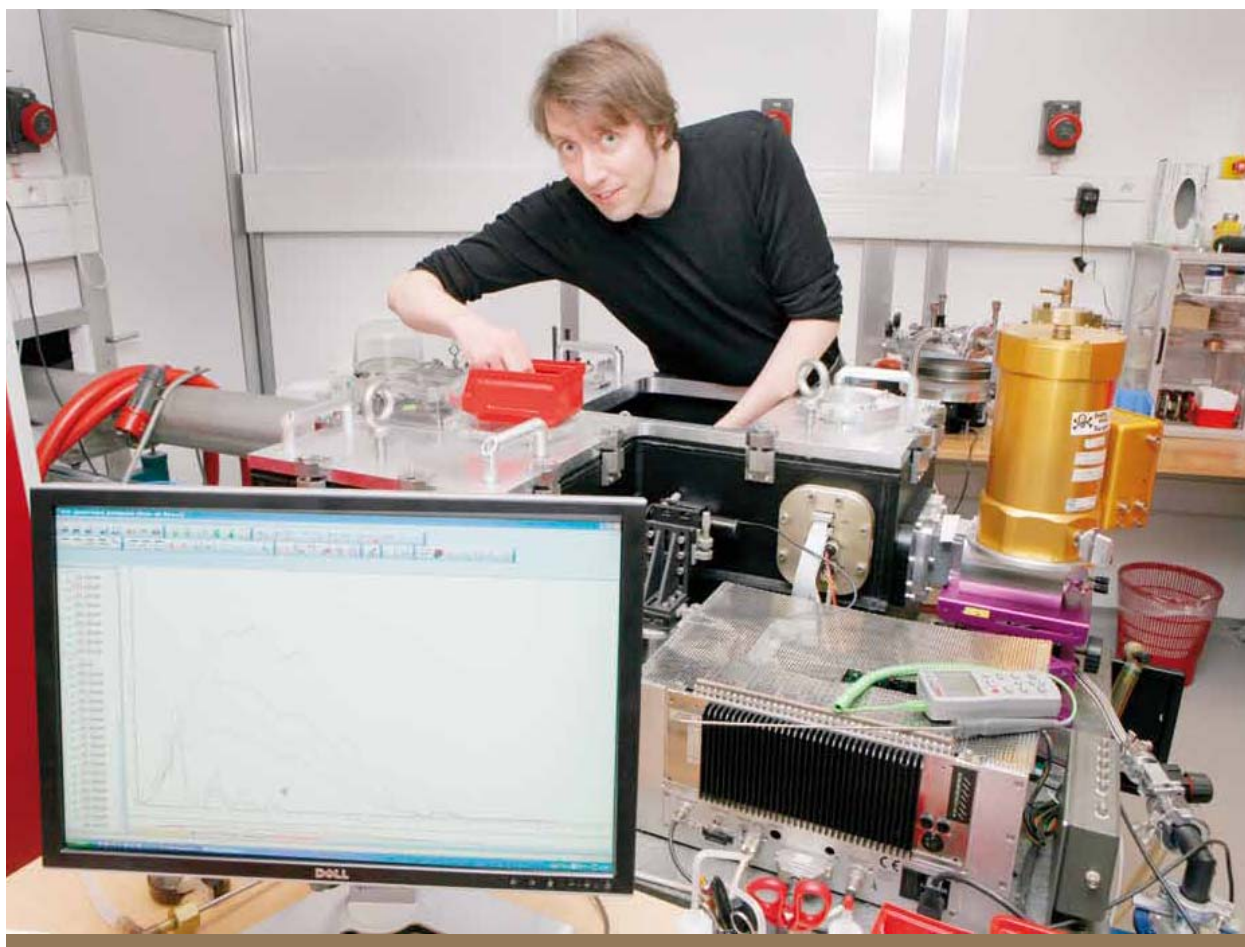


## METALLOPROTEINS AND OXIDATIVE STRESS

**The contribution** of Terahertz

More than one third of the enzymes contain a metal playing a key role at the active site. Metalloenzymes are involved in essential cellular processes, such as respiration, electron transfer, detoxification processes or DNA synthesis. AILES beamline proposes offers an approach particularly adapted to the study of these metallic sites

Jean-Blaise Brubach, scientist in the AILES team, preparing the Fourier transform spectrophotometer.



