Triggered infrared spectroscopy for investigating metalloprotein chemistry

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Recent developments in infrared (IR) spectroscopic time resolution, sensitivity and sample manipulation make this technique a powerful addition to the suite of complementary approaches available for the study of time-resolved chemistry at metal centres within proteins. Application of IR spectroscopy to proteins has often targeted the amide bands as probes for gross structural change. This article focuses on the possibilities arising from recent IR technical developments for studies that monitor localized vibrational oscillators in proteins—native or exogenous ligands such as NO, CO, SCN\(^-\) or CN\(^-\), or genetically or chemically introduced probes with IR-active vibrations. These report on the electronic and coordination state of metals, the kinetics, intermediates and reaction pathways of ligand release, hydrogen-bonding interactions between the protein and IR probe, and the electrostatic character of sites in a protein. Metalloprotein reactions can be triggered by light/dark transitions, an electrochemical step, a change in solute composition or equilibration with a new gas atmosphere, and spectra can be obtained over a range of time domains as far as the sub-picosecond level. We can expect to see IR spectroscopy exploited, alongside other spectroscopies, and crystallography, to elucidate reactions of a wide range of metalloprotein chemistry with relevance to cell metabolism, health and energy catalysis.

Keywords: infrared spectroscopy; time-resolved; metalloprotein; spectroelectrochemistry

1. Introduction and scope

Recent developments in mid-infrared (IR) spectroscopy in time resolution, sensitivity and sample manipulation make this technique a powerful addition to the suite of complementary approaches available for the study of time-resolved chemistry at metal centres within proteins. Application of IR spectroscopy to proteins has often targeted the amide I and II bands (1700–1600 and 1580–1480 cm\(^{-1}\)) of the polypeptide backbone as probes for gross structural change (Siebert & Hildebrandt 2008). The amide I band arises predominantly from

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stretches of the peptide C=O unit, whereas the amide II band arises from a combination of NH bending and C–N stretching. The exact shape and position of these bands vary with the secondary protein structure and the presence of hydrogen-bonding interactions. For very small oligopeptides, it is sometimes possible to assign individual amide I bands to specific peptide C=O units (Hamm et al. 1999), but for most proteins, the amide bands represent a superposition of the vibrational bands arising from each residue and thus report only on global structure in the protein, in particular, the extent of α-helix, β-sheet or random coil. This article focuses particularly on the possibilities arising from recent IR technical developments for studies that monitor identifiable, localized vibrational oscillators in proteins (figure 1). Vibrational bands arising from native or exogenous ligands such as NO, CO, SCN− or CN− are particularly rich sources of information, reporting on changes to the electronic or coordination state of metals, and kinetics, intermediates and reaction pathways of ligand binding and release. The frequencies for the vibrational bands of these groups...
coordinated to metal centres generally occur in regions of the IR spectrum that are distinguishable from the amide bands and thus can be identified readily (figure 2a). Other interesting opportunities arise from localized probes that give rise to IR-active vibrations, such as the non-natural amino acid cyanophenylalanine introduced into a protein (Schultz et al. 2006) or cysteine converted to a thiocyanate (Fafarman et al. 2006; figure 1d). These could be exploited to examine hydrogen-bonding interactions between the protein and IR probe, the electrostatic character of sites in a protein or possibly to confirm proximity between residues or follow protein–protein interactions or folding.

Data from IR spectroscopic studies of metalloproteins are often presented as difference spectra to highlight changes in vibrational features in response to a reaction trigger. Triggers for chemical changes in metalloproteins include light/dark transitions, an electrochemical step, a temperature change, and a
change in solute composition or equilibration with a new gas atmosphere (figure 2b). Providing that the response of the entire protein sample to the trigger is fast, modern lasers and detectors now facilitate instrumentation that can provide information on fast changes in the IR spectrum in response to these triggers down to the sub-picosecond time regime. The focus of this review is on the scientific possibilities rather than the technical aspects of these developments, which have been reviewed elsewhere (Osawa 2002; Groot et al. 2007; Towrie et al. 2009). Parallel advances in genetic strategies to obtain native and mutant forms of metalloproteins in higher yield and greater purity and to attach proteins to surfaces open up IR methods to a wider range of metalloproteins. These advances are timely, as roles for CO and NO in cell signalling and regulation continue to be revealed (e.g. Miller et al. 2009), and the availability of genome sequences for a wide range of organisms is revealing genes encoding yet more proteins that bind small ligands with IR-active vibrations. An example is the unexpected presence of genes encoding homologues of a CO/CO$_2$ cycling carbon monoxide dehydrogenase enzyme and its haem-protein activator at the gene level, CooA, in the genome of the nitrogen-fixing soil bacterium Azotobacter vinelandii (Setubal et al. 2009). The stage is set for IR spectroscopy to play a key role in elucidating details of the mechanism and chemistry of such proteins as they undergo physiologically relevant catalysis or reactions.

