable population distribution to relatively few discrete contributions. Often there is not enough information to be unequivocal about the number of components to use; too few and the fit will be poor, too many improves the fit but with large error estimates that can render the characteristic parameters for each component meaningless. Imperfect data blurs the boundary between too few and too many. The Boltzmann-statistics approach thereby extracts a relatively model-free distribution of internuclear distances present in a sample measured by REDOR, and allows NMR time to be spent obtaining better signal-to-noise on fewer data points.

Gehman JD, Separovic F, Lu K & Mehta AK. (2007) *Journal of Physical Chemistry*, **111:** 7802-11.

Chiral selection in metabolism studied by NMR of anisotropic media

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L-amino acids are used almost exclusively in ribosomal protein synthesis; this is a well-known example of the 'chiral bias' of living systems. The reason for this is the 'three-point attachment' phenomenon that is expressed in enzymes (*e.g.*, Kuchel & Ralston, 1998). A subtle situation involving chirality exists in human red blood cells (RBCs) whereby L-lactate is produced *via* glycolysis using NAD/NADH as the redox pair, and D-lactate is produced via the glyoxalase pathway using the reductant glutathione (Rae *et al.*, 1990).

NMR spectra of racemic mixtures of many solutes can be resolved if they are constituted in chiral media, and more structural information can be obtained if the medium is aligned with the magnetic filed of the NMR spectrometer as well (Emsley, 1996). Gelatin, which is chiral, can be set inside a silicone-rubber tube (Kuchel *et al.*, 2006; Naumann *et al.*, 2007) and variably stretched. Thus we can elicit, in NMR spectra, a range of residual dipolar splittings of spin = ½ nuclides, and residual quadrupolar splittings of spin > ½ nuclides. RBCs set and stretched in the device can be studied with respect to transmembrane exchange and metabolism of chiral solutes. D- and L-lactate give clearly resolved spectra; hence the simultaneous measurement of parallel fluxes in glycolysis and the glyoxalase pathway has been made possible.

Kuchel PW & Ralston GB. (1998) In: *Schaum's Outline of Theory and Problems of Biochemistry*, 2nd edn, eds. Kuchel PW & Ralston GB. p369. New York: McGraw-Hill.

Rae C, Berners-Price SJ, Bulliman BT & Kuchel PW (1990) *European Journal of Biochemistry*, **193:** 83-90.

Emsley J (1996) In: *Encyclopaedia of Nuclear Magnetic Resonance*. eds. Grant DM and Harris RK. pp 2788-2799. London: Wiley

Kuchel PW, Chapman BE, Müller N, Bubb WA, Philp DJ & Torres AM (2006) *Journal of Magnetic Resonance*, **180:** 256-65.

Naumann C, Bubb WA, Chapman BE & Kuchel PW. (2007) *Journal of the American Chemical Society*, **129:** 5340-1.

Enhanced protein stability through disulfide engineering

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The CSK-homologous kinase (CHK) is an endogenous inhibitor of Src-family protein tyrosine kinases (SFKs). Excessive SFK activity contributes to cancer formation and progression, thus endogenous SFK-inhibitors play a vital tumour-suppressor function in normal cells (reviewed in Chong *et al.,* 2005). The cellular function of CHK is modulated by its subcellular localisation, which is controlled by the ligand-binding properties of its Src homology-2 (SH2) and Src homology-3 (SH3) domains. For some years we have been studying the structure and function of CHK, including NMR studies of the SH2 and the SH3 domains (Mulhern *et al.,* 2002; Chong *et al.,* 2004; Chong *et al.,* 2006). Our initial attempt at NMR analysis of the CHK SH3 domain was hampered by the intrinsic instability of the recombinant protein, which resulted in slow irreversible unfolding at ambient temperatures. We adopted a protein engineering approach to generate a CHK SH3 construct more suitable for structural and functional analysis. We have successfully enhanced the stability of the CHK SH3 domain through an engineered disulfide bond. The mutant protein (DS-SH3) has a melting temperature (T_m) more than 20°C higher than that of the wild type SH3 domain. This increase in T_m is considerably greater than that reported for other comparable examples of disulfide engineering. The structural integrity of the domain was confirmed by circular dichroism (CD) spectropolarimetry and nuclear magnetic resonance (NMR) spectroscopy. A full-length version of CHK carrying the DS-SH3 mutations was expressed in insect cell culture and purified for functional analysis.

Chong YP, Chan AS, Chan KC, Williamson NA, Lerner EC, Smithgall TE, Bjorge JD, Fujita DJ, Purcell AW, Scholz G, Mulhern TD & Cheng HC (2006) *Journal of Biological Chemistry*, **281:** 32988.

Chong YP, Mulhern TD & Cheng HC (2005) *Growth Factors*, **23:** 233.

Chong YP, Mulhern TD, Zhu HJ, Fujita DJ, Bjorge JD, Tantiongco JP, Sotirellis N, Lio DSS, Scholz G & Cheng HC (2004) *Journal of Biological Chemistry*, **279:**, 20752-66.

Mulhern TD, To C & Cheng HC (2002) *Journal of Biomolecular NMR*, **24:** 363.

Dynamics of the troponin molecular switch in the thin filament using SDSL-EPR

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Troponin (Tn) is a heterotrimeric protein comprised of TnC, TnI and TnT subunits, which acts as the molecular switch of striated muscle contraction. The proposed mechanism of this calcium dependent regulation is based on static structural models. The Tn complex interacts *in vivo* with both the Tropomyosin and actin constituents of the thin filament. However, current structural models available of the Tn complex were made with experimental data collected in the absence of these two important binding partners. Site-Directed Spin Labeling Electron Paramagnetic Resonance (SDSL-EPR) is a technique that can be used to study protein structure and dynamics. It requires the insertion of cysteine residues into the protein backbone onto which spin labels can be specifically attached. Spin labels provide local environment structural and dynamic information. The advantage of SDSL-EPR over other techniques in the analysis of Tn is that binding partners Tropomyosin and actin can be included in the investigation. Additional advantages are that measurements can be made at physiological conditions allowing the dynamics of the Tn complex to be monitored in both regulation states, i.e. in the presence and absence of Ca^{2+}. A series of single cysteine substitution mutants, labeled with the nitroxide spin label MTSSL (1-oxyl-2,2,5,5-tetramethylpyrroline-3-methyl-methanethiosulfonate) were constructed to explore the dynamics of four functionally significant regions of the cardiac TnI subunit. These regions include the TnI structural core (Iso132Cys), the 'primary inhibitory peptide' (Leu144Cys), the 'switch peptide' (Ala151Cys) and the controversial second actin-binding domain termed the 'mobile domain' (Gln175 to Arg186, each substituted to Cys in turn). The current model suggests that there should be significant domain movement observed in the selected regions with the exemption of the structural core. EPR mobility experiments demonstrate these Tn domain movements in the thin filament, as proposed from the static models.