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he variety of reactions catalyzed by metalloenzymes (see ref. 1) results not only from the nature of the metal present in the active site (Fe, Cu, Mn, Zn, for example) but

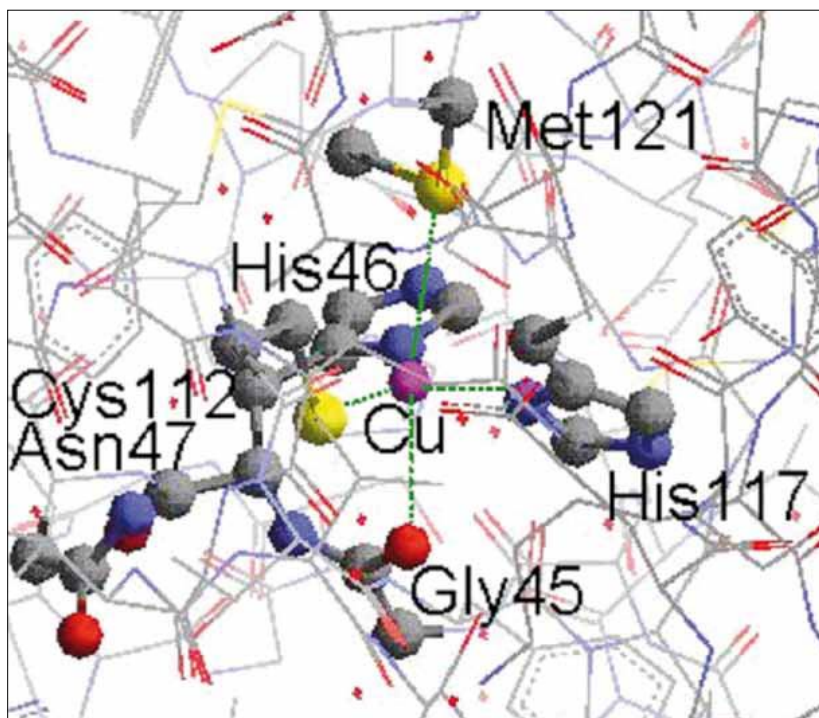
also and very importantly from the fact that the protein structure exerts a fine tuning on the metal centre properties. Metal site reactivity is controlled by the nature

of its ligands, the amino acid side chains at the first coordination sphere, and also by specific (electrostatic) interactions involving structural motifs of the protein at larger distance from the metal (the second coordination sphere). It is then of first importance to study in details the parameters controlling metal-sites reactivity and specificity in metalloproteins.

### When oxygen can become toxic

Oxidative stress is a major problem that concerns all cells living in the presence of dioxygen. It is characterized by the production of highly toxic reactive oxygen species (ROS), which are generated inevitably during aerobic metabolism as side products of aerobic respiration. These ROS correspond to the intermediates in reduction pro-

Figure 1: Cu active site of azurin



cesses of  $O_2$  and include superoxide radicals, hydrogen peroxide and hydroxyl radicals. Fortunately, cells have developed very efficient antioxidant enzyme systems, specific in the elimination of each type of ROS. However, any deficiency or overload of these antioxidant systems can result in severe cellular damages, which can ultimately lead to cell death. It is now well documented that oxidative stress is involved in the development of several diseases, (cancers, Alzheimer, Parkinson), age-related macular degeneration and also ageing.