2. Addressing challenges in infrared studies of metalloproteins

Many metalloproteins are difficult to obtain in large quantities: for bacterial proteins, several litres of cell culture may yield only nanomoles of a given metalloprotein and the isolation can take many hours or days. It is often difficult to concentrate sensitive proteins much beyond 1 mM without causing aggregation and or denaturation, and solution IR spectroscopic studies of proteins are often carried out close to the achievable concentration limit to maximize absorption from the protein against the water background. Much of the developmental work on new IR approaches has been done on readily available (often commercially available) haem proteins such as myoglobin, haemoglobin and cytochrome c which can often be concentrated as highly as 10 mM. Small-volume spectroscopic cells are important for working with proteins that are difficult to isolate, and approaches that require highly concentrated samples are undesirable. This situation has been improved by the possibility of over-expressing proteins and genetically introducing poly-histidine and streptavidin-type affinity tags to facilitate purification, meaning that IR studies have become accessible even for site-directed mutant proteins which tend to be less stable and therefore isolated in lower yields than wild-type proteins.

Water exhibits a broad, intense stretching band in the IR spectrum centred at about 3400 cm$^{-1}$, a weaker broad band centred at 2200 cm$^{-1}$ and a sharper weak band at 1650 cm$^{-1}$. Since proteins must almost always be studied in water, the background solvent absorption presents a considerable challenge, particularly for studies targeting the amide bands or bands above 2800 cm$^{-1}$. The small pathlengths chosen for studies of proteins in transmission mode (usually around 10–50 μm) represent a compromise between minimizing solvent background absorption and maximizing absorption from the protein at the
achievable concentration. Fortuitously, many ligands for metalloproteins have vibrational bands in regions of the spectrum that are fairly clear of the water and amide bands and therefore can easily be assigned (figure 2a). The accessible spectral window is altered by exchanging protein samples into D$_2$O (figure 2a), although this leads to shifts in any absorption bands of the sample which are sensitive to solvent exchangeable protons.

3. Instrumentation

Standard modern laboratory IR spectrometers are almost always Fourier transform IR (FTIR) instruments. In their Michelson interferometer, a beam splitter reflects 50 per cent of the beam from the source to a fixed mirror, whereas 50 per cent is transmitted to a second, moveable mirror perpendicular to the first. The beams reflected from the mirrors recombine, but with a phase shift dependent on the position of the moveable mirror, leading to constructive or destructive interference. The reflected beams pass through the sample to the detector where an interferogram is recorded: intensity as a function of the optical path difference. Finally, the spectrum is assembled from the interferogram by a Fourier transform operation. Many experiments on metalloproteins can be performed using fairly routine FTIR instruments equipped with a sensitive detector (usually a liquid-nitrogen-cooled mercury cadmium telluride (MCT) detector), and creative sample handling has offered some recent advantages, particularly for samples immobilized on metal surfaces. Gas-tight cells are often important because many metalloenzymes are sensitive to O$_2$ and many of the ligands with IR-active vibrations are gaseous. Incorporation of temperature control may also be important. Water-insoluble IR-transparent windows must be chosen for work with proteins in aqueous solutions, and CaF$_2$ is a common choice.