Up to now, only two different types of metalloenzymes have been described to catalyze the detoxification of the highly toxic superoxide radical species. These are the well known superoxide dismutase (SOD) and the more recently discovered superoxide reductase (SOR). Cu,Zn-SOD is ubiquitous, and represents almost 90 % of total SOD activity in human cells. Dysfunction or mutations of SOD have been associated with several diseases and notably familial amyotrophic lateral sclerosis. Protection against

degenerative diseases linked with oxidative stress, triggers intense research for the development of antioxidant molecules with SOD activity. However, the results in such approaches have remained scarce because of the difficulties in synthesizing small molecules, metal complexes, with efficient SOD activities.

### The assets of difference spectroscopy

Superoxide reductase, which also efficiently detoxifies superoxide, exhibits different reaction mechanism and different metal active centers than SOD. Although SOR has been found only in prokaryote cells, it presents potentially important applications in human health. It could constitute a model for new antioxidant molecules, alternative to SOD mimics, which could detoxify superoxide radical with a SOR activity. Accordingly, in that aim, several groups, mainly in the United States, are developing chemical synthesis to model the SOR active site.

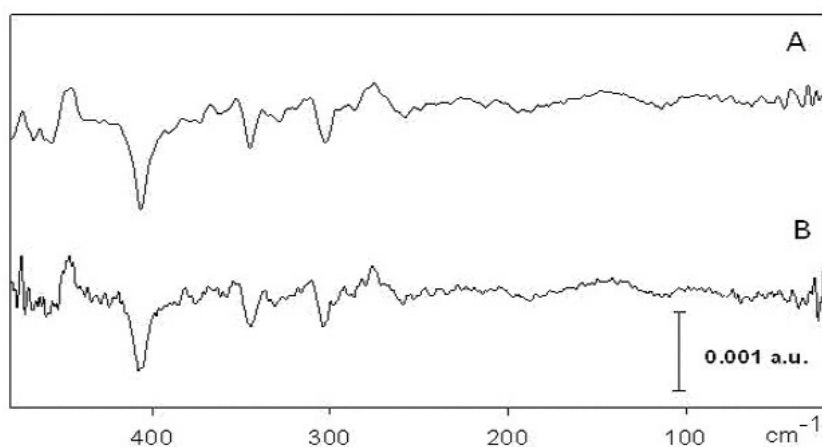
All these synthesis approaches require knowing the essential cha-

racteristics of the ligand to metal bonds that have to be reproduced in the metal complex, in order to mimic the SOD or SOR activity. Such information is not trivial and far to be completed for both the SOR and SOD enzymes. The fundamental study carried out on AILES beamline on SOD and SOR by Far-IR spectroscopy aims at a better characterization of these ligand-to-metal bonds properties, in relation with superoxide reactivity. One application of the results could be to improve the design of more active SOD and SOR mimics.

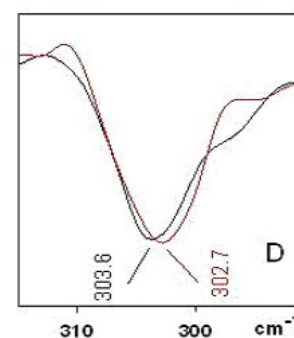
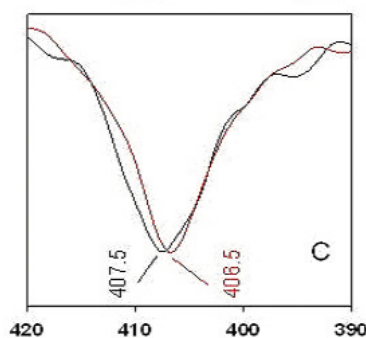
Vibrational spectroscopy is extremely useful to determine the properties of metal-ligand interactions in particular because this is one of the very few techniques which allow to directly investigate the strength of each coordination bond. Infrared absorption occurs when a change in the dipole moment is induced by exciting a vibrational mode, therefore almost all vibrations from any chemical groups in a protein can absorb in the infrared. Hence, the richness of information can become a weakness and specific information about a given bond can be masked by the other absorption in this same energy range. By using difference spectroscopy, however, detecting vibrations from single vibrators, specifically perturbed by the reaction becomes possible, by the triggering of a highly specific reaction in the protein (see ref. 2).