Two approaches to time-resolved spectroscopy are possible using fairly standard FTIR instruments. Rapid scan is limited to the millisecond time range. In a step-scan approach, micro- to nanosecond time resolution is possible. Instead of obtaining data for a full set of interferometer mirror positions after a reaction trigger, the mirror is moved step-wise, so that time-course data are collected after a reaction trigger at each mirror position. Interferograms and then spectra are assembled when the mirror series is complete. In general, the step-scan approach is only applicable if the reaction induced by the trigger is fully reversible because the trigger must be repeated many times. A microfluidic flow cell was employed in a recent step-scan study of the protein movements following photolysis of CO from myoglobin for which restoration of the starting state is irreversible on the time scale of the reactions being interrogated (Schleeger et al. 2009). The requirement for 20–200 μl (depending on the spectral range studied) of 4 mM protein would still be impractical for many difficult-to-isolate proteins. An alternative is to mount the sample on a moveable x–y stage (Gerwert 2002) and use a focused IR beam. Achieving time resolution also requires that the sample responds rapidly to the trigger. There have been many studies of proteins using rapid and step-scan approaches with a light trigger, but the approaches have also been successfully employed for immobilized enzyme responding to a direct electrochemical trigger in surface-enhanced studies on metal electrodes (see below). Significant further
advances in time resolution using rapid scan or step-scan methodologies are now unlikely because of limitations in speed of moving parts or detector response time.

4. Recent developments in infrared instrumentation and methodology which open up new opportunities for studying metalloproteins

(a) Time resolution

Further advances in time resolution down to the pico- and femtosecond time ranges have been made possible for light-triggered reactions by developments using pulsed lasers as the IR source (reviewed in Grills & George 2002). This approach uses a visible ‘pump’ pulse as the reaction trigger, followed at a specific time interval by the ‘probe’ IR pulse which reports on vibrational changes initiated by the pump pulse (figure 3), and is analogous to flash photolysis experiments in UV/visible spectroscopy. The pump and probe pulses are focused so that they spatially overlap, while variation of the separation time between the pulses makes it possible to build up a time series of data points. Again, the reaction must be fully reversible to permit collection of large data series, or the portion of sample interrogated must be renewed between pulse sequences. Since tuneable narrowband lasers were used in initial experiments of this type, pulse sequences had also to be repeated many times to build up a picture of the response of the sample over a wide spectral range. Despite the complications of long collection times, this method led to a highly productive series of studies in the late 1980s and 1990s in which CO docked within myoglobin or haemoglobin was observed at about 100 ps after photolysis from the haem centre, and associated protein structural rearrangements following photolysis were monitored (e.g. Moore et al. 1987; Lim et al. 1995). Experiments were carried out on highly concentrated protein samples, usually in the range 4–14 mM. Data collection times have now been vastly improved by application of broadband pulses (e.g. sampling more than 100 cm\(^{-1}\) at once) and multi-channel detection. In parallel, signal-to-noise ratio has been improved by higher photon flux lasers and superior time resolution has been made possible by shorter laser pulse times (Grills & George 2002). In addition to studies of CO photolysis from proteins, light-triggered time-resolved IR methods have been applied to a range of small-molecule systems including transition metal carbonyl complexes (Butler et al. 2007) and photodamaged DNA bases (Kuimova et al. 2006).

A related temperature-jump method makes use of a laser-induced temperature jump in the sample, followed at variable time intervals by an IR probe pulse, to obtain time-resolved IR data on temperature-triggered spectral changes, such
as protein unfolding studied by Phillips et al. (1995). Another related suite of methods is building up around the concept of two-dimensional IR in which both the pump and probe pulses are in the IR region, and coupling between vibrational modes in a protein can be deduced from cross-peaks in the two-dimensional plot, analogous to two-dimensional nuclear magnetic resonance methods. Using ultrafast lasers, it has been possible to target coupled vibrational transitions which relax within picoseconds. This was applied by Hamm et al. (1999) to analyse through-space or through-bond coupling between amide I bands of peptide units in a small cyclic pentapeptide for which all amide I transitions could be resolved in the spectrum, to obtain structure-dependent information. Extension to larger peptides and proteins is much more difficult because of overlapping amide I bands which cannot be assigned to specific residues, but the approach may have exciting applications for distance/structure determination within proteins or for pairs of interacting proteins that have been labelled with vibrationally active probes at strategic locations.

(b) Surface-enhanced infrared absorption

A signal enhancement for samples in close contact with a nanostructured metal surface was identified in the early 1980s and has been suggested to arise from coupling of photons with the metal and oscillating dipoles induced in the metal by the adsorbed sample (Osawa 2002). This can give a 10- to 100-times signal enhancement for species at distances less than 10 nm from the metal, but decays strongly with distance. The surface-enhanced infrared absorption (SEIRA) effect has been exploited widely in studies of adsorbates on metal surfaces, for example, in mechanistic studies of formic acid oxidation on Pt (Samjeské et al. 2006). Immobilization of proteins on bare metals is rarely compatible with retention of native structures (often resulting in denaturation), but attachment of proteins to a self-assembled monolayer (SAM) of short alkanethiols on gold provides a protective interface and has been shown to keep the protein in close enough proximity to the metal for significant signal enhancement, overcoming the problem of sensitivity for addressing sub-monolayer protein films on surfaces. Since only protein molecules in close contact with the surface contribute to the spectral response, formation of protein films on metal electrodes can be monitored by following the growth of the amide bands. Surface-enhanced IR spectroscopy, implemented for protein attached to a thin gold layer coated on an attenuated total reflectance (ATR) element, has been exploited in several recent studies of metalloprotein chemistry in which the trigger is provided by a gas change (Wisitrungsakul et al. 2009) or an electrochemical step (Ataka & Heberle 2004; Wisitrungsakul et al. 2008).

(c) Polarization-modulation infrared reflection adsorption spectroscopy

A direct electrochemical trigger should also be possible for immobilized protein films in a reflection–adsorption mode (figure 4). In practice, this is difficult because of poor signal to noise for low-coverage films and strong absorption from the water layer. Employment of a polarization-modulation approach potentially confers an advantage because of different attenuation of s- and p-polarized light by species at the electrode surface. This provides a convenient method for subtracting out the IR absorption of isotropic solvent, water, from spectra.
Polarization-modulation methods employ a photoelastic modulator in the beam path—a crystal (often ZnSe) that has an isotropic refractive index until stress applied along one axis induces anisotropy in the refractive index which causes rotation of the polarization of the beam. High-frequency oscillations in stress on the photoelastic modulator crystal, therefore, result in oscillations in polarization of the IR beam, and it is possible to switch rapidly between s- and p-polarized light. The polarization-modulated IR beam is directed via the reflective surface with adsorbed sample to a detector and a lock-in amplifier. Reflection–adsorption IR spectroscopy in this mode has been coupled with electrochemistry to study surface films such as phospholipid bilayers on gold (Zawisza et al. 2004). It should be possible to extend this approach to potential-triggered studies of proteins immobilized on reflective surfaces, providing that technical challenges arising from the need to work with thin electrolyte films and large (more than 1 cm²) reflective electrode surfaces (Skoda et al. 2009) can be overcome.

(d) Modification of proteins with site-specific infrared probes

One further area that offers promise in IR studies of localized probes within metalloproteins is the chemical or genetic attachment of IR-active groups at strategic locations. Schultz et al. (2006) reported introduction of a genetically encoded artificial amino acid with a strong $\nu$(CN) stretch close to the haem centre of myoglobin (figure 1d(i)). Since the frequency of the vibrational band of the probe is sensitive to local environment, IR spectra of the modified protein reported on ligand binding to the haem. Chemically modified cysteine residues converted to thiocyanate in a method reported by Fafarman et al. (2006) offer similar possibilities. In conjunction with two-dimensional IR methods, introduction of probes by chemical or genetic modification may allow resolution of structural factors and interactions between proteins, paralleling spin probes introduced into proteins for distance detection in electron paramagnetic resonance methods.

5. Studies of metalloprotein chemistry

Examples in which recent developments in IR spectroscopy and sample handling have been exploited in studies of chemistry occurring within metalloproteins are now discussed according to the nature of the reaction trigger.
CO slowly rebinds to the haem after photolysis so the depletion band diminishes in intensity. Several short-lived growth bands indicate CO docked in pockets in the protein.

\[ \Delta A \text{ (mOD)} \]

\[ \begin{align*}
\text{bound CO} & \quad 3 \text{ ns} \\
\text{‘free’ CO} & \quad 1 \text{ ns} \quad \gg \text{ ns}
\end{align*} \]

1900 1950 2000 2050 2100 2150

wavenumber (cm\(^{-1}\))

Figure 5. Spectra obtained in a visible pump–IR probe methodology during photolysis of CO-inhibited cytochrome P450. Reprinted in part with permission from Rupenyan et al. (2009). Copyright 2009 American Chemical Society.