### Cu-azurin as a model protein

First, the teams of LIPM (CNRS-CEA, Marseille), LGBP (CNRS-CEA, Marseille) and AILES demonstrated the feasibility of electrochemistry coupled to FTIR difference spectroscopy in the Far-Infrared domain to identify IR bands from single vibrators at the active site of Cu-azurin. We have selected the Cu-metalloprotein azurin, because of its small size (14kDa), and because of the coordination sphere of Cu, possessing Cys and His ligands, similar to the ligands in SOD and



**Figure 2:**  
Reduce-minus-Oxidized FTIR difference spectra recorded with Azurin at pH=8,5.  
A ) 2cm<sup>-1</sup> resolution  
B) 1cm<sup>-1</sup> resolution.  
C) and D) Superimposition of spectra recorded with <sup>63</sup>Cu (black) and <sup>65</sup>Cu (red) azurin.



SOR. Azurin is a small metallo-protein (14 kDa) involved in electron transfer reactions. The redox Cu ion is coordinated to the thiolate group of a cysteine (Cys112) and to two histidine nitrogens (His46 and His117) in a trigonal planar geometry. Two axial ligands, a methionine thioether (Met121) and a glycine amide oxygen (Gly45), complete the Cu coordination sphere (Figure 1). The protein environment strongly influences the properties of the Cu centre and in particular H-bonds formed between His46-Asn47 and the sulphur atom of Cys112.

This model is used as a benchmark to develop an integrated spectroscopic and theoretical methodology for interpretation of Far-IR spectra of metalloproteins such as SOR and SOR.

In this study, the scientists focussed on the identification of Cu-ligand vibrations in the model protein azurin. Towards this aim, they recorded on the AILES beamline electrochemically-induced FTIR difference spectra in the 500-20 cm<sup>-1</sup> region, on samples of azurin reconstituted with <sup>63</sup>Cu and <sup>65</sup>Cu (Figure 2). Due to the high brilliance and of the high stability of AILES, the spectral quality is higher -for a much shorter acquisition time- than that obtained with

an internal IR source, and the spectral range is broader. This will be of prime importance to study proteins that cannot be used as concentrated. To obtain a sufficient signal to noise, the results from 15 to 20 electrochemical cycles have been averaged, which takes about 20 hours.

### Early results justify the chosen approach

Substitution of Cu by <sup>63</sup>Cu and <sup>65</sup>Cu is expected to alter specifically IR bands involving the Cu, i.e. metal-ligand bond vibrations. These frequency shifts are expected to be very small. Indeed, using a 2 cm<sup>-1</sup> resolution and long accumulation times (about 20 hours), band shifts of less than 1 cm<sup>-1</sup> have been identified on some of the bands detected in the reduced-minus-oxidized spectra (Figure 2). The shift of about 1 cm<sup>-1</sup> detected for the band at 406 cm<sup>-1</sup> confirms that this band corresponds to the CysS-Cu<sup>2+</sup> stretching mode. This band is part of a band massif detected in resonance Raman spectroscopy and assigned to Cys-Cu<sup>2+</sup> vibrations. A second band at 303 cm<sup>-1</sup> was shifted

by -0.8 cm<sup>-1</sup> upon <sup>63</sup>Cu/<sup>65</sup>Cu exchange. This band is thus assigned to the ν<sub>as</sub>(HisN-Cu<sup>2+</sup>-NHis) vibration. In contrast other bands were not sensitive to the Cu labelling. These preliminary experimental results are the first definitive assignment of metal-ligand IR vibrations in the Cu-protein, including Cu-His vibrations. We will also compare results obtained with samples in H<sub>2</sub>O and <sup>2</sup>H<sub>2</sub>O to identify IR bands from the protein involving exchangeable protons. All these results set the bases for an analysis by means of quantum chemical calculations of the vibrational spectra.

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→ **Contacts :**  
catherine.berthomieu@cea.fr  
jean-blaise.brubach@synchrotron-soleil.fr  
rainer.hienerwadel@univmed.fr

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