(a) Light triggered reactions

Building on the body of work carried out on photolytic release of CO from myoglobin and haemoglobin, there have been a number of elegant recent studies of ultrafast ligand release in metalloenzymes in response to a light trigger using the visible pump–IR probe approach. Spectra shown in figure 5 from the work of Rupenyan et al. (2009) reveal that CO is trapped in pockets in cytochrome P450 following release from the haem centre, just as it is in myoglobin. In the set-up used in this work, the probe pulse diameter was 120–150\(\mu\)m, meaning that it was possible to mount the sample on a moveable stage and address a fresh portion during each pulse sequence to avoid complications from long-term damage from the repeated photoreaction (a full dataset, incorporating 80 time points, may take several hours). Each reading represents a difference spectrum for pump illuminated sample against a background of unmodified sample obtained from a preliminary probe pulse with no prior pump pulse.

Given appropriate sophisticated optical and laser instrumentation, pump–probe experiments can now be carried out with relative ease, and this should lead to more widespread application in bio-inorganic chemistry. There is increasing interest in attaching enzymes to photoreceptors to enable light-triggered catalysis. This holds particular importance for hydrogenases where light-driven H\(_2\) production has been demonstrated by Ihara et al. (2006) who attached a hydrogenase via a fusion protein to cyanobacterial photosystem I, or by Reisner et al. (2009) who immobilized hydrogenase onto dye-sensitized TiO\(_2\). Hydrogenases conveniently have endogenous CO and CN\(^{-}\) ligands at their active
sites (figure 1c), and enzyme–photoreceptor constructs could yield interesting time-resolved details of hydrogenase active site chemistry during catalysis if studied by pump–probe IR methods.

(b) Gas exchange or solute exchange as a reaction trigger

Difference spectra triggered by gas exchanges have been used in a number of studies of hydrogenases (H₂/N₂/air exchange), in combination with mediated electron transfer from a gold minigrid electrode in a transmission cell. These investigations have addressed various active and inactive forms of the enzyme which can be distinguished on the basis of the frequencies of IR bands arising from active site CO and CN⁻ (De Lacey et al. 2007). Slow diffusion of gases through water means that time resolution is necessarily low (many seconds). Gas or solute triggers using stop-flow mixing approaches are limited by mixing times and therefore generally limited to the ms time domain. However, stop-flow mixing was used productively in conjunction with a conventional FTIR instrument set up in an anaerobic glove box by George et al. (1997) for a series of studies of CO binding to the nitrogenase enzyme. One way to take time resolution into the μs domain in solute concentration triggered experiments is the use of caged starting materials which are released in response to a light pulse, in conjunction with time-resolved IR step-scan methods (Cheng et al. 2002). The release of caged solutes implemented within a pump–probe experiment may allow time resolution to be improved further, providing that release of the required solute from the caged starting material is fast, and the caged molecule is attached to the protein of interest to minimize diffusion time.

(c) Electrochemical triggers (spectroelectrochemistry)

Experiments in which the trigger is a potential step at a working electrode have routinely been applied to small molecule systems such as small molecule metal carbonyl cluster mimics of hydrogenase active sites (Best et al. 2008). For proteins larger than about 10 000 Da, diffusion-dependent electron exchange with an electrode is too slow, and small molecule redox mediators are generally included to facilitate electron transfer. Fine semi-transparent metallic grid electrodes have been employed in most transmission IR redox-mediated spectroelectrochemical studies of protein solutions, using a cell developed by Moss et al. (1990) with very few subsequent modifications (De Lacey et al. 2007). An alternative approach used by Iwaki et al. (2005) involves electrodes located in a solution mixture of protein and mediators in contact with an ATR prism which internally reflects the IR beam. Use of a cocktail of redox mediators provides access to a range of potentials, but potential control is indirect in these studies, and the rate of electron transfer may be limited by diffusion of the mediators, particularly for enzymes engaging in fast catalytic turnover.

Direct electron transfer between an immobilized redox protein and an electrode is now well established as the technique of protein film electrochemistry (Vincent et al. 2007) and provides much more precise control over redox chemistry of centres in proteins. Several approaches have been attempted for coupling direct electrochemical control over redox enzymes with IR spectroscopic sampling. Optically transparent electrodes are an attractive possibility because they are compatible with simple transmission cell designs. Boron-doped diamond has been
shown to be effective for electrochemistry of blue copper proteins (McEvoy & Foord 2005) but unfortunately transmits poorly in the IR above about 1800 cm$^{-1}$. The most promising approach so far for spectroelectrochemistry involving direct electron transfer between an immobilized protein and an electrode is SEIRA implemented within an ATR configuration for proteins attached to a modified thin gold layer coated onto the ATR element (figure 6a).

Developments in IR spectroelectrochemistry involving direct electron transfer have been taken furthest for the small, commercially available haem protein, cytochrome c, for which direct electrochemically triggered SEIRA spectroscopy on gold was reported by Ataka et al. (2004) and Wisitruangsakul et al. (2008) as shown in figure 7a,b. Potential-induced evolution of spectral changes in the amide region provides information on structural changes that accompany electron transfer, and permits determination of a reduction potential for the immobilized protein.
Figure 7. (a) Potential-triggered spectra obtained for cytochrome c on a mercaptoethanol-modified gold electrode in an ATR cell: difference spectra calculated with a spectrum of fully reduced enzyme \((-0.10 \text{ V})\) as the reference. Adapted with permission from Ataka et al. (2004). Copyright 2004 American Chemical Society. (b) cyclic voltammogram for cytochrome c on a mercaptoundecanoic acid SAM on gold. Reproduced from Wisitruangsakul et al. (2008) by permission of the PCCP Owner Societies. (c) Spectra obtained for \textit{Desulfovibrio vulgaris} Miyazaki F hydrogenase in solution (lower trace) and on a gold electrode (upper trace) and (d) cyclic voltammograms for the enzyme on gold in the SEIRA cell at different \(\text{H}_2\) levels. Reprinted and adapted in part with permission from Millo et al. (2009). Copyright 2009 American Chemical Society.

protein. Particularly for enzymes, the direct electrochemical control of this method means that such experiments should eventually permit determination of potentials for redox centres under catalytic turnover conditions which cannot readily be obtained from solution potentiometric titrations. Since electron transfer is direct and there is no dependence on diffusion of either mediators or the protein of interest, the SEIRA approach is suited to time-resolved rapid-scan

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(seconds) or step-scan (milliseconds) methods. Orientation-selective attachment of proteins to the gold surface is advantageous for homogeneity in the film and efficient electron transfer, and Jiang et al. (2008) and others have made use of SAMs capped with a nickel nitriloacetic acid complex to selectively coordinate poly-histidine of ‘His-tagged’ proteins (figure 6b).

The endogenous active site CO and CN$^{-}$ ligands of hydrogenases (figure 1) provide a convenient vibrational probe for different states of the enzyme generated by electron transfer, ligand exchange or catalysis. Figure 7c shows that the spectrum for a [NiFe]-hydrogenase in solution corresponds well to the spectrum measured in a SEIRA experiment for the same enzyme immobilized by electrostatic interactions on a SAM on a gold electrode (Millo et al. 2009). Activation of the immobilized enzyme in response to introduction of H$_2$ gas was examined spectroscopically at open circuit potential. Since this reaction is slow (requiring many minutes), the time taken for gas equilibration was not a problem in this case. The possibility of electrochemical control over the enzyme film was demonstrated through cyclic voltammograms which also reveal inactivation/re-activation reactions (figure 7d), but electrochemically triggered SEIRA difference spectra were not reported. Since the enzyme is immobilized, solutes can readily be exchanged in the solution in contact with the film, and it should be possible to exploit a combination of electrochemical, gas and solute exchange triggers in SEIRA measurements. The superior signal/noise and sensitivity to surface species should make this a powerful approach for future electrochemically triggered studies of redox enzymes providing that they can be immobilized in electroactive conformations on metal surfaces.

Graphite, rather than metals, has been the surface of choice for direct adsorption of many proteins for electrochemical study (Vincent et al. 2007). While the reflectivity of graphite makes it less suitable than polished metals for external reflection–adsorption IR spectroscopic methods, we have established that a thin film of micrometre-dimension graphite particles in a polymer electrolyte, coated onto a silicon ATR prism, functions as a viable electrode for IR spectroelectrochemical study of species trapped in the film (figure 6c). We are therefore proceeding to exploit networks of particles modified with enzyme as high-surface-area electrodes in studies of direct electrochemically triggered IR spectroscopy of metalloproteins—particularly hydrogenases—on graphite.

(d) Temperature triggered studies

The use of a temperature change within the cryogenic range to trigger ligand release, rebinding or rearrangement is well established in IR studies of metalloproteins. A study of rebinding of NO trapped in a pocket near the haem centre of a haem–copper oxidase as the temperature is raised from 30 to 60 K is one recent example (Hayashi et al. 2009). Nienhaus et al. (2003) have used temperature ramps to study the thermodynamics of CO rebinding and movement between specific sites near the haem of myoglobin after photolysis at 3 K. Data are presented as contour plots, where correlation between increased absorbance at one wavenumber and decreased absorbance at a different wavenumber at a specific temperature indicates movement or rebinding of CO (figure 8). This provides an example of IR spectroscopy used alongside site directed mutagenesis,
Figure 8. Experiments on wild type myoglobin, and genetic variants of the protein in which the bulky amino acid tryptophan (W) has been introduced in place of leucine (L) or isoleucine (I) to modify sites where Xe has been observed to dock in crystallographic studies. (a) Spectra recorded 1s after photolysis of CO-inhibited protein at 3K. (b, c) Contour plots showing increasing absorbance (solid lines) or decreasing absorbance (dotted lines) in the wavenumber range associated with bound (i) and docked (ii) CO as the temperature is increased from 3 to 100K. Temperature was increased at a rate of 0.005Ks\(^{-1}\), and absorbance changes were recorded at 1K intervals. Adapted in part with permission from Nienhaus et al. (2003). Copyright 2003 American Chemical Society.
and interpreted alongside other time- and temperature-resolved spectroscopic and crystallographic approaches to understand chemistry at the metal centre within a protein. Similar IR methods were used recently alongside molecular dynamics simulations to address ligand binding and movement in another haem protein, neuroglobin (Lutz et al. 2009). Temperature triggers have also been used elegantly in time-resolved laser temperature jump studies to study protein folding or unfolding; in a study of RNase unfolding, Phillips et al. (1995) achieved an increase of about 10K in around 100 ps. This approach should also be able to provide time-resolved information on thermally induced ligand release, rebinding or movement within metalloproteins.

((e) Future directions)

Significant technical developments in IR spectroscopy for metalloproteins come at a time in which CO and NO binding haem proteins are increasingly being attributed roles in cell regulation and signalling (Lee et al. 2009; Miller et al. 2009), bringing up interesting questions about how a tiny diatomic ligand leads to gross structural change in a protein. There is also considerable current interest in understanding the active site chemistry of metalloproteins that carry out small-molecule activation reactions relevant to energy catalysis, including H₂ oxidation by hydrogenases, CO/CO₂ cycling by carbon monoxide dehydrogenase, and CO₂ reduction to formate by formate dehydrogenase. Gas access in these metalloenzymes is also an important question: there has been much debate about whether the ability of hydrogenases to oxidize or produce H₂ in the presence of O₂ is dependent on selective filtering in gas channels through the protein. Similarly, the large enzyme complex, carbon monoxide dehydrogenase/acetyl coenzyme A synthase, is believed to possess long range gas channels to transport CO from the site of its production without release of the toxic gas into cells. Following on from the substantial body of work on CO as a probe for gas movement in myoglobin in IR studies, it should be possible to obtain time- and temperature-resolved information on movement of gases through a range of metalloproteins using sensitive pump–probe IR methods. Studies of CO movement will continue to be particularly powerful when used in combination with genetic mutagenesis in which pockets and putative gas channels in proteins are selectively modified to reveal their role in stabilizing gases. Non-natural amino acids that give rise to IR-active vibrational bands are likely to play a valuable role in determining the electrostatic characteristics of protein pockets and revealing proximity between sites or interactions between proteins. With precise potential control provided by direct electronic communication between redox enzymes and electrode surfaces, it should be possible to obtain time-resolved information on states of enzymes generated transiently during catalysis. We can expect to see a suite of IR spectroscopic approaches, alongside other spectroscopies and crystallography, playing a key role in elucidating metalloprotein chemistry related to health, cellular metabolism and future energy solutions.

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